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CARACTERIZACION DE LOS DOMINIOS FUNCIONALES DE LA PROTEINA VP4 DE LOS ROTAVIRUS

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P R E S E N T A :

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PROGRAMA DE MAESTRIA Y DOCTORADO
EN CIENCIAS BIOQUIMICAS

CLAUDIA SELENE ZARATE GUERRA
P R E S E N T E:

Por este conducto me permito informarle que la Comisión Académica acordó asignarle el siguiente jurado de examen para obtener el grado de Doctora en Ciencias.

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Sin mas por el momento me es grato enviarle un cordial saludo.

A T E N T A M E N T E
"POR MI RAZA HABLARA EL ESPIRITU"
CUERNAVACA, Mor. a 30 de abril de 2002

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RESUMEN

La entrada de diferentes familias virus a su célula huésped es un proceso de varios pasos, en el que diversas proteínas, tanto celulares como virales, están involucradas. Además, durante la entrada ocurren una serie de cambios conformacionales en la partícula viral, que finalmente le permiten ingresar a la célula. Los rotavirus, principal agente etiológico de gastroenteritis infantil, también utilizan una variedad de moléculas celulares como receptores por lo que su entrada involucra varias interacciones con la superficie celular; estas interacciones son llevadas a cabo por las proteínas virales que conforman la capa externa, llamadas VP7 y VP4. Para activar la infectividad viral es necesario que la proteína VP4 sufra un corte proteolítico, lo que da lugar a los polipéptidos VP8 y VP5.

Entre las proteínas celulares que han sido identificadas como receptores para rotavirus se encuentran la integrina $\alpha 2\beta 1$ (8), la integrina $\alpha v\beta 3$ (29) y la proteína de choque térmico hsc70 (28). En este trabajo caracterizamos las proteínas virales, y las regiones dentro de éstas, responsables de las interacciones con las tres proteínas celulares arriba mencionadas. Así, encontramos que los rotavirus se unen a la integrina $\alpha 2\beta 1$ a través de los aminoácidos DGE localizados en la proteína viral VP5. En esta misma proteína viral encontramos otro dominio de unión a la superficie celular, responsable de la interacción de los rotavirus con la proteína hsc70. Por otro lado, encontramos que los rotavirus interaccionan con la integrina $\alpha v\beta 3$ a través de una región de la glicoproteína VP7, que contiene la secuencia CNP. Este hallazgo no solamente nos permitió establecer por primera vez una función específica para la proteína VP7, sino que además nos permitió proponer un nuevo motivo de interacción con la integrina $\alpha v\beta 3$, que podría ser utilizado por otros ligandos de ésta.

En conjunto, los datos obtenidos en este trabajo nos han permitido identificar en la partícula viral varios dominios de interacción con la célula y proponer un modelo de interacciones secuenciales del virus con la superficie celular, que culminarían en la entrada de los rotavirus a la célula para establecer la infección en el citoplasma.

SUMMARY

The entry of a virus into its host cell is a multistep process that involves several proteins of cellular, and viral origin. Generally, this process induces conformational changes in the viral particle, that allow the virus to reach the cytoplasm. Rotaviruses, the most important agent of viral gastroenteritis, interact with several cell surface molecules, and the entry process involves sequential interactions with its receptors; these interactions are mediated by the outermost layer of the virion, which is composed by two proteins, VP7 and VP4. The entry of the virus depends on the proteolytic treatment of the particle with trypsin, which specifically cleaves the VP4 protein into polypeptides VP8 and VP5.

Several cellular molecules are known to be involved on rotavirus infectivity, among them are the integrin $\alpha 2\beta 1$ (8), the integrin $\alpha v\beta 3$ (29), and the heat shock cognate protein hsc70 (28). In this work we characterized the viral proteins, and the domains within them, that are responsible for the interactions of rotavirus with the cellular proteins mentioned above. We found that rotaviruses bind to the integrin $\alpha 2\beta 1$ through the integrin binding motif DGE, located in the viral protein VP5. We also found that there is an additional binding domain in VP5 , that is used by rotaviruses to interact with the hsc70 protein.

On the other hand, we found that rotaviruses interact with the integrin $\alpha v\beta 3$ through a region, within the VP7 protein, that contains the aminoacid sequence CNP, this finding not only allowed us to establish for the first time a role for the VP7 protein, but led us to propose a novel integrin binding motif that could be used by other proteins to interact with integrin $\alpha v\beta 3$.

Altogether, the data obtained in this work allowed us to describe several cell surface interacting domains in the outermost layer of the viral particle; in addition we have proposed a model for the early interactions of the virus with its host cell that consists in sequential steps that finally allow rotaviruses to establish a productive infection.

INTRODUCCION

1. Generalidades

Los rotavirus del grupo A son el principal agente etiológico de las diarreas deshidratantes severas en niños menores de dos años. Se ha estimado que una vacuna efectiva contra estos virus podría evitar cerca de 800,000 muertes cada año. La infección por rotavirus es muy común, pues se ha observado que a la edad de 5 años el 95 % de los niños ya han sido infectados por estos virus. La mayor incidencia de la enfermedad se situa entre los 6 meses y los 2 años de edad, siendo esta población la que presenta un mayor riesgo de sufrir una diarrea severa que requiera hospitalización. Si bien la mortalidad debida a las infecciones por rotavirus es mucho mayor en países en desarrollo que en países desarrollados, la frecuencia de infección es muy similar en todo el mundo; ésto indica que aún niveles de higiene avanzados son incapaces de controlar significativamente las infecciones ocasionadas por estos virus (41). Por todo lo anterior, existe un interés considerable para desarrollar estrategias efectivas de vacunación que prevengan este tipo de infecciones.

Los rotavirus se clasifican dentro de la familia *Reoviridae*; estos virus carecen de envoltura lipídica y su diámetro es de aproximadamente 100 nm. El genoma viral está compuesto por once segmentos de RNA de doble cadena, que codifican para 6 proteínas estructurales y 6 proteínas no estructurales. La partícula viral está formada por tres capas concéntricas de proteínas. La capa más interna está formada por 60 dímeros de la proteína VP2, dentro de este "core" viral se encuentran el genoma y pequeñas cantidades de las proteínas VP1 y VP3, las cuales tienen actividad de RNA polimerasa y de guanilil transferasa, respectivamente. La capa intermedia de la partícula viral está formada por 260 trímeros de la proteína VP6, que es la proteína más abundante del virión. La capa más externa de los rotavirus está formada por dos proteínas: la glicoproteína VP7 (780 copias), que forma la superficie lisa del virión y por 60 dímeros de la proteína VP4, que se proyectan en forma de espículas desde la superficie de la partícula viral (19) (Figura 1).

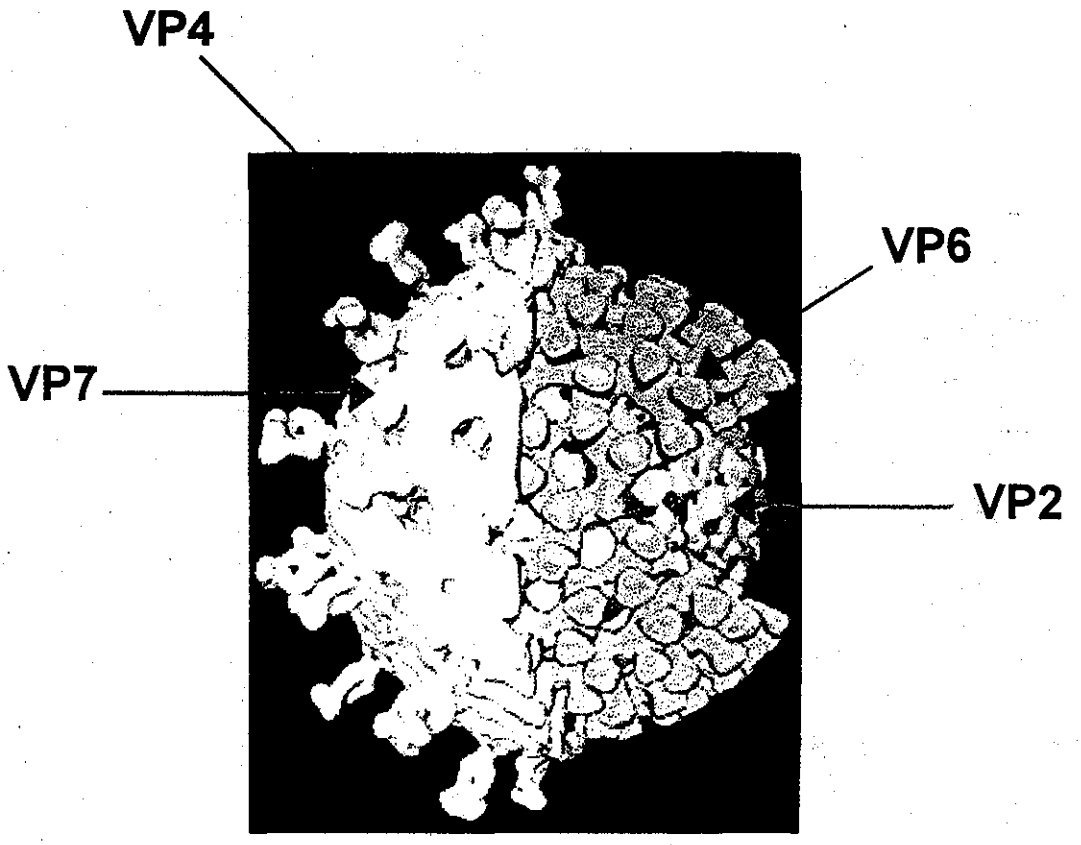

Figura 1. Representación esquemática de rotavirus. Se muestra la estructura de la partícula viral. En gris se muestra el "core" viral formado por la proteína VP2. La capa intermedia, constituida por VP6, está representada en azul. La capa externa, que se muestra en amarillo, está formada por la proteína VP7 y las espículas de VP4.

Los rotavirus poseen un tropismo muy específico, *in vivo* infectan únicamente los enterocitos de las puntas de las vellosidades del intestino delgado e *in vitro* sólo son capaces de infectar líneas celulares derivadas de epitelio renal e intestinal (41). Los rotavirus inician su ciclo de infección mediante el reconocimiento de receptores específicos en la membrana de su célula huésped, estos receptores les permiten acceder al citoplasma mediante un

mecanismo que aún no ha sido dilucidado. Durante su paso hacia el citoplasma, o bien una vez que se encuentran en éste, los rotavirus pierden las proteínas de capa externa; este evento señala el inicio de la transcripción viral. Los RNAs mensajeros se utilizan en la síntesis de proteínas virales y como templado durante la replicación del genoma. La replicación y la morfogénesis de las partículas virales inmaduras ocurre en regiones densas del citoplasma, compuestas por proteínas y material genético virales, a las cuales se denomina viroplasmas. Las partículas inmaduras, con dos de las tres capas protéicas, geman hacia el lumen del retículo endoplásmico, adquiriendo una membrana lipídica transitoria; dicha membrana es sustituida por las proteínas virales VP7 y VP4 (mediante un proceso que aún no ha sido caracterizado),
dando como resultado la formación de partículas virales maduras. Finalmente los viriones maduros son liberados mediante lisis celular (18).

2. Eventos inciales de la infección.

A. Corte con tripsina.

Sabemos que la infectividad de los rotavirus aumenta y muy probablemente depende de que el virus haya sido tratado con tripsina. Este tratamiento proteolítico resulta en el corte específico de VP4 (776 aa) que genera dos polipéptidos de menor peso molecular, llamados VP8 (aa 1-231) y VP5 (aa 247 a 776) (4, 48). El corte de VP4 no afecta la unión del virus a la célula y más bien ha sido asociado con la entrada del virus, por penetración directa (40, 68).

El mecanismo por el cuál el tratamiento de las partículas virales con tripsina incrementa la infectividad no se conoce aún, pero se han propuesto dos posibles mecanismos: por un lado, los extremos generados por el corte de VP4 podrían promover la penetración del virus o la interacción con otras moléculas celulares; o bien, el corte con tripsina podría inducir un cambio conformacional en VP4, que expusiera nuevos dominios de interacción con la superficie celular.

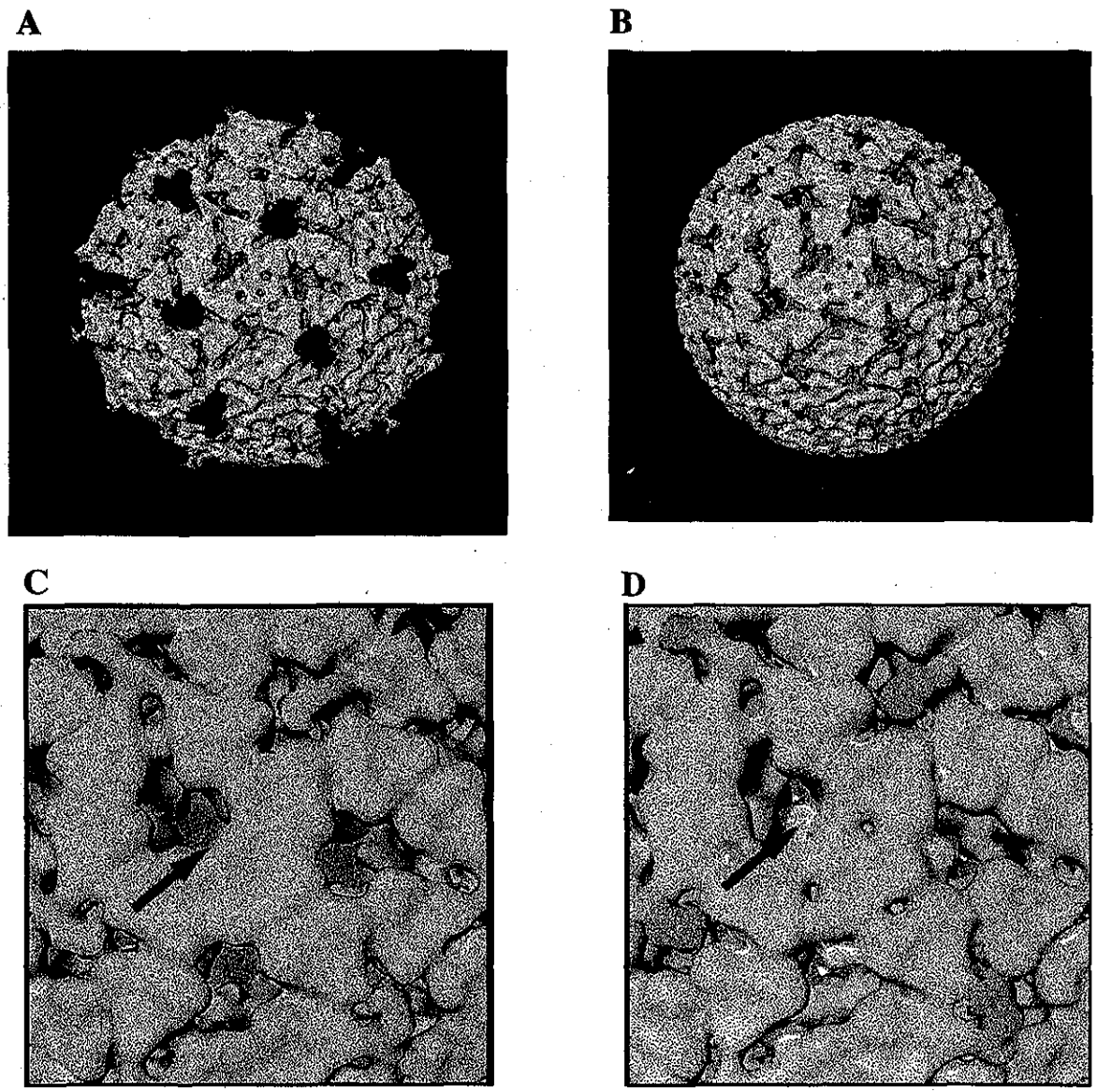

Figura 2. Criomicroscopía electrónica comparativa entre rotavirus cortados (A y C) o no cortados (By D) con tripsina. En los páneles A y B se muestra el aspecto de la partícula completa. En C y D se muestran los cambios en la conformación de la proteína VP7 (en amarillo) señalados con una flecha roja. Tomado de Crawford y col. (2001).

Recientemente un estudio de criomicroscopía electrónica mostró que existen diferencias estructurales entre las partículas virales tratadas o no con tripsina. Así, se observó que en las partículas virales no cortadas con tripsina, las espículas de VP4 aparecen difusas, lo que indica que no se encuentran ordenadas y que pueden adoptar una serie de conformaciones distintas; al tratar a estos virus con tripsina las espículas se hacen más evidentes, indicando que el tratamiento con tripsina da lugar a espículas con una conformación ordenada (Figura 2A y 2B). Además, se observó que también existen diferencias en la conformación de la proteína VP7 entre los virus tratados o no con tripsina (Figura 2C y 2D). Sin embargo, aún no se ha determinado si este cambio en VP7 es un efecto directo de la tripsina, o bien, si es un reflejo de los cambios que ocurren en la conformación de VP4 (9). Las diferencias estructurales encontradas entre las partículas virales cortadas o no con tripsina, sugieren que este tratamiento proteolítico induce cambios conformacionales en la partícula viral que posiblemente le permiten ser competente para inciar la penetración hacia el citoplasma celular.

B. Dependencia de ácido siálico.

En cultivo, la infectividad de varios rotavirus de origen animal es sensible al tratamiento de las células con neuraminidasa (NA), lo cual indica que es necesaria la presencia de ácido siálico (AS) en la superficie celular para que estos virus sean capaces de infectarla (25). Además, al preincubar a estos virus con proteínas sialidadas (por ejemplo glicoforina) se inhibe su infectividad (42, 78), lo que indica que si en estos virus se bloquea el sitio de interacción con AS, se previene su infectividad. Sin embargo, posteriormente se ha encontrado que la interacción con AS no es esencial para la infectividad de los rotavirus, pues se han aislado variantes virales que son resistentes al tratamiento de las células con NA y no requieren de la presencia de AS para infectar a la célula (55); además, existen cepas de rotavirus animales que son naturalmente resistentes al tratamiento de las células con NA, al igual que la mayoría de las cepas aisladas de humano (6, 57).

Tradicionalmente las cepas de rotavirus han sido clasificadas como dependientes e independientes de AS, con base en su resistencia al tratamiento con NA; sin embargo, recientemente se ha visto, en ensayos de cromatografía en capa fina, que algunos rotavirus, típicamente considerados como independientes de AS, se unen al ganglíosido GM1 que posee AS internos que no son removidos por el tratamiento con NA (11). Por otra parte, Guo y col demostraron que la infectividad de algunos rotavirus de origen humano, considerados como independientes de AS, es inhibida al preincubar las células con la subunidad B de la tóxina de cólera, que se une al gángliósido GM1. Con base en estos resultados recientes, consideramos que la clasificación de los rotavirus basada en la dependencia de AS debe ser reevaluada y por el momento la clasificación más apropiada debe de considerar si la infectividad de estos virus es resistente o no al tratamiento de las células con NA.

C. La entrada de rotavirus es un proceso de varios pasos.

La interacción inicial de un virus con sus receptores celulares da lugar a una serie de eventos dinámicos que finalmente le permiten al virus entrar a la célula. La interacción virus-receptor es, en sí misma, un proceso de varios pasos, pero además, los virus pueden utilizar distintos receptores de manera secuencial, o bien un grupo diferente de ellos dependiendo del tipo celular que estén infectando. Se ha encontrado que virus de distintas familias utilizan cuando menos dos tipos de receptores para interaccionar con su célula huésped: un receptor de unión, que en general le permite al virus anclarse de manera rápida a la superficie celular y un segundo tipo de receptores que le permiten la entrada a la célula, a estos receptores se les ha llamado co-receptores o receptores post-unión (70).

La entrada de los rotavirus también parece ser un proceso complejo que involucra varios pasos, evidencia de ésto es que cepas de rotavirus aisladas de humano, naturalmente resistentes al tratamiento con NA, son capaces de competir la infectividad de cepas sensibles al tratamiento con NA de manera no recíproca. Lo anterior sugiere que distintos rotavirus

utilizan receptores diferentes para unirse inicialmente a la superficie celular, aunque comparten al menos una de las interacciones posteriores (57).

El término entrada, entendido como el primer paso del ciclo de infección de un virus, describe la manera en la que un virus puede llegar al citoplasma celular; ésto incluye una gran variedad de mecanismos para distintos tipos de virus. Entre los que se han descrito se encuentran la fusión independiente de pH en la superficie de las células, la fusión dependiente de pH en las vesículas endocíticas, la endocitosis dependiente de receptor y los cambios conformacionales que llevan al desnudamiento del genoma viral (70). Para varios virus, como influenza y HIV, se ha propuesto un modelo de entrada que requiere que la proteína viral encargada de unirse al receptor sufra un cambio en la estructura nativa hacia una conformación que sea competente para la entrada; este cambio puede ser inducido por el contacto con el receptor inicial o por un cambio en el pH del medio. Este modelo se basa en la idea que la proteína se encuentra atrapada en un estado metaestable y que el cambio conformacional sería, en consecuencia, energéticamente favorable, facilitando el proceso de fusión (5). En el caso de los rotavirus se desconce el mecanismo mediante el cual estos virus entran a la célula, pero se ha propuesto que durante este proceso la partícula viral podría sufrir cambios conformacionales que explicarían el uso secuencial de receptores que ha sido observado para estos virus.

D. Receptores de rotavirus

Existen varias moléculas celulares que han sido identificadas como receptores para rotavirus. Los rotavirus sensibles al tratamiento con NA interaccionan en primer lugar con una molécula celular que contiene AS que aún no ha sido identificada, aunque se ha propuesto al ganglióside GM3 (31, 66); y, como se mencionó anteriormente, para algunos rotavirus resistentes al tratamiento de las células con NA, se ha propuesto como receptor inicial al ganglióside GM1 (cuyos AS son resistentes a NA) (31). También se ha encontrado que las integrinas $\alpha 2\beta 1$, $\alpha 4\beta 1$ (8, 33) y $\alpha v\beta 3$ (29) están involucradas en los primeros

eventos de la infección por rotavirus, así como la proteína de choque térmico hsc70 (28). Esta variedad de moléculas celulares (ninguna de las cuáles parece ser capaz, por sí sola, de mediar la infectividad de los rotavirus), que han sido propuestas como receptores para estos virus, sugiere que durante la entrada de los rotavirus ocurre más de una interacción con la superficie celular. Además estas interacciones son secuenciales, puesto que se ha encontrado que algunas de estas moléculas celulares funcionan como receptores post-unión.

Las integrinas forman parte de una familia de proteínas transmembranales que están involucradas tanto en procesos de adhesión célula-célula y célula-matriz extracelular, como en la señalización de procesos tan diversos como migración, proliferación, diferenciación y sobrevivencia (35, 39, 71). Estas proteínas están formadas por dos subunidades, denominadas α y β, que están unidas entre sí de manera no covalente. Hasta el momento se han descrito 18 subunidades α diferentes, así como 8 subunidades β; la combinación de estas subunidades determina la especificidad de ligando de cada integrina. En general, los ligandos naturales de la integrinas son componentes de la matriz extracelular tales como fibrinógeno, fibronectina, vitronectina y colágeno. Las secuencias de aminoácidos utilizadas por estas proteínas para unirse a las integrinas han sido identificadas, así, se ha observado que generalmente el tripéptido RGD, presente en la vitronectina, funciona como motivo de unión a la integrina $\alpha v\beta 3$ (34); el tripéptido DGE es usado por el colágeno de tipo I para unirse a la integrina $\alpha 2\beta 1$ (72); la secuencia IDA, que se encuentra en la fibronectina, sirve como motivo de unión a la integrina $\alpha 4\beta 1$ (59); la secuencia GPR, presente en el fibrinógeno, es reconocida por la integrina $\alpha x\beta 2$ (47) y el tripéptido LDV es el sitio de unión utilizado por el fibrinógeno para interaccionar con la integrina $\alpha 4\beta 1$ (43).

En células en reposo las integrinas están presentes en una conformación latente o inactiva; después de la activación celular, estas proteínas sufren una serie de cambios conformacionales que las convierten en receptores de alta afinidad para sus ligandos específicos. Las integrinas pueden señalizar a través de la membrana celular en cualquier dirección: la unión extracelular de las integrinas es regulada desde el interior de las células

(señalización dentro-fuera), mientras que la unión de sus ligandos produce señales que son transmitidas hacia el interior (señalización fuera-dentro). En la mayoría de los procesos de señalización mediados por integrinas, el dominio citoplásmico de la subunidad β es esencial. La activación de tirosina cinasas iniciada por integrinas está bien documentada (76), y se ha visto que para el caso de adenovirus y virus adeno-asociados, la activación de la cinasa P13, dependiente de integrinas, es necesaria para que el virus pueda entrar a la célula (45, 69).

Recientemente se ha reportado que virus pertenecientes a distintas familias pueden utilizar a las integrinas como receptores; entre ellos están el virus de la fiebre aftosa (37), el papilomavirus (20), los adenovirus (77), el coxsakievirus A9 (65), los hantavirus (26) y los rotavirus (8, 29, 33). Algunos de estos virus utilizan los motivos de unión a integrinas presentes en los ligandos naturales de estas proteínas; en general, estos motivos están presentes en las proteínas virales de superficie. Sin embargo, algunos de estos virus no poseen estos motivos de unión a integrinas en ninguna de sus proteínas de superficie, y las secuencias que utilizan para interaccionar con las integrinas se desconocen (62).

En el caso de los rotavirus, se ha encontrado que éstos poseen los siguientes motivos de unión a integrinas en sus proteínas de superficie: en la proteína VP4 se encuentran los motivos DGE e IDA de unión a las integrinas α2β1 y α4β1, respectivamente; mientras que en VP7 se han reportado los tripéptidos LDV y GPR de unión a las integrinas α4β1 y αxβ2 (8, 33). Además se ha encontrado que en células MA104 anticuerpos dirigidos contra las subunidades α2, β2, y α4, y péptidos que mimetizan a los ligandos de estas integrinas bloquean la infectividad del rotavirus SA11, el cuál depende de AS, y del rotavirus humano RV5, que es resistente al tratamiento con NA (8). También se ha encontrado que las integrinas α2β1 y α4β1 están involucradas en la unión y la entrada del rotavirus SA11 en la línea celular K562 (33). Más recientemente se encontró que la integrina αvβ3 también está involucrada en la infectividad de los rotavirus en un paso posterior a la unión inicial del virus con su célula huésped (29).

Por otro lado, otra de las proteínas celulares que ha sido asociada con la infectividad de los rotavirus es la proteína de choque térmico hsc70. Hsc70 es una proteína celular de localización citoplasmática y nuclear muy abundante, que es miembro de la familia hsp70 de chaperonas moleculares. Las proteínas integrantes de esta familia son ATPasas altamente conservadas que han sido asociadas con gran número de funciones, entre las que resaltan el doblamiento de proteínas, la translocación de polipéptidos a través de membranas, y el ensamble y desensamble de complejos oligoméricos, entre otras (63). En respuesta a diferentes condiciones de estrés, estas proteínas previenen la formación de agregados proteícos estabilizando los intermediarios mal doblados, que posteriormente son plegados correctamente al estado nativo, o bien son degradados (32, 54, 58). En particular, se ha encontrado que hsc70 participa en el transporte de proteínas a través de las membranas de los organelos, se une a polipéptidos nacientes y disocia la clatrina de las vesículas endocíticas cubiertas con esta proteína (63). La proteína hsc70 está compuesta por dos dominios principales: el dominio N-terminal (44 kDa) posee actividad de ATPasa, y el dominio C-terminal (30 kDa) contiene el sitio de unión a péptidos y es capaz de formar complejos con polipéptidos desnaturalizados. La unión y la liberación de los polipéptidos es regulada por la unión e hidrólisis de ATP; la velocidad de intercambio de ATP es regulada a su vez por co-chaperonas. El extremo C-terminal, cuya secuencia es EEVD, es esencial para que hsc70 se asocie con las co-chaperonas hsp40 y Hop (12, 23) y se ha visto que la supresión o la mutación de estos cuatro residuos incrementa la actividad intrínseca de ATPasa de hsc70 y previene su asociación estable con los sustratos (23). A pesar de que hsc70 no tiene una secuencia señal de exportación, se encuentra en la superficie de varios tipos celulares (60). La presencia de esta proteína en la superficie de células MA104 se comprobó por citometría de flujo e inmunofluorescencia de células no permeabilizadas y su papel en la infectividad de los rotavirus se determinó utilizando anticuerpos monoclonales dirigidos contra hsc70 que son capaces de bloquear la infectividad de estos virus (28).

E. Proteínas virales de superficie

Las interacciones iniciales de un virus con su célula huésped involucran el reconocimiento y la unión a un receptor adecuado. Estas interacciones son llevadas a cabo por las proteínas que se encuentran en la superficie de los viriones. Como ya se mencionó, la capa externa de los rotavirus está compuesta por las proteínas VP4 y VP7. Se ha visto que la proteína VP4 de los rotavirus tiene funciones esenciales para las primeras interacciones del virus con la célula, incluyendo la unión inicial y la penetración hacia el citoplasma (51, 55, 67, 80). Por lo tanto las propiedades de esta proteína son determinantes importantes del rango de huésped, virulencia, tropismo, e inducción de inmunidad protectora (18). La proteína VP7 es altamente inmunógena e induce la producción de anticuerpos neutralizantes (18), sin embargo, el papel de VP7 durante las primeras interacciones del virus con la célula no es claro, aunque se ha demostrado que es capaz de modular algunos de los fenotipos virales mediados por VP4, incluyendo el de unión al receptor (56).

E.1 VP4

VP4 es una proteína no glicosilada de 776 aa (88 kDa); se han caracterizado varios dominios funcionales en esta proteína (Figura 3): 1) como se mencionó anteriormente, se encontró que al tratar, *in vitro,* a los virus con tripsina ocurre un corte específico en las argininas 231, 241 y 247 de VP4; este corte da lugar a los polipéptidos VP8 (28 kDa) y VP5 (60 kDa) e incrementa la infectividad viral (4, 17, 48) (Figura 3); 2) el dominio de unión a AS presente en VP4 está localizado entre los aminoácidos 93 al 208 del polipéptido VP8 (24), siendo los aminoácidos 101, 155 y 188 a 190, los que juegan un papel esencial en la unión de VP8 al AS (15, 36); 3) la proteína VP5 es capaz de mediar la unión de los rotavirus a la superficie celular (80).

Ahora bien, se han descrito además dos regiones en VP5 que podrían ser relevantes en las primeras interacciones del virus con la célula huésped, una de ellas es el tripéptido DGE localizado entre los aa 308 al 310 de VP5. Se ha propuesto que esta región podría mediar la interacción entre los rotavirus y la integrina $\alpha 2\beta 1$, puesto que anticuerpos dirigidos

contra esta integrina, y péptidos sintéticos que mimetizan esta región son capaces de bloquear la infectividad de los rotavirus (8).

La otra región de VP5 que podría ser relevante para las etapas iniciales de la infección es una región hidrofóbica (aa 390-401) que guarda similitud con los péptidos de fusión de los alphavirus (53). Estudios recientes han mostrado que la proteína VP5 de los rotavirus es capaz de permeabilizar liposomas (13) y que mutaciones puntuales en esta región hidrofóbica son capaces de abatir la actividad de permeabilización de esta proteína (16), sin embargo, aún no se ha demostrado que el virus completo posea esta actividad, ni tampoco se sabe cual es la relevancia de esta función durante la infección.

Los análisis de predicción de estructura secundaria de la proteína VP4 sugieren que existen por lo menos dos dominios distintos en esta proteína. Los dos primeros tercios de la proteína tienen, predominantemente, una estructura de hojas β, mientras que el tercio carboxilo terminal tiene un alto contenido de hélices α, incluyendo una región en la que se predice la formación de una estructura de tipo "coiled-coil" (36, 50). El uso de los anticuerpos monoclonales (AcM) 7A12 y 2G4 (dirigidos contra el dominio de hemaglutinación y la región hidrofóbica de VP4, respectivamente) en estudios de criomicroscopía electrónica, permitió localizar estas dos regiones en la espícula formada por VP4. Así, se encontró que la punta de la espícula está formada principalmente por VP8, mientras que la región de hojas β de VP5 forma el cuerpo de la espícula y la región de hélices α forma su base (75). Más recientementre se reportó la estructura cristalográfica de VP8, que ha permitido constatar que su estructura está formada principalmente por hojas β y se han determinado directamente los aminoácidos que realizan los contactos con el AS (15).

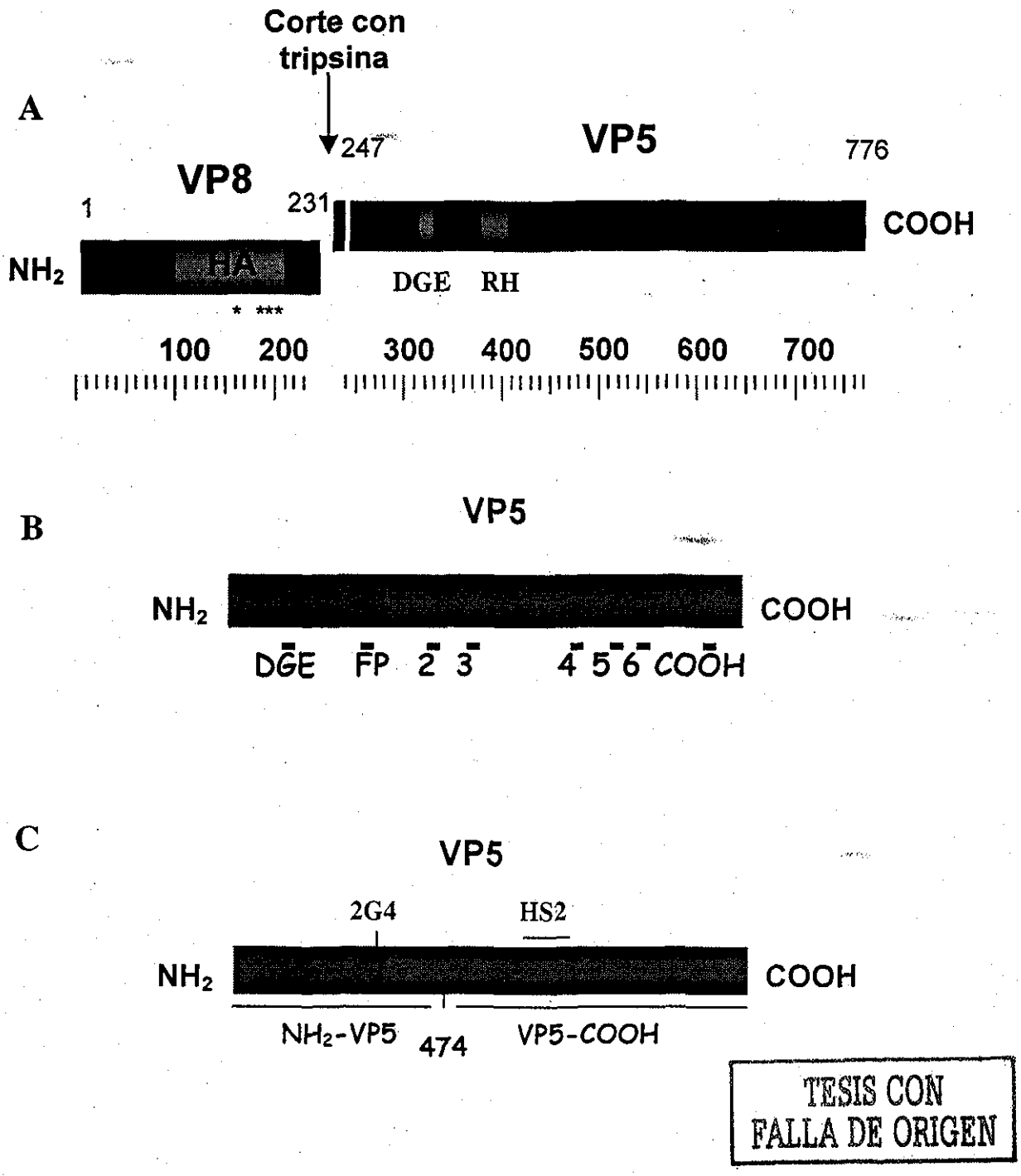

Figura 3. Representación esquemática de VP4. (A) Se muestra el sitio de corte con tripsina, la región DGE de interacción con la integrina $\alpha 2\beta 1$ y la región hidrofóbica RH. (B) Se muestran los sitios que corresponden a los péptidos sintéticos utilizados en este trabajo. (C) Se muestran las construcciones NH2-VP5 y VP5-COOH y los sitios reconocidos por los AcM 2G4 y HS2 (ver más adelante)

E.2 VP7

VP7 es la segunda proteína más abundante del virión, es altamente inmunogénica e induce la producción de anticuerpos neutralizantes específicos de serotipo (18). En la figura 4 se muestra un diagrama con las características de VP7. La secuencia del gene predice una proteína de 326 aa. En el extremo amino terminal de la proteína se encuentran dos regiones hidrofóbicas (H1 aa 6-23 y H2 aa 33-44) que son capaces de dirigir a la proteína VP7 hacia el retículo endoplásmico. Estas regiones hidrofóbicas se procesan, dando lugar a la proteína madura, la cual inicia en la glutamina 51 (73). La proteína VP7 es retenida en el retículo endoplásmico siendo posiblemente los residuos ITG, localizados en la posición 59 a 61, los responsables de esta retención (52). VP7 posee un solo sitio de glicosilación localizado en el aminoácido 69 (3). Además de la glicosilación y del procesamiento, la conformación correcta de esta proteína requiere de la formación de puentes disulfuro intramoleculares y de la presencia de calcio (74). En la partícula viral, la proteína VP7 se encuentra organizada en trímeros, se sabe que la presencia de calcio es indispensable para la formación de estos trímeros, aunque no se conocen la región de VP7 responsable de la interacción con calcio (14). Por otro lado, recientemente se encontró que la glicoproteína VP7 contiene la secuencia GPR, que es un ligando de la integrina $\alpha x \beta 2$, y el motivo LDV que se une a la integrina $\alpha 4 \beta 1$ (8), aunque no se ha demostrado que estos motivos de interacción con integrinas sean funcionales.

F. Primeras interacciones de los rotavirus con su célula húesped

Como modelo de estudio de las primeras interacciones de los rotavirus con su célula huésped, en el laboratorio hemos utilizado dos cepas de rotavirus: un rotavirus aislado de simio, RRV, cuya infectividad es sensible al tratamiento de las células con NA y una variante derivada de RRV, resistente a NA, a la cuál hemos denominado nar3 (55). En algunos casos también se ha empleado la cepa de origen humano Wa, cuya infectividad es resistente al tratamiento de las células con NA.

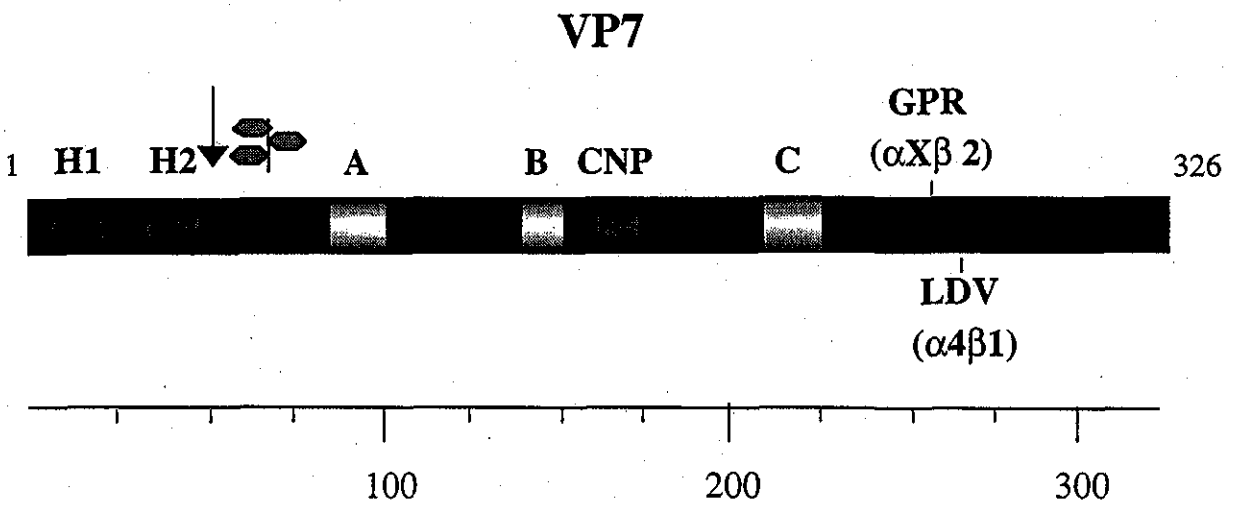

Figura 4. Representación esquemática de VP7. La proteína VP7 posee dos dominios hidrofóbicos H1 y H2 (aa 6-23 y 33-44). Se muestra el sitio de procesamiento en el retículo endoplásmico ↓, en la Gln 51. El sitio de glicosilación se encuentra en el aa 69. Las regiones marcadas como A (86-101), B (142-152) y C (206-221) son epítopes de neutralización. Las secuencias GPR y LDV, que podrían mediar la interacción con integrinas se localizan entre los aa 253-255 y 269-271, respectivamente. La región CNP descrita en este trabajo se localiza entre los aa 161-169.

Aún no ha sido identificada la molécula celular que contiene AS con la que los rotavirus interaccionan, pero sabemos que esta interacción es mediada por el dominio VP8 de la proteína VP4 (24, 36), mientras que la variante independiente de AS, llamada nar3 (55), se une a la superficie celular a través del dominio VP5 de la proteína VP4 (80). También hemos mostrado que la entrada del virus silvestre RRV requiere de las dos interacciones arriba descritas, las cuáles ocurren de una manera secuencial; es decir, el virus silvestre se une inicialmente a AS en la superficie celular a través del polipéptido VP8 y después de esta interacción el virus se une a otro receptor celular a través de VP5 (80). Los rotavirus de origen humano también se unen a las células mediante un receptor resistente a NA que, sin embargo, es distinto al receptor inicial de nar3 (57). Recientemente hemos encontrado que sin importar qué molécula utilizan los virus sensibles o resistentes a NA en su primera

interacción con la célula, ambos tipos de virus interaccionan, en un paso posterior a la unión inicial, con la integrina αvβ3 (29) y con la proteína de choque térmico hsc70 (28). Todos estos datos apuntan hacia el hecho que la entrada de los rotavirus es el resultado de una serie de interacciones de la partícula viral con la superficie de la célula huésped.

OBJETIVOS

OBJETIVO GENERAL

Definir cuales son las proteínas de rotavirus, y los dominios específicos de éstas, que están involucradas en el contacto con los receptores celulares

OBJETIVOS PARTICULARES

1. Definir cuales son las proteínas virales involucradas en la unión de dos cepas de rotavirus a la superficie de la célula: la cepa de simino RRV, cuya infectividad depende de la presencia de AS, y la variante nar3, que es independiente de la presencia de AS.
2. Determinar los dominios de las proteínas virales responsables de las interacciones de los rotavirus con con cada uno de los receptores identificados en nuestro laboratorio.
3. Evaluar el papel del corte con tripsina en la unión inicial de los rotavirus a la superficie celular.
4. Determinar a que nivel (unión inicial o post-unión) se llevan a cabo la interacción de los rotavirus con los receptores hasta ahora identificados.

Como estrategia general, realizamos ensayos de unión de los virus a la célula a 4 °C (a esta temperatura los virus se unen a la célula, pero no pueden penetrar hacia el citoplasma), en presencia de anticuerpos dirigidos contra las proteínas virales y celulares involucrads en este proceso, también se utilizaron proteínas virales recombinantes y péptidos sintéticos que mimetizan regiones importantes de las proteínas virales para la unión a los receptores.

También utilizamos ensayos de infectividad, que se llevan a cabo a 37 °C, y que nos permiten encontrar interacciones que no están involucradas en la unión inicial, pero que son inportantes para la infectividad de estos virus.

MATERIALES Y MÉTODOS.

Células y virus. En este trabajo utilizamos la línea celular MA104, que proviene de células epiteliales de riñon de mono. Las células se crecieron en el medio mímino esencial de Eagle (MEM) (GibcoBRL), suplementado con 10 % de suero fetal bovino. El virus RRV se obtuvo de H. B. Greenberg, Universidad de Stanford, Palo Alto, California, EEUU. El virus nar3 es una variante derivada de RRV que se ha descrito anteriormente (55). Los virus RRV y nar3 se propagaron en células MA104; las células se lavaron dos veces con MEM sin suero y se añadió el lisado viral (previamente activado con 10 µg/ml de tripsina por 30 min a 37 °C), incubando las células por 1 h a 37 °C. Posteriormente se añadió MEM sin suero y se dejó proseguir la infección hasta obtener efecto citopático. Para obtener virus purificado las células infectadas se congelaron y descongelaron dos veces y los lisados se peletearon por centrifugación durante 1 h a 25,000 rpm a 4 °C en el rotor SW28 (Beckman). El pellet se resuspendió en la solución TNC (10mM Tris-HCl [pH 8], 140 mM NaCl, 10 mM $CaCl_2$), se sonicó por 20 s, se extrajo con freón y se sometió a centrifugación isopícnica en CsCl como se ha descrito previamente (17). Los virus cortados o no con tripsina se prepararon haciendo las siguientes modificaciones al protocolo arriba descrito: después de añadir el lisado viral activado a la monocapa de células e incubar esta mezcla por 1 h a 37 °C, se lavaron las células tres veces con MEM sin suero para eliminar el exceso de tripsina, se agregó MEM sin suero y se dejó proseguir la infección hasta que se observó efecto citopático. Las células infectadas se congelaron y descongelaron dos veces y el lisado se dividió en dos partes. Una parte se trató con 100 µg/ml de tripsina por 30 min a 37 °C, mientras la otra parte se mantuvo en hielo. Los lisados se peletearon y se purificaron como está descrito en el párrafo anterior.

Anticuerpos. Los anticuerpos monoclonales (AcM) dirigidos contra rotavirus, 2G4 (específico para VP5), 7A12, 1A9, M11 y M14 (específicos para VP8), 159, 4F8, 57-8, 60 y

129 (específicos para VP7) y 255/60 (específico para VP6), utilizados en este trabajo, fueron proporcionados por H. B. Greenberg, Universidad de Stanford, California. El AcM no neutralizante HS2 (dirigido contra VP5) fue descrito por Padilla-Noriega y col. (64). Los AcMs dirigidos contra las integrinas α2 (P1E6), α4 (P1H4) y αvβ3 (LM609), se compraron a Chemicon y se utilizaron a una concentración de 10 μg/ml. El anticuerpo policlonal dirigido contra un epitope localizado en el extremo amino de la subunidad de integrina β3 se obtuvo de Santa Cruz Biotechnology y se utilizó a 20 μg/ml. El anticuerpo dirigido contra la proteína hsc70 es un suero policlonal de conejo que se produjo en el laboratorio utilizando a la proteína hsc70 recombinante expresada en bacteria como antígeno. El anticuerpo se purificó y se utilizó a una concentración de 80 μg/ml.

Proteínas. La clonación y expresión de las proteínas GST-VP8, GST-VP5, GST-VP5^{D308A} y GST-VP5^{G400D}, ha sido descrita previamente (36, 79, 80). Las proteínas se purificaron por cromatografía de afinidad utilizando el protocolo descrito por Isa y col (36). La proteína NH_2-VP5 comprende los aminoácidos 248-474 de VP4 y la proteína VP5-COOH contiene del aminoácido 474 hasta el aminoácido 776 de VP4. Las proteínas VP5his, NH_2-VP5, VP5-COOH y hsc70 se expresaron con una extensión de seis histidinas en el extremo COOH terminal, utilizando el sistema de expresión pET 28 (Novagen) y se purificaron por cromatografía de afinidad utilizando el sitema de purificación AKTA y las columnas HiTrap chelating (Pharmacia).

Péptidos. Los péptidos utilizados fueron sintetizados químicamente por Research Organics, excepto los péptidos RGD y RGE que se adquirieron de GIBCO y fueron utilizados a las concentraciones indicadas en cada experimento. La secuencia de los péptidos utilizados se encuentra en la Tabla 1.

Tabla I. Secuencias de los péptidos sintéticos utilizados en este trabajo.

Péptido	Proteína de la que se deriva	Secuencia*
1	VP5	^{453}AYPNNGKEYYE^{463}
2	VP5	^{490}RQDLERQLGELREEF^{504}
3	VP5	^{548}MKKFFKSGLNAS^{559}
4	VP5	^{595}STQITDVSSSVSSISTQT^{612}
5	VP5	^{642}KTKIDRSTQISPNTLPD^{658}
6	VP5	^{676}INNDEVFEAGTDGRY^{690}
COOH	VP5	^{738}RQQAFNLLRSDPRVLRE^{754}
DGE	VP5	^{300}KPANYQYTYTRDGEDVTAHTCC^{321}
RH	VP5	^{383}TGGDYSFALPVGQWPVMTGGA^{404}
S5		TIRPSITPKDKQTNSLD
CNP	VP7	^{161}NEWLCNPMD^{169}
HANTA	G1G2	^{759}NSWACNPPD^{767}
sCNP		WPENNCDLM
RGD		GRGDSP

* Los números indican la posición de los péptidos en la proteína de la que se derivan.

Biotinilación de péptidos. Para facilitar la biotinilación de los péptidos CNP y sCNP se añadieron los aminoácidos KYG a la secuencia de éstos, en el extremo amino para el péptido CNP y en el carboxilo para el péptido sCNP. La biotinilación se llevó a cabo incubando 100 µl de péptido (10 mg/ml en PBS) con una concentración final de 10 µg/µl de Sulfo-NH-LC-Biotina (Pierce) durante 2 h a temperatura ambiente (TA). La biotina libre (no acoplada) se neutralizó añadiendo 50 µl de glicina (100 mM) durante 2 h a TA. El péptido biotinilado se separó de la biotina libre utilizando la resina sepharosa G10 (Sigma) mediante

centrifugación. Para determinar si los péptidos se habían biotinilado se realizó un ELISA en el que los péptidos se unieron a una placa de 96 pozos para ELISA, diluidos en una solución de bicarbonato (50 mM $NaHCO_3$ [pH 9]) durante toda la noche a 4 °C. Posteriormente se añadieron 50 µl de estreptavidina-peroxidasa (diluída 1:1500) y la placa se incubó por 1 h a 37 °C. Finalmente se añadió el sustrato de peroxidasa (0.1M solución de citratos [pH 4.5], 1 mg/ml de ortofenilendiamina (Sigma), 4 µl/10 ml H_2O_2) y la placa se incubó hasta que se desarrolló el color; la reacción se paró añadiendo 100 µl de H_2SO_4 0.1M y se determinó la absorbancia a 490 nm en un lector automático de microplacas EL311 (Bio-Tek).

Ensayo de infectividad. Monocapas confluentes de células MA104, crecidas en placas de 96 pozos, se infectaron durante 1 h a 37 °C utilizando virus previamente activado con tripsina. Posteriormente se retiró el inóculo viral, las células se lavaron dos veces con MEM sin suero y se dejó proseguir la infección por 14 h a 37 °C. Las células se fijaron y se tiñeron siguiendo el protocolo descrito por Arias y col (2). El número de células infectadas se cuantificó utilizando el sistema semiautomático Visiolab (30).

Ensayos de bloqueo de la infectividad. Células MA104 crecidas en placas de 96 pozos se preincubaron con los anticuerpos especificados en cada caso, después de esta incubación las células se lavaron y se añadieron 2000 unidades formadoras de focos (FFUs) de virus por pozo. Se permitió que el virus se adsorbiera a la superficie de la célula y posteriormente se retiró el inóculo viral. Se dejó proseguir la infección por 14 h a 37 °C y las células se trataron como se describe en el párrafo anterior.

Ensayo de unión de células en suspensión. La unión de los rotavirus a la superficie celular se determinó mediante un ensayo no radioactivo, descrito previamente (80). Brevemente, células MA104 crecidas en monocapa se incubaron con una solución 0.5 mM de EDTA en PBS por 20 min a 37 °C para desprender las células y obtener una suspensión uniforme.

Estas células en suspensión se lavaron dos veces con MEM sin suero, centrifugando a 1000 rpm durante 1 min a 4 °C y se determinó su concentración utilizando un hematocitómetro. Para el ensayo de unión se mezclaron 5 $X10^4$ células con la concentración de virus o proteína recombinante (previamente sonicados y centrifugados 2 min a 14,000 rpm) indicada en cada caso, en una solución de 1 % de albúmina bovina (BSA) en MEM; el volúmen final de incubación fue de 200 µl. Esta mezcla se incubó por 1 h a 4 °C, posteriormente las células se lavaron 3 veces con una solución fría de 0.5 % de BSA en PBS y finalmente se lisaron en 50 µl de solución de lisis (50 mM de Tris-HCl [pH 7.5], 150 mM NaCl, 0.1% Tritón X-100). El virus o las proteínas recombinantes presentes en este lisado se cuantificaron mediante un ELISA.

Ensayo de unión en monocapa. Se crecieron células MA104 en placas de 48 pozos hasta que alcanzaron confluencia. Se lavaron las células y se incubaron en MEM sin suero por 30 min a 37 °C, se retiró el medio y, para evitar el pegado inespecífico del virus al plástico, las células se incubaron con 500 µl de una solución de 1% de BSA en PBS, por 1 h a TA. Posteriormente se lavaron las células con una solución fría de 0.5 % de BSA en PBS y se incubaron, durante 1 h a 4 °C, con la cantidad de virus o proteína (diluidos en un volúmen final de 200 µl de MEM) especificada en cada caso. Pasado este tiempo las células se lavaron 3 veces con PBS para retirar el exceso de virus o proteína y finalmente se añadieron 120 µl de solución de lisis (ver el párrafo anterior). Las células se congelaron y descongelaron dos veces para lisarlas, y la cantidad de virus o proteína unida se determinó mediante un ELISA.

ELISA para detección de virus o proteínas. Para detectar el virus se utilizaron sueros policlonales de cabra y conejo contra rotavirus como anticuerpos de captura (diluido 1:10,000) y de detección (diluido 1:1,500), respectivamente. Las proteínas fusionadas a GST fueron capturadas con el suero anti-rotavirus de cabra y detectadas con un suero policlonal

de conejo dirigido contra GST (diluido 1:1,500). Las proteínas expresadas con colas de histidinas se capturaron con el suero anti-rotavirus de cabra y se detectaron con los AcM 2G4 (para NH_2-VP5) o HS2 (en el caso de VP5his y VP5-COOH), dirigidos contra la proteína VP5 (diluidos 1:1,000). Para revelar el ELISA se utilizaron los sueros anti-inmunoglobulina conjugados con fosfatasa alcalina (Kirkergaard and Perry) correspondientes y el sustrato Sigma 104 (1 mg/ml) disuelto en una solución de dietanolamina (100 mM dietanolamina [pH 9.4], 1 mM $MgCl_2$, 5 mM azida de sodio). Se midió la absorbancia a 405 nm en un lector automático de microplacas EL311 (Biotek). El ensayo se realizó como ha sido descrito anteriormente (80).

ELISA de unión a la proteína hsc70 y a la integrina αvβ3. Para estudiar la interacción de las proteínas virales con sus posibles receptores, se llevaron a cabo ensayos de ELISA de acuerdo con el protocolo utilizado por Guerrero y col. (28). Brevemente, las placas de ELISA (Costar) de 96 pozos se cubrieron con 500 ng/pozo de la proteína hsc70 (obtenida en el laboratorio) en PBS, o bien con 100 ng de la integrina αvβ3 (Chemicon). Posteriormente las placas se incubaron con las cantidades indicadas de péptidos sintéticos, proteínas recombinantes, o de virus, y en cada caso se detectó la presencia de la proteína, o del virus unidos mediante anticuerpos específicos. Para detectar al virus se utilizó un suero policlonal de conejo dirigido contra rotavirus (diluido 1:1500). La proteína NH_2-VP5 se detectó con el AcM 2G4 (diluido 1:1000) y las proteínas VP5his y VP5-COOH con el AcM HS2 (diluido 1:1000); los péptidos bitotinilados CNP y sCNP se detectaron utilizando estreptavidina conjugada con peróxidasa (ver arriba).

RESULTADOS

1. El dominio VP5 de VP4 puede mediar la unión de los rotavirus a las células.

El interés inicial del presente trabajo fue la caracterización de la unión de los rotavirus a la supericie de células MA104. Los resultados de este trabajo se publicaron en el artículo "The VP5 domain of VP4 can mediate attachment of rotaviruses to cells" y las figuras a las que se refiere el texto a continuación están contenidas en éste. Como ya habíamos mencionado utilizamos como modelo de estudio dos cepas de rotavirus diferentes en su sensibillidad al tratamiento de las células con NA: la cepa de simio RRV, cuya infectividad es sensible a NA, y el virus mutante nar3. Nar3 es una variante derivada de RRV cuya infectividad es insensible al tratamiento de las células con NA, esta mutante se seleccionó a partir de virus crecido en células tratadas con NA (55); la secuenciación de su genoma permitió identificar los aminoácidos responsables del cambio observado en su fenotipo. Se encontró que todos los cambios se encuentran en la proteína VP4, en los aminoácidos 37 (L→P), 187 (K→R), y 267 (Y→C), sin embargo el análisis de revertantes indicó que, aparentemente, el cambio más relevante es el que se produce en la posición 187, ya que reversiones en esta única posición dan como resultado la pérdida del fenotipo de resistencia al tratamiento con NA (56).

Para hacer la caracterización de la unión de estos rotavirus a la superficie celular, se utilizaron dos tipos de ensayos. El ensayo de unión se basa en la observación que a 4 °C los rotavirus son capaces de unirse de manera específica a la superficie celular pero no son capaces de entrar al citoplasma; por ello este ensayo nos permite estudiar la primera interacción del virus con la célula huésped. El otro ensayo que utilizamos es el de infectividad, en el que cuantificamos el número de células que, después de la infección, producen antígeno viral, detectado por un ensayo de inmunoperoxidasa *in situ* sobre las monocapas celulares; en este tipo de ensayo lo que observamos es el efecto de diferentes condiciones experimentales, como antcuerpos, péptidos sintéticos y proteínas recombinantes, en el proceso de infección. Sin embargo, este ensayo no nos permite discriminar si el paso

que está siendo bloqueado es la unión, la entrada, el desnudamiento o la síntesis de antígeno viral. Para la interpretación de los resultados consideramos los bloqueos, con anticuerpos, péptidos sintéticos o proteínas recombinentes, observados en los ensayos de unión como bloqueos de la interacción inicial del virus con la célula y los bloqueos obaervados en los ensayos de infectividad, que no correlacionan con bloqueos en unión, los interpretamos como interacciones post-unión.

Utillizamos los virus RRV y nar3 purificados por gradiente de CsCl para hacer ensayos de unión en células MA104 en suspensión. Encontramos que ambos virus se unen a la célula de manera saturable y dependiente de la cantidad de virus añadida (Fig. 1, art. 1). Al preincubar los virus con AcM dirigidos contra las proteínas de superficie de rotavirus VP8, VP5 y VP7, y contra la proteína interna VP6 utilizada como control, encontramos que los AcM dirigidos contra VP8 bloquean la unión de RRV a la superficie de la célula; ésto es consistente con el hecho que el virus RRV se une a AS a través del dominio de hemaglutinación que está presente en VP8. Los AcM dirigidos contra VP5 y VP7 no afectan la unión de RRV, si bien son capaces de neutralizar su infección; ésto indica que dichos AcM neutralizan al virus RRV en un paso posterior a su unión inicial a la superficie celular. Por otro lado el AcM 2G4, dirigido contra la proteína VP5 bloquea la unión de la variante nar3, lo que sugiere que este virus utiliza el domino VP5 para unirse a la célula. El AcM 159, dirigido contra VP7 no afecta la unión de los virus RRV y nar3, pero si neutraliza la infectividad de estos virus, sugiriendo nuevamente el bloqueo de un paso posterior a la unión inicial del virus con la célula. Estos resultados se muestran en la figura 2 del artículo 1.

Las proteínas VP8 y VP5 se expresaron en bacteria, como fusiones a GST, con la idea de utilizarlas en nuestros ensayos de unión. Encontramos que ambas proteínas se unen a la superficie de la célula de manera saturable y dependiente de la concentración de proteína añadida (Fig. 3, art. 1). Para determinar si la manera en que estas proteínas se unen a la célula es equivalente a lo que habíamos observado con los virus, preincubamos las proteínas de fusión con los AcM dirigidos contra VP8, VP5 y VP7, éste último utilizado como control de

especificidad. Posteriormente medimos la capacidad de unión de estas proteínas a la superficie celular. Encontramos que los AcM dirigidos contra VP8 bloquean la unión de la proteína recombinante GST-VP8, pero no afectan la unión de la proteína GST-VP5, el AcM 2G4 bloquea la unión de GST-VP5, pero no afecta la unión de la otra proteína, y el AcM 159, dirigido contra VP7 no tiene ningún efecto sobre la unión de estas dos proteínas (Fig. 4, art.1).

Una vez que habíamos determinado que la unión de las proteínas recombinantes es específica y para estudiar si estas proteínas son capaces de competir con el virus por la unión a la superficie celular, preincubamos las células con las proteínas de fusión durante 1 h a 4 °C y después de retirar el exceso de proteína añadimos una cantidad fija del virus RRV o nar3. Encontramos que la proteína GST-VP8 bloquea la unión de RRV pero no la de nar3, mientras que GST-VP5 bloquea la unión de nar3 y no la del virus RRV. Al preincubar las células con una mezcla de estas proteínas no se observa ningún efecto sumatorio en el bloqueo de la unión (Fig. 5, art. 1). Todos estos resultados confiman que existen dos dominios de unión a la célula presentes en VP4; uno es el dominio de unión a AS, que se encuentra en VP8 y que ya ha sido caracterizado; este dominio es utilizado por el virus RRV para unirse a la superficie celular. El otro dominio se encuentra en la proteína VP5 y es utilizado por la variante nar3 para unirse a la célula.

Finalmente probamos el efecto de las proteínas recombinantes GST-VP8 y GST-VP5 en la infectividad de los rotavirus, y encontramos que la proteína de fusión GST-VP8 bloquea la infectividad de RRV pero no la de nar3, mientras que la proteína GST-VP5 bloquea la infectividad de la variante nar3 (Fig. 6A, art. 1), como era de esperarse. Además, probamos el efecto de las proteínas recombiantes en la infectividad de los virus en células tratadas con NA; este tratamiento disminuye en gran medida la infectividad del virus RRV, por lo que se aumentó 10 veces la cantidad de virus RRV utilizada. En células tratadas con NA encontramos que GST-VP8 áun bloquea la infectividad de RRV, pero en proporción menor que en células no tratadas, indicándo que el tratamiento con NA no remueve por

complero el AS de la superficie de las células, probablemente debido a que algunos AS son resistentes al tratamiento con NA (ref). Por otro lado GST-VP5, que en células no tratadas sólo disminuye la infectividad de nar3, en células tratadas reduce el 75 % de la infectividad residual de RRV(Fig 6B, art. 1). Este resultado sugiere que RRV es capaz de interaccionar, a través de VP5, con un receptor que no contiene ácido siálico, aunque con una eficiencia mucho menor que nar3, o bien que en RRV existe una población minoritaria de virus capaz de unirse a la superficie celular a través de VP5.

En conjunto, estos resultados nos llevaron a proponer el siguiente modelo: los virus dependientes de AS (o sensibles al tratamiento de las células con NA) se unen inicialmente a una molécula celular que contiene AS, a través del dominio de hemaglutinación presente en VP8; suponemos que esta interacción ocasiona un cambio conformacional en la partícula viral, que ahora le permite interaccionar con un segundo receptor, a través de VP5, ya que si bien el AcM contra VP5 no afecta la unión de este virus, si neutraliza su infectividad. Este segundo receptor es resistente al tratamiento con NA. Por otro lado la variante nar3 posee este cambio conformacional por lo que no interacciona con el receptor que posee AS, sino que se une directamente al segundo receptor a través de VP5. La representación del modelo se muestra en la figura 7 del artículo 1.

ARTICULO 1
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The VP5 Domain of VP4 Can Mediate Attachment of Rotaviruses to Cells

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Some animal rotaviruses require the presence of sialic acid (SA) on the cell surface to infect the cell. We have isolated variants of rhesus rotavirus (RRV) whose infectivity no longer depends on SA. Both the SA-dependent and -independent interactions of these viruses with the cell are mediated by the virus spike protein VP4, which is cleaved by trypsin into two domains, VP5 and VP8. In this work we have compared the binding characteristics of wild-type RRV and its variant nar3 to MA104 cells. In a direct nonradioactive binding assay, both viruses bound to the cells in a saturable and specific manner. When neutralizing monoclonal antibodies directed to both the VP8 and VP5 domains of VP4 were used to block virus binding, antibodies to VP8 blocked the cell attachment of wild-type RRV but not that of the variant nar3. Conversely, an antibody to VP5 inhibited the binding of nar3 but not that of RRV. These results suggest that while RRV binds to the cell through VP8, the variant does so through the VP5 domain of VP4. This observation was further sustained by the fact that recombinant VP8 and VP5 proteins, produced in bacteria as fusion products with glutathione *S*-transferase, were found to bind to MA104 cells in a specific and saturable manner and, when preincubated with the cell, were capable of inhibiting the binding of wild-type and variant viruses, respectively. In addition, the VP5 and VP8 recombinant proteins inhibited the infectivity of nar3 and RRV, respectively, confirming the results obtained in the binding assays. Interestingly, when the infectivity assay was performed on neuraminidase-treated cells, the VP5 fusion protein was also found to inhibit the infectivity of RRV, suggesting that RRV could bind to the cell through two sequential steps mediated by the interaction of VP8 and VP5 with SA-containing and SA-independent cell surface receptors, respectively.

The initial interaction of a virus with its host cell involves the recognition of, and a stable binding to, an appropriate receptor on the surface of the cell. Even though a great amount of work has been invested in the study of rotaviruses, little is known about the initial interactions of these viruses with their host cells.

Rotaviruses are the leading cause of morbidity and mortality due to acute gastroenteritis in children younger than 2 years (23). These viruses belong to the *Reoviridae* family and are composed of a genome of 11 segments of double-stranded RNA surrounded by three concentric layers of protein. The outermost layer is formed by VP7, a 37-kDa glycoprotein, which forms a smooth layer, and by VP4, an 88-kD protein, which forms the spikes that extend from the surface of the particle (11).

It has been shown that VP4 has essential functions in the early virus-cell interactions, including receptor binding and cell penetration (1, 5, 28, 31, 36). The infectivity of rotaviruses is greatly enhanced by and apparently is dependent on the trypsin treatment of the viral particle; this proteolytic treatment results in the specific cleavage of VP4 into polypeptides VP5 and VP8 (10, 12, 27). The cleavage of VP4 does not affect cell binding but has been associated with the entry of the virus into the cell (3, 15, 22).

In vivo, rotavirus infection is highly restricted to the mature tip cells of the small intestine (23). The infection in vitro is also restricted, being most permissive in a variety of epithelial cell lines of renal and intestinal origin (11). The high selectivity of these viruses suggests the presence of specific receptors in the surface of susceptible cells, which might be at least one of the factors responsible for determining their selective tropism.

Some rotaviruses of animal origin bind to the cell surface through a sialic acid (SA)-containing cell receptor (2, 14, 24, 31). Human rotaviruses, in contrast, do not require SA to infect the cells (14). Recently, we isolated variants of a SA-dependent rhesus rotavirus (RRV) which no longer depend on the presence of SA to bind and thus to infect the cell (31). The characterization of these variants indicated that binding to SA is not an essential step in infection of cells by animal rotaviruses. It also showed that the initial interaction with SA, which is probably nonspecific, can be superseded by an interaction with a secondary receptor (SA independent), which might be responsible at least in part, for the tropism of these viruses. We have also shown that the SA-independent interaction of the RRV variants is mediated by VP4, through a site in the viral protein different from the SA-binding domain, located in VP8 (32).

To characterize the domains of the VP4 protein that interact with the surface of the host cell which ultimately lead to penetration of the virus into the cell, we have compared the binding characteristics of RRV and one of its SA-independent variants, nar3, to MA104 cells. We found that while wild-type (wt) RRV initially binds to the cell through VP8 (13, 21, 36), the SA-independent variant interacts with the cell through VP5. This finding supports our previous suggestion that the interaction of animal rotaviruses with the cell surface might involve at least two sites on the VP4 protein and directly assigns a novel cell interaction role to VP5.

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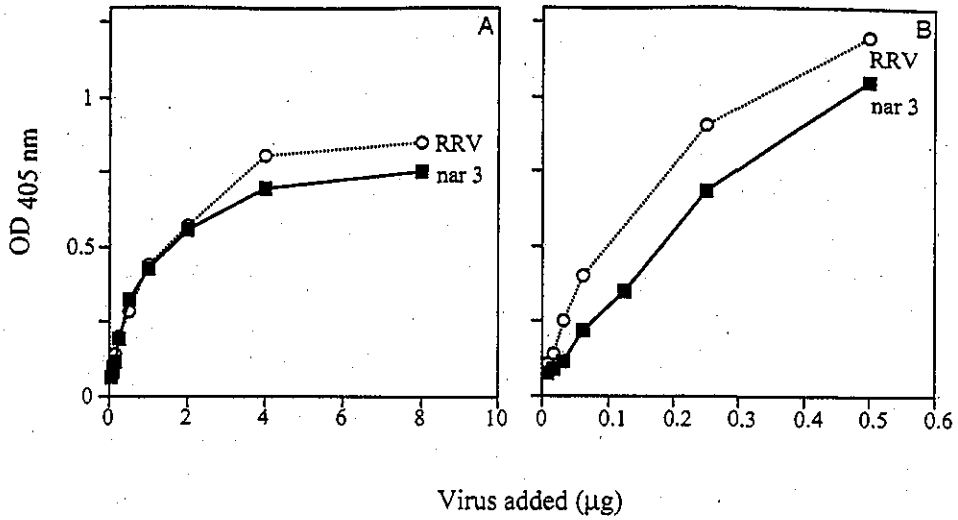

FIG. 1. Binding of RRV and nar3 to MA104 cells. The indicated amounts of purified viruses were incubated with 5×10^4 MA104 cells in suspension for 1 h at 4°C, and the amount of cell-bound virus was determined by an ELISA as described in Materials and Methods. The total amount of viral particles added in each assay (in micrograms) is plotted against the OD_{405} reading obtained in the ELISA plate. (A) Readings obtained in the binding assays. (B) Readings obtained when the viral particles were assayed directly in an ELISA.

MATERIALS AND METHODS

Cells, viruses and monoclonal antibodies. MA104 cells were cultured in Eagle's minimal essential medium (MEM) supplemented with 10% fetal bovine serum. RRV was obtained from H. B. Greenberg, Stanford University, Stanford, Calif., and rotavirus variant nar3 has been described previously (31). RRV and nar3 were propagated in MA104 cells as previously described (9).

To prepare purified virus, virus-infected cells were harvested after complete cytopathic effect was attained, the cell lysate was frozen and thawed twice, and the virus was pelleted by centrifugation for 60 min at 25,000 rpm at 4°C in an SW28 rotor (Beckman). The virus pellet was resuspended in TNC buffer (10 mM Tris-HCl [pH 7.5], 140 mM NaCl, 10 mM $CaCl_2$), extracted with Freon, and subjected to isopycnic centrifugation in CsCl as previously described (10). The protein content of the purified triple-layered particles was determined by the Bradford protein assay (Bio-Rad).

The infectious titer of the trypsin-activated (10 μg of trypsin per ml for 30 min at 37°C) viral preparations was determined by an immunoperoxidase focus assay with MA104 cells grown in 96-well tissue culture plates, as previously described (26). Titers are expressed as focus-forming units (FFU) per milliliter. When indicated, cells were treated with 20 mU of neuraminidase (NA) from *Arthrobacter ureafaciens* (Sigma Chemical Co.) per ml for 1 h at 37°C. After two washes with phosphate-buffered saline (PBS), the cells were infected as described previously (31).

Monoclonal antibodies (MAbs) 2G4 (specific for VP5), 7A12, 1A9, M11, and M14 (specific for VP8), 159 (serotype G3 specific), and 255/60 (subgroup I specific) used in this work were kindly provided by H. B. Greenberg.

Binding assays. Rotavirus binding was determined by a nonradioactive binding assay. Confluent monolayers of MA104 cells were washed and brought into a single-cell suspension by incubation with 5 mM EDTA in PBS for 10 min at 37°C and dispersed by gentle pippeting. The cell suspensions were centrifuged at $82 \times g$ for 1 min at 4°C, washed, and resuspended in MEM without serum, and the cell concentration was determined with a hemocytometer. For the binding assay, 5×10^4 cells were mixed with either virus or recombinant proteins (previously sonicated and centrifuged for 2 min in the Eppendorf centrifuge) in MEM–1% bovine serum albumin (BSA) in a final volume of 200 μl and incubated for 1 h at 4°C with gentle mixing. The cell-virus complexes were washed three times with ice-cold PBS containing 0.5% BSA and then lysed in 50 μl of lysis buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 0.1% Triton X-100). During the last wash, the cells were transferred to a fresh Eppendorf tube. The virus and recombinant proteins present in the lysates were quantified by enzyme-linked immunosorbent assays (ELISAs). In all the binding assays, of either virus or recombinant proteins, controls of binding without cells were performed. When the recombinant proteins or the viruses were directly detected by ELISA, they were diluted in lysis buffer.

Capture ELISAs for rotavirus and GST fusion rotavirus proteins. To detect the virus, goat and rabbit polyclonal sera to rotavirus were used as capture (diluted 1:10,000) and detection (diluted 1:1,500) antibodies, respectively. The rotavirus proteins fused to glutathione *S*-transferase (GST) were captured with the goat anti-rotavirus serum and detected with a rabbit serum to GST (diluted 1:1,500). Similarly, the nonfused, control GST protein was captured with a goat anti-GST antibody (Pharmacia; diluted 1:100) and detected with the GST-specific rabbit serum. In the competition assay, where both rotavirus particles and GST rotavirus fusion proteins were present in the same sample, the virus was detected with a MAb directed to RRV VP7 (MAb 159). In general, the ELISA was performed as follows. Polystyrene 96-well plates were coated for 2 h at 37°C with 100 μl of the capture antibody diluted in PBS. Residual free protein binding *sites were blocked by incubation with 200 μl of 1% (wt/vol) BSA in PBS for 2 h* at 37°C. Incubation with 50 μl of viral or protein antigen sample per well in lysis buffer for 1 h at 37°C was followed by incubation with 50 μl of the appropriate detection antibody (see above) per well diluted in 1% BSA in PBS. Finally, 50 μl of the respective alkaline phosphatase-conjugated anti-immunoglobulin serum (goat anti-rabbit immunoglobulin G or goat anti-mouse immunoglobulin G [diluted 1:1,500]; Kirkergaard and Perry) per well was incubated for 1 h at 37°C, and then Sigma 104 phosphatase substrate, diluted in diethanolamine buffer (100 mM diethanolamine [pH 9.4] 1 mM $MgCl_2$, 5 mM sodium azide), was added, and the absorbance at 405 nm was recorded in a Microplate Autoreader EL311 (Bio-Tek Instruments).

Cloning, expression, and purification of GST fusion proteins. The cloning and expression of the RRV VP8 protein as a fusion protein with GST has already been described (21). The DNA fragment encoding VP5 was obtained from the cDNA clone of RRV VP4 (6) by digestion of the gene with *Ban*II and *Xba*I; the ends were made blunt with T4 DNA polymerase and the Klenow fragment, and the DNA fragment containing nucleotides 749 to 2347 of the RRV VP4 gene was cloned into the *Sma*I site of the pGEX 4T-2 vector (Pharmacia). The resultant fusion protein, GST-VP5, contained 226 amino acids from the GST protein, the thrombin recognition site, 3 amino acids resulting from translation of part of the vector polylinker, and 529 amino acid residues of the VP4 protein (from amino acid 248 to 776), resulting in a fusion protein of approximately 84 kDa. The expression and purification of the GST-fusion proteins were performed essentially as described by Isa et al. (21).

RESULTS

Binding characteristics of RRV and nar3 to MA104 cells. The binding of RRV and nar3 to MA104 cells was determined by a direct, nonradioactive assay, in which increasing amounts of CsCl gradient-purified viruses were incubated with a constant number of MA104 cells in suspension for 1 h at 4°C. The cell-bound virus was separated from free virus by three washes with PBS–0.5% BSA and detected in the final cell pellet by an ELISA with a goat polyclonal anti-rotavirus serum as the capture antibody and a rabbit polyclonal anti-rotavirus serum as the detecting antibody. Similar nonradioactive binding assays have been described previously (17, 19, 20).

The binding of purified RRV and nar3 to MA104 cells as measured by this direct assay was dose dependent and saturable (Fig. 1A). To show that the saturation observed in the

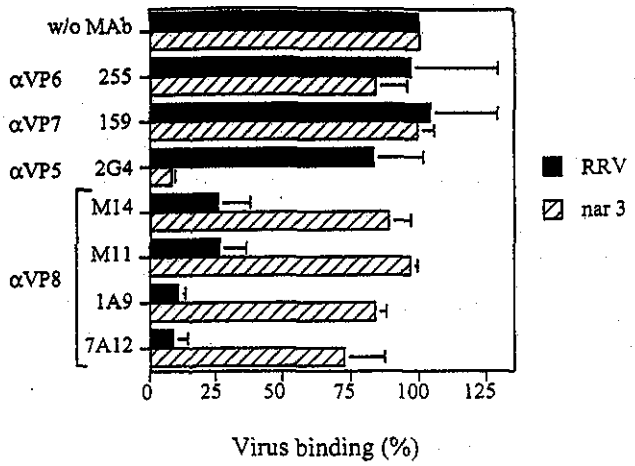

FIG. 2. Binding of RRV and nar3 in the presence of antirotavirus MAbs. Purified RRV (A) or nar3 (B) viral particles (300 ng) were preincubated for 1 h at room temperature with the indicated concentrations of the MAbs (see below) in a final volume of 100 μl. After incubation with the MAbs, the virus-antibody mixture was added to a suspension of MA104 cells (5×10^4 cells/binding assay) and incubated for 1 h at 4°C with gentle shaking. The amount of cell-bound virus was determined by an ELISA as described in Materials and Methods. Data are expressed as the percentage of the virus binding obtained when the virus particles were preincubated with PBS as a control (w/o MAb). The arithmetic means and standard deviations for two independent experiments performed in duplicate are shown. The concentrations of MAbs used were as follows: 255, 159, 2G4, 1A9, and 7A12, 75 μg/ml; M11, 50 μg/ml; and M14, 100 μg ml.

binding curves for both viruses was due to saturation of the attachment sites in the cell surface and was not the result of saturation of the detection system, a direct ELISA of the purified viruses was performed in parallel to the binding assay; the optical density (OD) readings of the direct ELISA kept increasing up to 1.2 (Fig. 1B). No virus was detected in the ELISAs of control experiments where no cells were added during the binding assay (data not shown).

MAbs to VP5 and VP8 differentially prevent the binding of nar3 and RRV. We have previously shown that in contrast to wt RRV, the SA-independent variant nar3 is not neutralized by MAbs directed to the VP8 domain of VP4 (MAbs 7A12, 1A9, M11, and M14), even though these MAbs bind to the variant as efficiently as to the wt virus, as judged by HA inhibition and ELISA (31). On the other hand, MAb 2G4, which is directed to the VP5 domain of VP4 (29), inhibits the infectivity of both wt and variant viruses (31). It has been shown that MAbs directed to VP8 are able to neutralize the infectivity of RRV by preventing the initial attachment of the virus to the cell (36) while MAb 2G4 inhibits its infectivity at a not yet defined postbinding step.

Since RRV and the variant nar3 apparently have distinct requirements for cell binding, we characterized the effect of MAbs directed to the outer-shell proteins VP7, VP8, and VP5, on the attachment of these viruses to MA104 cells. We also used, as control, a MAb directed to the inner-shell protein, VP6. In these assays, a fixed amount of purified virus (300 ng) was preincubated with the different MAbs for 1 h at room temperature, this mixture was added to cells in suspension, and the cell-bound virus was quantitated by ELISA, as described above. Figure 2 shows that while MAbs 255 and 159, directed to VP6 and VP7, respectively, did not affect significantly the binding of either nar3 or RRV, the MAbs directed to VP8 or VP5 did have a differential effect, depending on the virus tested. While the VP8 MAbs reduced the attachment of RRV to cells, as previously reported (36), the binding of the SA-independent variant nar3 was not affected. In contrast, the VP5 MAb 2G4 prevented the binding of the nar3 variant, although it did not affect the binding of wt RRV. To discard the possibility that the MAbs bound to the virus affected its recognition by the capture antibody in the ELISA, a fraction of the virus-antibody complexes was added directly to the detection ELISA. No differences in the OD readings between the virus-MAb mixtures and the controls viruses without MAbs were observed (data not shown).

Since MAbs 7A12, 1A9, M11, M14, and 2G4 are able to bind equally well to RRV and its variant nar3 (31), these results confirm that wt RRV binds to the cells through the VP8 domain of VP4 and strongly suggest that the variant nar3 does so through VP5.

Binding of the recombinant VP5 and VP8 GST fusion proteins to MA104 cells. To confirm that both domains of VP4 are able to interact with the surface of the cells, we expressed wt RRV VP5 and VP8 in bacteria as fusion proteins with GST. Both fusion polypeptides, as well as the GST moiety alone, were purified by affinity chromatography with glutathione-agarose beads (inset in Fig. 3B) and tested for their ability to bind to MA104 cells. In this case, the cell-bound recombinant proteins were detected by ELISA with a goat antirotavirus serum (or goat anti-GST serum for the GST control protein) as the capture antibody and a rabbit anti-GST serum as the detection antibody for all three proteins.

In these binding assays, we found that both GST-VP8 and GST-VP5, but not the control protein GST, were able to bind to the surface of the cells in a dose-dependent and saturable manner (Fig. 3A). A direct ELISA of the recombinant proteins showed that this assay was not saturated up to an OD reading of 1.5 (Fig. 3B). No protein was detected in the control ELISAs where no cells were added to the binding assay mixtures (data not shown).

To confirm the specificity of binding of GST-VP5 and GST-VP8 to MA104 cells, the recombinant proteins were preincubated with MAbs directed to both domains of VP4 and to VP7. The binding of GST-VP5 and GST-VP8 was decreased by incubation only with the MAbs directed to the corresponding VP4 domain and not by incubation with other MAbs (Fig. 4). Also, to discard the possibility that the MAbs bound to the fusion proteins affected their recognition by the capture antibody in the ELISA, a fraction of the recombinant protein-antibody complexes was added directly to the detection ELISA. Again, no significant differences in the OD readings between the recombinant protein-MAb mixtures and the controls GST-VP5 and GST-VP8 without MAbs were observed (data not shown).

These results indicate that the attachment of these proteins to MA104 cells is specific and also show that GST-VP5 and GST-VP8 are correctly folded, since the MAbs used in these assays are known to be sensitive to the protein conformation (21, 29, 30).

The attachment of nar3 and RRV is differentially prevented by the VP5 and VP8 domains of VP4. Since both fusion proteins were able to bind specifically to the cells, we next asked whether their attachment to the cell surface would prevent the binding of nar3 and RRV. For this, MA104 cells in suspension were preincubated with the bacterially expressed proteins, a fixed concentration of the purified viruses was added, and the virus binding assay was performed as described above. In the ELISA used for these competitions, the VP7 MAb 159 was used to detect the viruses; in this way we were able to distinguish between the bound viruses and the bound GST-VP8, GST-VP5, and GST proteins. To monitor the binding of the recombinant proteins in this assay, a parallel ELISA in which

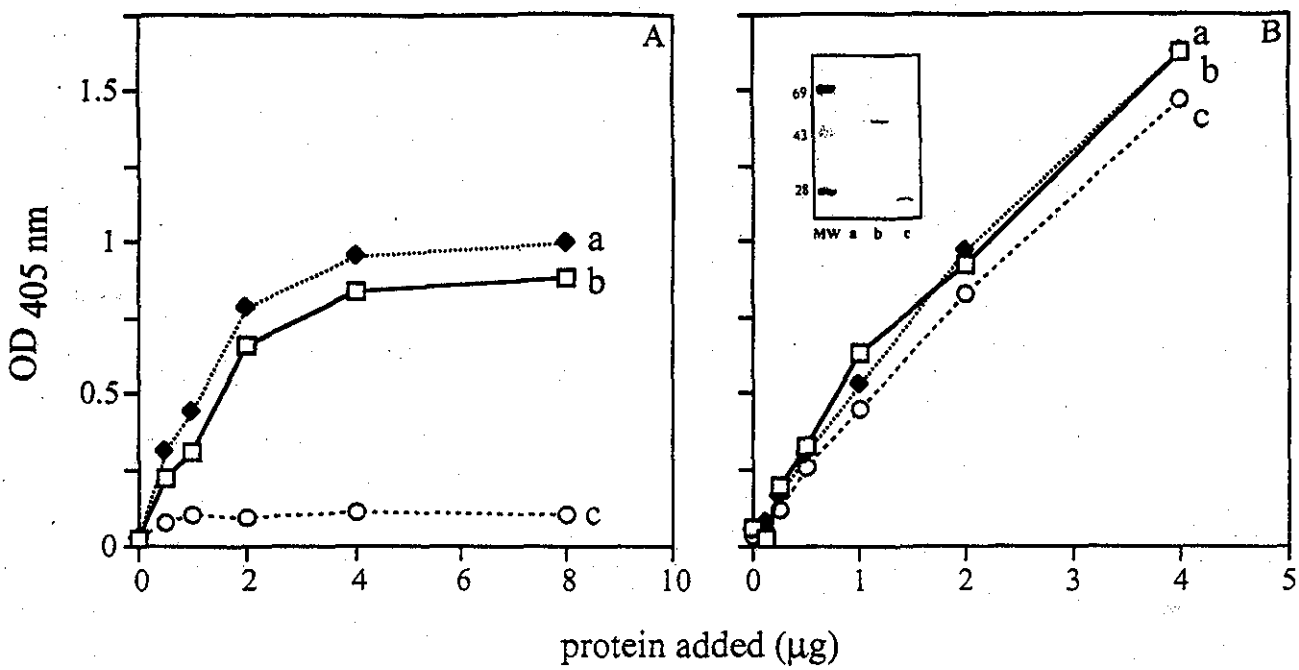

FIG. 3. Binding of the recombinant proteins GST-VP8, GST-VP5, and GST to MA104 cells. (A) The indicated amounts of affinity-purified GST-VP5 (a), GST-VP8 (b), and GST (c) were incubated with 5×10^4 MA104 cells in suspension for 1 h at 4°C. The amount of cell-bound protein was determined by an ELISA as described in Materials and Methods. The total amount of recombinant protein added to each assay mixture is plotted against the OD_{405} reading obtained in the ELISA plate. (B) OD readings obtained when the indicated amounts of recombinant proteins were directly assayed in an ELISA. The inset shows the SDS-PAGE analysis of the affinity-purified fusion proteins used in the ELISA and binding assays. MW, molecular weight in thousands.

the detection antibody was a rabbit anti-GST antibody was performed (data not shown).

The recombinant proteins were found to compete the binding of RRV and nar3 in a selective manner (Fig. 5). GST-VP8 decreased the binding of RRV by 75% compared to the binding of the virus in the absence of the recombinant protein. whereas it did not alter the attachment of the nar3 variant. Conversely, GST-VP5 was able to displace the binding of nar3 but had no effect on the binding of RRV. Preincubation of the cells with a mixture of GST-VP5 and GST-VP8 fusion proteins did not increase the inhibitory effect observed with the individual proteins. The GST protein, used as a control, did not

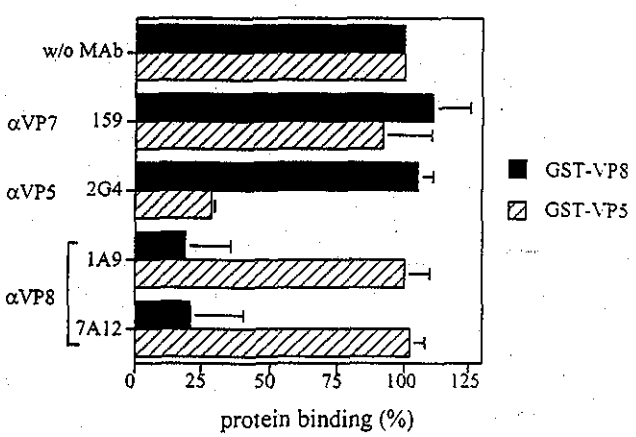

FIG. 4. Binding of the recombinant proteins GST-VP5, GST-VP8, and GST in the presence of anti-rotavirus MAbs. Affinity-purified GST-VP5, GST-VP8, or GST alone (1.5 μg of each) were preincubated for 1 h at room temperature with the indicated MAbs (75 μg/ml) in a final volume of 100 μl. After incubation with the MAbs, the fusion protein-antibody mixture was added to a suspension of MA104 cells (5×10^4 cells/binding assay) and incubated for 1 h at 4°C with gentle shaking. The amount of cell-bound protein was determined by an ELISA as described in Materials and Methods. Controls of protein binding without cells were used in each experiment (results not shown). Data are expressed as the percentage of the recombinant protein binding obtained when the fusion proteins were preincubated with PBS as a control (w/o MAb). The arithmetic means and standard deviations for two independent experiments performed in duplicate are shown. The specificity of the MAbs used is shown on the left.

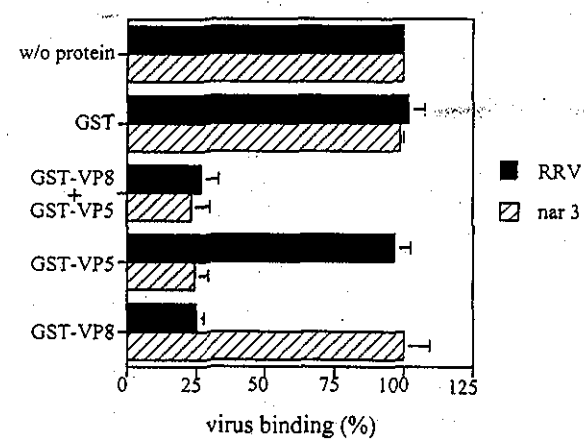

FIG. 5. Effect of the recombinant proteins GST-VP8, GST-VP5, and GST on the binding of RRV and nar3 viral particles to MA104 cells. Affinity-purified fusion proteins (1.5 μg of each) were preincubated with 5×10^4 MA104 cells in suspension for 1 h at 4°C. The excess unbound protein was removed, and then 300 ng of either RRV or nar3 viral particles was added, and the mixture was further incubated for 1 h at 4°C. The amount of virus or fusion protein bound to cells was determined by an ELISA as described in Materials and Methods. Data are expressed as the percentage of the virus binding obtained when the cells were preincubated with PBS as a control. The arithmetic means and standard deviations for two independent experiments performed in duplicate are shown. In the ELISAs, where the fusion proteins were detected, no significant differences in the binding of the recombinant proteins to the cells were found (results not shown).

modify the binding of either virus. Taken together, these results indicate that nar3 and RRV bind to MA104 cells through two different domains of VP4. The fact that the mixture of the two fusion proteins did not further decrease the binding of either virus suggests that wt RRV binds initially to the cell mainly through VP8 while nar3 does so mainly through VP5.

The infectivity of nar3 and RRV is decreased by the recombinant VP5 and VP8 proteins. Since the GST fusion proteins specifically and selectively inhibited the binding of nar3 and RRV, we evaluated how these recombinant proteins influenced the infectivity of these viruses. To do this, MA104 cells in 96-well plates were preincubated with the recombinant proteins or PBS as control and then a fixed amount of the virus was added. After 1 h of binding at 4°C, the nonbound virus was removed and the infection was left to proceed for 16 h, at which time the cells were fixed and stained for the presence of viral antigen.

The GST-VP8 protein was found to reduce the infectivity of RRV by 60%, while it did not affect that of nar3, as compared to control wells where no protein was added (taken as 100% infectivity) (Fig. 6A). When GST-VP5 was tested in this assay, the reverse was found; the infectivity of the SA-independent variant was reduced by 50% whereas that of RRV was not significantly affected. Preincubation of the cells with a mixture of the two recombinant proteins resulted in an inhibition of infectivity similar to that observed when either the GST-VP5 for nar3 or the GST-VP8 for RRV was individually tested. Preincubation of the cells with GST did not affect the infectivity of either virus. These results suggest that VP8 and VP5 block the infectivity of RRV and nar3, respectively, by blocking their binding to the cell surface.

The recombinant VP5 protein decreases the infectivity of RRV in NA-treated cells. We also characterized the effect of the recombinant proteins on the infectivity of RRV and nar3 in MA104 cells that had been treated with NA. Since the infectivity of RRV decreases up to 80% in NA-treated cells compared to that in untreated cells (31), the amount of RRV used in these experiments was increased sixfold to maintain a similar number of FFU per well (~2,000 FFU/well) under both conditions. MA104 cells in 96-well plates were treated with NA as described in Materials and Methods and then preincubated with the recombinant proteins. Under these conditions, GST-VP5 reduced the infectivity of both nar3 and RRV by 75% (Fig. 6B). The GST-VP8 protein had a less pronounced effect on RRV, reducing its infectivity to 65% of that observed in controls, whereas it did not significantly affect the infectivity of nar3 compared to the GST control protein. When a mixture of GST-VP5 and GST-VP8 was added to the cells, the infectivity of RRV was reduced to 15% while that of the SA-independent variant nar3 was reduced to about 50%, findings not significantly different from those obtained when the individual proteins were tested. Taken together, these results indicate that on cells treated or not treated with NA, the SA-independent variant binds preferentially through the VP5 domain of VP4 while RRV binds to untreated cells through VP8 and to NA-treated cells mainly through VP5.

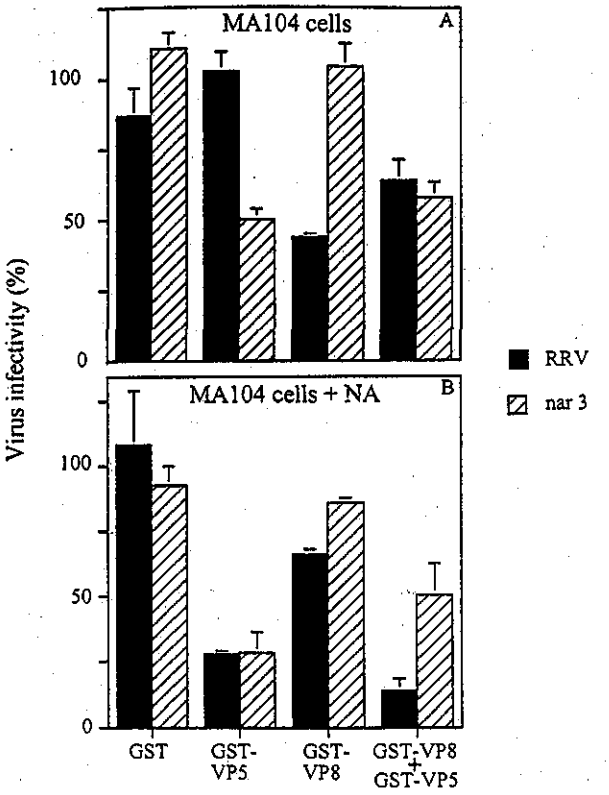

FIG. 6. Effect of the recombinant proteins GST-VP8, GST-VP5, and GST on the infectivity of RRV and nar3 viral particles. Affinity-purified fusion proteins (35 μl of a 50 ng/μl solution) were added to monolayers of MA104 cells in 96-well plates, either treated (B) or not treated (A) with NA, for 30 min at 4°C. Then, 2×10^5 FFU of RRV or nar3 (A) or 1.2×10^4 FFU of RRV, or 2×10^5 FFU of nar3 (B) was added per well, and after a 30-min adsorption period at 4°C, the inoculum was removed and the infection was left to proceed for 16 h at 37°C. At this time, the cells were fixed and immunostained as described in Materials and Methods. Data are expressed as the percentage of the virus infectivity obtained when the cells were preincubated with PBS as a control. The arithmetic means and standard deviations for three independent experiments performed in duplicate are shown.

DISCUSSION

The attachment of a virus to its cellular receptor is the first step in infection and may control the efficiency of virus entry. A detailed understanding of the molecular interactions between the viral attachment proteins and the cellular proteins involved is a prerequisite for understanding the translocation of the virus into the cytoplasm of the cell.

In this work we have studied by a direct, nonradioactive binding assay the cell attachment characteristics of the SA-dependent rotavirus RRV and of its variant nar3, which no longer requires SA to infect the cell. The binding of these viruses to NA-treated cells could not be evaluated by this kind of assay, since the treated cells in suspension tend to aggregate, giving unreliable results.

We found that these two viruses initially attach to the cell surface of untreated MA104 cells through different domains of VP4. It has been previously shown (13, 21, 26) that RRV binds to a SA-containing molecule through VP8, while here we report that the SA-independent variant binds to an asialo receptor through VP5. We have previously shown that although this variant does not need SA to infect the cells, it retains its ability to agglutinate erythrocytes in a SA-dependent manner (31). However, apparently the variant binds to a SA-independent cell surface molecule even in normal, untreated cells, since the attachment of these viruses to untreated cells is blocked by preincubation of the cells with GST-VP5 and not by preincubation with GST-VP8. In addition, MAbs to VP8, which efficiently block the RRV cell attachment, do not block nar3 binding, while the reverse was observed with the VP5 MAb 2G4. These results also suggest that the binding to SA on the

surface of erythrocytes and the surface of MA104 cells might not represent the same type of interaction.

While in VP8 the amino acids that might be in contact with the SA moiety of the receptor have been located (21), the region of VP5 that interacts with the cell surface has not been determined. The putative fusion region between amino acids 384 and 404 (29) and the integrin binding site located between amino acids 308 and 310 (4) might be good candidates to play this role.

Sequence analysis of the VP4 gene of the SA-independent variants showed that the gene product had three amino acid changes, at positions 37 (Leu-Pro), 187 (Lys-Arg), and 267 (Tyr-Cys), with respect to the parental RRV gene product (32). Recently, we reported that the new Cys at position 267 is involved in the formation of an alternate disulfide bridge with the Cys at position 318 in the VP4 of a SA-independent variant, and we proposed that this alternate disulfide bond, by altering the conformation of the VP5 protein, might allow the variant virus to directly interact with an asialo molecule in the cell membrane, superseding the initial interaction with the SA-containing receptor (6). In the assays reported here, we used a VP5 fusion protein derived from the wt virus; nevertheless, this fusion protein was able to efficiently attach to the cell surface, and block the binding and infectivity of the variant virus. These results suggest that the recombinant wt VP5 protein, when expressed out of the context of the virus structure, can adopt the proper conformation needed to interact with the SA-independent receptor on the surface of MA104 cells.

Since the recombinant GST-VP8 and GST-VP5 proteins were able to compete for the attachment of RRV and nar3, respectively, we investigated whether these fusion proteins were able to block their infectivity; we found that in untreated cells, GST-VP8 was able to decrease the infectivity of RRV while the GST-VP5 protein decreased the infectivity of nar3, consistent with our findings in the binding assays. On the other hand, in NA-treated cells, the recombinant proteins had a different effect; GST-VP5, which in untreated cells inhibited the attachment and infectivity only of nar3, was now able to reduce by 75% the residual (20% of that observed in untreated cells) infectivity of RRV (Fig. 6B). These results suggest that RRV is able to interact, through VP5, with the asialo receptor in NA-treated cells, albeit with lower efficiency than that of the variant nar3. The inhibition of GST-VP5 on the variant was consistent, although slightly more pronounced than in untreated cells. The GST-VP8 fusion protein blocked about 35% of the infectivity of RRV, indicating that the treatment of the cells with NA might leave a small amount of SA on the surface of the cells that might be resistant to this treatment and that this SA can still be used by the SA-dependent strain to attach and infect the cell. As expected, GST-VP8 did not inhibit significantly the infectivity of nar3. Taken together, these results suggest that the residual infectivity observed for SA-dependent strains in NA-treated cells (2, 31) might be the consequence of the interaction of the viruses with residual SA left on the cell surface, in addition to a direct interaction of VP5 with an asialo receptor, which would seem to be less efficient for these strains than the VP8-SA interaction.

The observation that the MAb 2G4, which binds to VP5, blocks the binding of the variant without altering the binding of wt RRV, while it is able to neutralize the infectivity of both viruses, together with the finding that GST-VP5 blocks the infectivity of both viruses in NA-treated cells, gives further support to the idea that there are at least two sequential interactions of animal rotaviruses with cell surface molecules. The first, which involves the initial binding of the virus, through the VP8 domain of VP4, to a SA-containing compound, followed by a second virus-cell interaction step which involves VP5 and a SA-independent molecule. The SA-independent variant nar3 apparently does not require the first interaction, since it is able to efficiently interact directly through VP5 with the second asialo molecule in the cell membrane (Fig. 7).

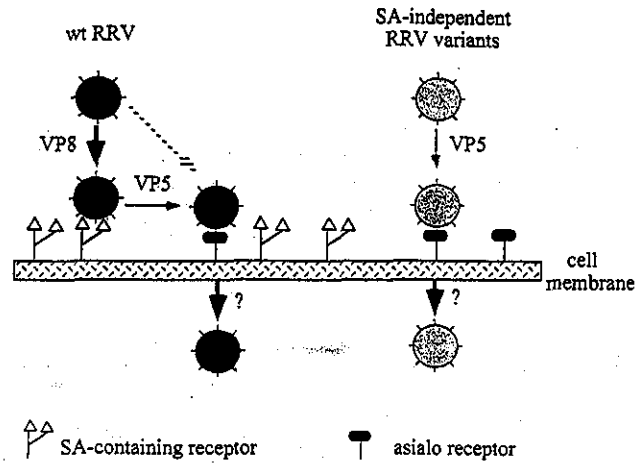

FIG. 7. Model for the early interactions of animal rotaviruses with MA104 cells. wt RRV interacts primarily with a SA-containing cell receptor through the VP8 domain of VP4 (thick arrow). After this initial interaction, which might induce a conformational change in VP4, the virus interacts with a second, SA-independent cell receptor, through the VP5 domain of VP4. This interaction might (?) facilitate the entry of the virus into the cell. A small proportion of the wt RRV virus can interact directly through VP5 with the SA-independent molecule (dashed arrow; see the text). The SA-independent variant nar3, due to the amino acid changes in its VP4 protein, interacts directly through VP5 with the asialo receptor. For the sake of clarity, the SA-containing and asialo cellular receptors are depicted in this model as two separate entities; however, they could be two domains of the same receptor molecule; this point remains to be clarified (see the text).

The results presented in this work also suggest that the two contacts of the virus with the cell surface are sequential, such that in wt RRV the initial contact with the SA-containing molecule might "facilitate" the interaction with the second, SA-independent molecule. In the variant, the amino acid changes in VP4 probably induce a conformational change in the protein, such that its VP5 domain can interact directly with the SA-independent molecule, surpassing the initial contact of VP8 with SA. Therefore, one would expect that GST-VP5, which binds to the asialo receptor, should be able to compete the secondary interaction of RRV with this molecule and thus block its infectivity. This inhibition was not observed; however, it could be explained if RRV, once attached to the cell through VP8, were able to efficiently displace the already bound recombinant VP5. This would not be the case for the nar3 variant (or for RRV in NA-treated cells), since the initial interaction of this virus is with the asialo receptor, and so when GST-VP5 is blocking this molecule, the variant cannot bind. In further support of the sequential interactions of RRV with two cell molecules is the finding that a MAb directed to the cell surface of MA104 cells, which blocks the cell binding of nar3 but not that of RRV, is able to inhibit the infectivity of both viruses (S. Lopez, R. Espinosa, P. Isa, S. Zarate, E. Mendez, and C. F. Arias, unpublished results).

Although the present data strongly support the existence of two different interactions between wt RRV and the cell surface, at this point it is not possible to establish whether two sites in the same cell surface molecule or two cell molecules are involved in the interactions with VP8 and VP5. The fact that in infection competition assays the wt and variant viruses com-

pete with each other reciprocally (33) suggests that if it is not the same cellular entity, the two cellular molecules might be in close proximity.

Recently, it was reported that a recombinant RRV-VP5 protein produced in bacteria is able to specifically permeabilize liposomes (8). The GST-VP5 protein used in our assays was not able to promote the coentry of α-sarcin into MA104 cells, which has been shown to correlate with virus entry (7, 25); thus, the two activities that have been reported for VP5 (reference 8 and this work) might represent two different functions of this protein and might reside in two different domains of this 529 amino acid polypeptide. This issue is currently under investigation.

The list of examples of viruses that have more than one interaction with the surface of the host cell is accumulating (16, 18, 34, 35), suggesting that virus attachment is a multistep process, more complex than the bimolecular virus-cell interaction previously envisioned. Multiple viral attachment proteins can bind to different cell receptors, or different binding sites in either the viral protein or the cell receptor, may act together to modulate each other or to contribute in complementary functions. The cell receptors that bind to different virus ligands might act sequentially; thus, binding of the virus to the first cellular component could cause conformational changes in the virus or in the host cell that are necessary before the second interaction can take place.

For rotaviruses, recent competition experiments with strains of human and animal origin together with the SA-independent variants suggest that there might be at least one other interaction, in addition to the two described in this work, between the virus and the cell surface (33). Further studies to characterize the cellular molecules and the viral protein domains involved in these interactions should provide insight into the highly selective tropism of rotaviruses.

ACKNOWLEDGMENTS

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2. La integrina α2β1 es el receptor inicial del virus nar3.

Como mencionamos anteriormente, encontramos que la variante nar3 se une a la superficie de la célula a través del dominio VP5 de la proteína VP4. Para definir cual es la región de VP5 responsable de la unión de nar3 a la célula, e identificar a la molécula celular que utiliza el virus nar3 como receptor inicial, realizamos el siguiente trabajo, cuyos resultados fueron publicados en el artículo, "Integrin α2β1 mediates the cell attachment of the rotavirus neuraminidase-resistant variant nar3"; las figuras mencionadas en el texto a continuación se encuentran en el artículo anexo.

Como se mencionó en la introducción, previamente habían sido descritas dos regiones de VP5 que podrían estar involucradas en los primeros contactos del virus con la célula huésped, una de ellas es una región hidrofóbica que es similar a los péptidos de fusión de la familia de los alphavirus (53), y la otra el tripéptido DGE que puede funcionar como motivo de unión a integrinas de tipo α2β1 (8).

Anteriormente habíamos encontrado que la proteína VP5, expresada como fusión a GST, es capaz de competir con nar3 por la unión a su receptor (ver fig. 6, art. 1). Para determinar si la región DGE o la región hidrofóbica estaban involucradas en la unión mediada por VP5, construimos dos proteínas VP5 mutantes. En una de ellas cambiamos el aspartato 308 por alanina ($VP5^{D308A}$), ya que se ha reportado que el cambio del tripéptido DGE por AGE elimina la capacidad de los ligandos para unirse a la integrina α2β1 (72). En la otra construcción cambiamos la glicina 400 por aspartato ($VP5^{G400D}$), pues en alfavirus se ha visto que un cambio equivalente dentro del péptido de fusión elimina por completo la actividad de fusión de estos virus (44). Las proteínas recombinantes se produjeron en E. coli, como fusiones a GST y se purificaron por cromatografía de afinidad. Posteriormente evaluamos la capacidad de estas proteínas para unirse a la superficie de la células y de competir la unión del virus nar3 en comparación con la proteína VP5 silvestre.

Encontramos que ambas proteínas mutantes se unen a la célula de manera similar a como lo hace la proteína silvestre (datos no mostrados). También encontramos que la proteína

mutada en la región hidrofóbica ($VP5^{G400D}$) compite la unión del virus nar3 al mismo nivel que lo hace la proteína silvestre, mientras que la proteína mutada en el dominio de interacción con la integrina $\alpha2\beta1$ ($VP5^{D308A}$) no es capaz de competir esta unión (Fig. 2, art. 2); esto indica que la proteína $VP5^{D308A}$ no es capaz de unirse al receptor de nar3 y por lo tanto este virus se une a su receptor aún en presencia de esta proteína mutante. Sin embargo la proteína $VP5^{D308A}$ se une a la superficie celular, indicando la posible presencia de otro dominio de unión a la célula, presente en VP5.

Anteriormente se había reportado que las integrinas $\alpha2\beta1$, $\alpha4\beta1$ y $\alpha x\beta2$ estan involucradas en la infectividad de los rotavirus (8, 33), por lo que probamos el efecto de anticuerpos dirigidos contra diferentes subunidades de las integrinas en la infectividad de los rotavirus RRV y nar3. Encontramos que los anticuerpos dirigidos contra las integrinas $\alpha2$, $\beta2$ y $\alpha4$ disminuyen entre 25 y 40 % la infectividad tanto de nar3 como de RRV (Fig. 1A, art. 2); a pesar de que el bloqueo observado es parcial, estos datos son consistentes con los que habían sido reportados previamente, indicando que el proceso de unión y entrada de los rotavirus involucra múltiples interacciones y la posibilidad de que algunos virus puedan utilizar rutas alternativas en este proceso.

Posteriormente, decidimos probar si los AcM dirigidos contra las diferentes integrinas tienen algún efecto sobre la unión de los virus RRV y nar3. Como se muestra en la figura 1B del artículo 2, los anticuerpos dirigidos contra las subunidades de integrinas $\beta2$ y $\alpha4$ no tienen ningún efecto sobre la unión de ninguno de los dos virus, mientras que el anticuerpo dirigido contra la subunidad $\alpha2$ disminuye en un 50 % la unión de nar3 sin afectar la unión de RRV. Estos datos sugirieron que la integrina $\alpha2\beta1$ es el receptor inicial de la variante nar3, y que esta molécula celular es utilizada por el virus RRV en un paso posterior a la unión a la célula.

Finalmente, para confirmar estos datos utilizamos péptidos sintéticos que representan diferentes regiones de VP5 para evaluar su efecto sobre la infectividad y la unión de los rotavirus RRV y nar3. Se utilizaron tres péptidos, uno que llamamos DGE, comprende la posible región de interacción con la integrina $\alpha2\beta1$, el segundo, llamado FP, contiene la

región hidrofóbica postulada como péptido de fusión, y un péptido que llamamos COOH que representa una región en el extremo carboxilo de la proteína VP5 (ver tabla I y figura 3B de la tesis). Como se muestra en la figura 3A del artículo 2, la infectividad, tanto de nar3 como de RRV, comparada con la obtenida en ausencia de péptido, se ve afectada entre un 45 a un 70 % por los péptidos DGE y FP, indicándo que estas regiones podrían ser importantes en los primeros eventos de la infección por rotavirus. Por otro lado, el péptido COOH no tiene ningún efecto sobre la infectividad de estos virus.

Ya que los péptidos sintéticos DGE y FP son capaces de inhibir la infectividad de los rotavirus RRV y nar3, probamos su efecto sobre la unión de los virus a la superficie celular. En la figura 3B del artículo 2, podemos observar que ninguno de los péptidos afectó la unión del virus RRV, lo que está de acuerdo con el hecho que este virus se une inicialmente a una mólecula que contiene AS, a través del polipéptido VP8. Por otro lado encontramos que el péptido DGE bloquea la unión del virus nar3 en un 60 %, mientras que el péptido FP tuvo un efecto marginal y el péptido COOH no tuvo ningún efecto.

Los resultados anteriores nos permitieron identificar a la integrina $\alpha 2\beta 1$ como el receptor inicial del rotavirus nar3, el cuál se une a ella a través del dominio DGE de la proteína VP5 (79).

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RAPID COMMUNICATION

Integrin $\alpha2\beta1$ Mediates the Cell Attachment of the Rotavirus Neuraminidase-Resistant Variant nar3

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It was previously reported that integrins $\alpha2\beta1$, $\alpha4\beta1$, and $\alpha X\beta2$ are involved in rotavirus cell infection. In this work we studied the role of integrin subunits $\alpha2$, $\alpha4$, and $\beta2$ on the attachment of rotaviruses RRV and nar3 to MA104 cells. Integrin $\alpha2\beta1$ was found to serve as the binding receptor for the neuraminidase-resistant virus nar3, whereas the neuraminidase-sensitive strain RRV interacted with this integrin at a postattachment step. It was shown that nar3 binds $\alpha2\beta1$ through the DGE integrin-recognition motif located in the virus surface protein VP5. Integrin subunits $\alpha4$ and $\beta2$ do not seem to be involved in the initial cell binding of either virus.

Rotaviruses have a genome of 11 segments of double-tranded RNA contained in a triple-layered protein cap-id. The smooth external surface of the virus is made of imers of the glycoprotein VP7, while 60 spike-like struc-ures, formed by dimers of VP4, protrude from this sur-ace (*6*). VP4 has essential functions during the early nteractions of the virus with the cell; it is used as the irus attachment protein (*12, 17*), and it is also important or virus entry, since for the virus to penetrate into the ell's cytoplasm VP4 has to be cleaved by trypsin into vo subunits, VP5 and VP8 (*2*).

The cell attachment of some rotavirus strains isolated om animals (other than humans), including rhesus ro-virus RRV, is greatly diminished by treatment of cells ith neuraminidase (NA), indicating the need for sialic cid (SA) on the cell surface. However, the interaction of ese strains with a SA-containing receptor does not eem to be essential, since variants which no longer eed SA to infect the cells have been isolated from RRV nd from the simian rotavirus strain SA11 (*3, 12, 14*).

The binding of RRV to sialic acids has been shown to e mediated by VP8, and the domain of the protein volved in this binding was previously described (*7, 10*). n the other hand, the NA-resistant RRV variant nar3 was found to attach to cells through the VP5 trypsin cleavage product of VP4 (*17*), although the region of VP5 responsible for this interaction and the cell molecule that functions as receptor for the virus have not yet been determined.

Recently, it was reported that the VP5 subunit of VP4 contains the tripeptide-sequence motifs DGE and IDA, which are known to interact with integrins $\alpha2\beta1$ and $\alpha4\beta1$, respectively, while VP7 contains the $\alpha X\beta2$ integrin ligand site GPR, and the $\alpha4\beta1$-binding motif LDV (*4, 9*). It was also shown that antibodies to integrin subunits $\alpha2$, $\alpha4$, and $\beta2$, as well as peptides that mimic the ligand sites for these integrins, blocked the infectivity of the NA-sensitive simian rotavirus strain SA11 and of the NA-resistant human rotavirus strain RV5. We recently described that antibodies to these three integrin subunits, and to integrin $\alpha v\beta3$, are able to block the infectivity of rotaviruses RRV and nar3, as well as that of human rotavirus strain Wa, and we showed that integrin $\alpha v\beta3$ interacts with these three rotavirus strains at a postattachment step (*8*). In this work we studied the role of integrins $\alpha2$, $\alpha4$, and $\beta2$ on the cell attachment of rotaviruses RRV and nar3. We found that integrin $\alpha2\beta1$ serves as the attachment receptor for the NA-resistant rotavirus strain nar3, through the interaction with the DGE motif present in the VP5 protein of the virus. This integrin was found to interact with RRV at a postattachment step, similar to integrin subunits $\alpha4$ and $\beta2$, which did not function as primary binding sites for these two viruses.

The infectivity and cell-binding blocking assays employed in this work were carried out essentially as de-

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scribed (*11*). For the infectivity assay, MA104 cells in 96-well plates were preincubated with MAbs P1E6 to $\alpha 2$, MHM23 to $\beta 2$, P4G9 to $\alpha 4$ (Dako, Carpinteria, CA), or FB12 to $\alpha 1$ (Chemicon, Temecula, CA), for 1 h at 37°C and the cells were then infected with 2000 focus-forming units (FFUs) of either RRV or nar3 viruses. After a virus adsorption period of 1 h at 37°C, the cells were washed and the infection was allowed to proceed for 14 h, after which the cells were fixed and the virus titer was determined by an immunoperoxidase focus assay (*1*). For the cell-binding blocking assay, MA104 cells in suspension were preincubated with the MAbs to the different integrins for 1 h at 4°C, and after the excess antibody was removed, 300 ng of CsCl-purified viruses nar3 or RRV was added to the cells. After an incubation period of 1 h at 4°C the cells were washed, and the cell-bound virus was determined by ELISA (*17*).

As previously observed (*8*), the infectivity of RRV and that of its variant nar3 were found to decrease 25 to 45% when the cells were incubated with MAbs to integrins $\alpha 2$, $\alpha 4$, and $\beta 2$, while they were not affected by MAb FB12, directed against integrin subunit $\alpha 1$ (Fig. 1A). This level of inhibition is in accordance with that found by Coulson *et al.* (*4*) and Hewish *et al.* (*9*) for SA11 and RV5 *rotaviruses*.

When the blocking activity of the integrin antibodies on the binding of the viruses was analyzed, a differential effect on RRV and nar3 was observed. Although the binding of RRV was not affected by any of the antibodies tested, MAb P1E6 to integrin subunit $\alpha 2$ reduced the cell attachment of nar3 by about 40%. The MAbs to the other integrin subunits did not modify the binding of nar3 (Fig. 1B).

These results suggest that the MAb to integrin $\alpha 2$ blocks the infectivity of RRV and nar3 through different mechanisms. It seems to inhibit the infectivity of nar3 by preventing its attachment to the cell surface, while RRV seems to be blocked at a postbinding step. Antibodies to $\alpha 4$ and $\beta 2$ integrins apparently inhibit a postattachment interaction of both viruses.

We recently reported that the NA-resistant variant nar3 binds to the surface of MA104 cells through the VP5 domain of VP4 (*17*). Since VP5 contains the tripeptide-recognition motif DGE for integrin $\alpha 2\beta 1$, and a MAb to the $\alpha 2$ integrin subunit blocks the binding of nar3 (Fig. 1B), we hypothesized that this virus binds to cells through the DGE motif, located at VP5 amino acid residues 308–310. To demonstrate the role of DGE in the binding of VP5 to cells, we used a recombinant RRV GST-VP5 fusion protein produced in bacteria, which was previously shown to bind specifically to MA104 cells (*17*). In addition, two modified versions of this protein were constructed by site-directed mutagenesis. In one, the aspartic acid residue in the triplet DGE was replaced by an alanine residue (GST-VP5^{D308A}). In the second, we altered the sequence of the putative fusion peptide

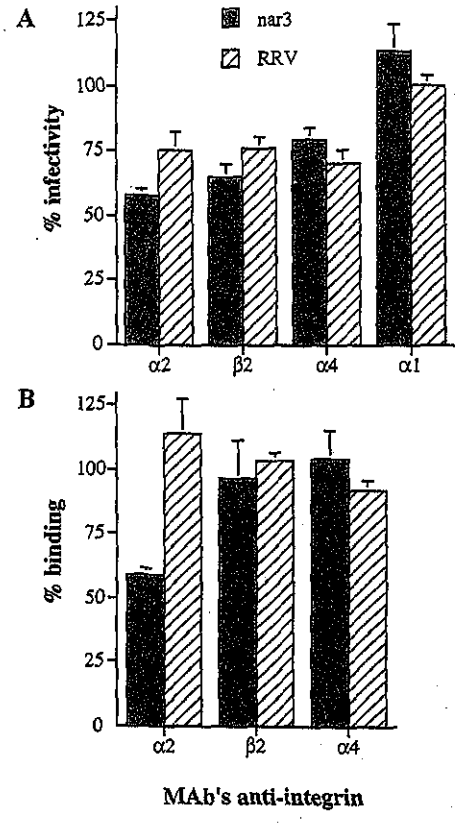

FIG. 1. Effect of MAbs to different integrin subunits on the infectivity and binding of RRV and nar3 viruses. (A) MA104 cells in 96-well plates were preincubated for 1 h at 37°C, with MAbs to integrin subunits $\alpha 2$, $\beta 2$, $\alpha 4$, or $\alpha 1$; after incubation with antibody the cells were washed twice with MEM, and then RRV or nar3 viruses (2000 FFUs per well) were adsorbed for 60 min at 4°C; after the adsorption period the inoculum was removed and the infection was allowed to proceed for 14 h at 37°C, after which the cells were fixed and immunostained. Data are expressed as the percentage of the virus infectivity obtained when the cells were preincubated with MEM as control. (B) MA104 cells in suspension (5×10^4 cells/assay) were preincubated with MAbs to integrins $\alpha 2$, $\beta 2$, and $\alpha 4$ for 1 h at 4°C. The excess, unbound antibody was removed, and then 300 ng of either RRV or nar3 purified virus particles was added, and the mixture was further incubated for 1 h at 4°C. The amount of virus bound to cells was determined by an ELISA as described (*17*). Data are expressed as the percentage of the virus binding obtained when the cells were preincubated with PBS as a control. The bars represent the standard error of at least three independent experiments performed in duplicate. The antibodies used were MAb P1E6 (3.2 μg/ml) to $\alpha 2$, MHM23 (41 μg/ml) to $\beta 2$, P4G9 (8.1 μg/ml) to $\alpha 4$, and FB12 (10 μg/ml) to $\alpha 1$.

present in VP5 (*13*); the glycine residue at amino acid position 400 was replaced by an aspartic acid, to yield the mutant protein GST-VP5^{G400D}.

Wild-type (wt) GST-VP5 and the mutant proteins were expressed in bacteria and affinity-purified essentially as described by Isa *et al.* (*10*). To assay the ability of these polypeptides to bind to the cell surface, different amounts of each fusion protein were incubated with MA104 cells for 1 h at 4°C, and the protein bound to cells was determined by ELISA (*17*). The inset to Fig. 2 shows that the binding to the cell surface of both mutant pro-

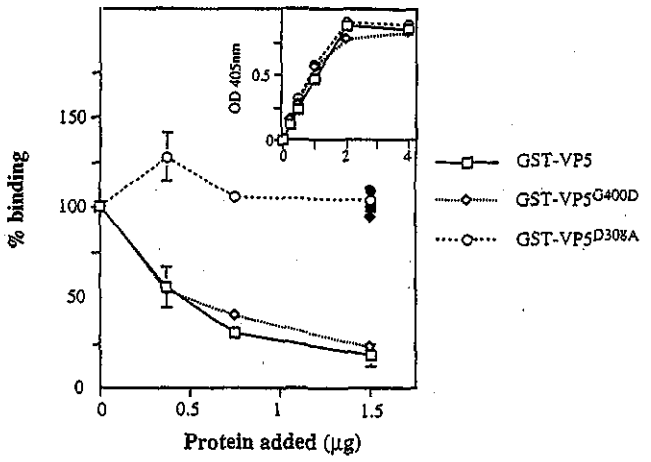

FIG. 2. Effect of recombinant proteins GST-VP5, GST-VP5^{G400D}, and GST-VP5^{D308A} on the binding of RRV and nar3 viral particles to MA104 cells. The indicated amounts of affinity-purified fusion proteins were preincubated with 5 × 10^4 MA104 cells in suspension for 1 h at 4°C. After the excess protein was removed, 300 ng of either RRV (closed symbols) or nar3 (open symbols) purified viral particles was added, and the mixture was further incubated for 1 h at 4°C. The amount of virus or fusion proteins that remained bound to the cells was determined by an ELISA. The total amount of recombinant protein added to each assay is plotted against the OD_{405} readings obtained in the ELISA plate (shown in the inset). The amount of cell-bound virus particles is expressed as the percentage of the virus binding obtained when the cells were preincubated with PBS as a control. The arithmetic means ± standard error from two independent experiments performed in duplicate are shown. For RRV virus only the highest amount of each recombinant protein was tested.

teins was very similar to that found for wt GST-VP5, indicating that the changes introduced did not impair the ability of the mutagenized polypeptides to attach to MA104 cells.

We previously described that the wt GST-VP5 protein is able to efficiently compete the binding of nar3, but not that of RRV (*17*); thus, we assayed the ability of both mutant proteins to compete the binding of RRV and nar3. For this, purified viruses were added to MA104 cells, which were previously incubated with different amounts of each recombinant protein for 1 h at 4°C. The virus that remained bound to the cells was determined as described above. While the GST-VP5^{G400D} protein competed the binding of nar3 as efficiently as did wt GST-VP5, the GST-VP5^{D308A} polypeptide did not affect the attachment of the virus at any of the concentrations used (Fig. 2). As expected, the attachment of RRV was not affected by any of the recombinant proteins tested (Fig. 2, shown only for the highest concentration of the proteins). These results suggest that binding through the DGE integrin motif is required for the GST-VP5 protein to compete the attachment of the variant nar3. On the other hand, the fusion peptide region of VP5 does not seem to be important for the binding of this virus, since the mutant GST-VP5^{G400D} retained its ability to block the attachment of the virus. The fact that the GST-VP5^{D308A} recombinant protein was not able to compete the binding of nar3, even though it binds to the cell surface as efficiently as wt GST-VP5, suggests the existence of an additional attachment site in VP5, independent of the DGE motif, and also probably independent of the integrin-recognition motif IDA present at residues 538–540 of VP5 (*4*), since the MAb to $\alpha 4$ did not affect the binding of nar3 (Fig. 1B). The possibility that GST-VP5^{D308A} might bind to the cell surface in a nonspecific manner, however, cannot be ruled out.

We also studied the effect of synthetic peptides that mimic different regions of VP5 on the infectivity and binding of nar3 and RRV. The peptides tested comprised amino acids 297 to 318, which contain the $\alpha 2$ integrin-binding site (peptide DGE); amino acids 380 to 401, which contain the putative fusion region of the protein (peptide FP); or amino acids 738 to 754 from the carboxy-terminal region of VP5 (peptide COOH). The ability of these peptides to block the infectivity and binding of the viruses was tested by preincubation of MA104 cells with 8 μg/ml of each peptide before addition of the virus. As shown in Fig. 3, peptide DGE was found to block the infectivity of both RRV and nar3 by about 40 and 70%, respectively, although it inhibited the binding only of the latter virus. Peptide FP also blocked the infectivity of both viruses, although to a lesser extent than did peptide DGE, and it did not affect, or only slightly affected, the binding of nar3 and RRV. Peptide COOH, which was used as a negative control, did not affect either the infectivity or the binding of the viruses. These results are consistent with the idea that the DGE motif present in VP5 is relevant for the initial binding of the nar3 variant, while it is not used by wt RRV to initially attach to the cells. The block in the infectivity of RRV caused by peptide DGE is again, most probably, at a postattachment step.

The inhibitory effect that peptide FP has on the viral infectivity of both RRV and nar3 viruses could be the result of a direct interaction of the peptide with the cell membrane, which in turn interferes with the virus infection at some point after the initial binding of the viruses to the cell surface. Recently, Dowling *et al.* (*5*) showed that mutations in this region of VP5 abrogate the membrane permeabilization activity of the protein. Our observation that a peptide that mimics the fusion peptide of VP5 is able to block the infectivity of rotaviruses at a postbinding step is in accordance with their findings.

Taken together, these results indicate that the NA-resistant variant nar3 binds to the surface of MA104 cells by interacting with the $\alpha 2\beta 1$ integrin through the DGE motif of VP5, while RRV interacts with this integrin after its initial binding to the cell surface with an as yet undetermined SA-containing receptor.

Recently, Hewish *et al.* (*9*) found that K562 cells, transfected with the genes for either $\alpha 2$ or $\alpha 4$ integrin subunits, bound more rotavirus SA11 than did untransfected cells, and this increased binding was blocked by antibodies to $\alpha 2$ and $\alpha 4$ integrins. In that work, an indirect

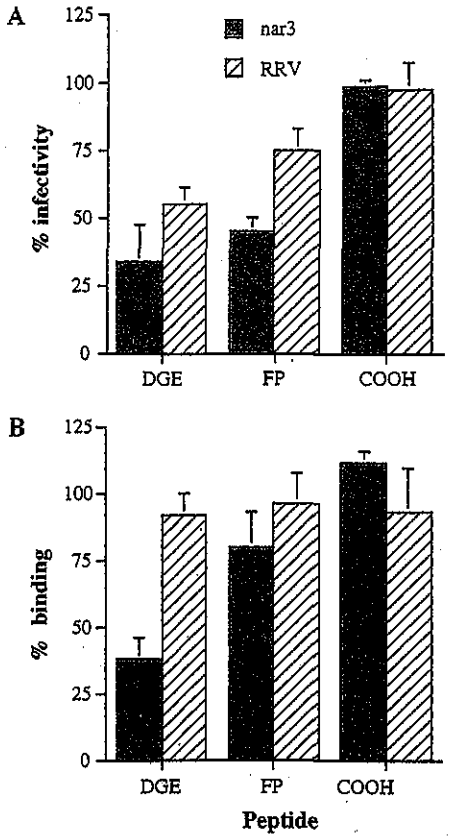

FIG. 3. Effect of synthetic peptides on the infectivity and binding of RRV and nar3 viruses. (A) MA104 cells in 96-well plates were preincubated for 1 h at 37°C with 8 μg/ml of each peptide, and then RRV or nar3 viruses (2000 FFUs per well) were adsorbed for 1 h at 4°C; after the adsorption period the inoculum was removed and the infection was allowed to proceed for 14 h at 37°C, after which the cells were fixed and immunostained. Data are expressed as percentage of the virus infectivity obtained when the cells were preincubated with MEM as control. The bars represent the standard error of two independent experiments performed in duplicate. (B) MA104 cells in suspension (5×10^4 cells per assay) were preincubated with 4 μg/ml of each peptide for 1 h at 4°C. The excess peptide was removed, the cells were washed, and 300 ng of either RRV or nar3 viral particles was added. The mixture was incubated for 1 h at 4°C and the amount of virus bound to the cell was determined by an ELISA. Data are expressed as the percentage of the virus bound to the cells when they were preincubated with PBS as a control. The bars represent the standard error of two independent experiments performed in duplicate.

binding assay, in which the K562-cell-bound virus was recovered by freeze–thawing of the cells and subsequently titered in MA104 cells, was used. In contrast to those results we found that antibodies to either α2 or α4 were not able to prevent the binding of the simian rotavirus RRV, which shares with SA11 not only the VP7 serotype but also its dependence on SA to infect cells. This discrepancy in the role of integrin subunits α2 and α4 as attachment receptors for SA-dependent viruses might be explained by the different binding assays used, the different cell lines employed, or the different rotavirus strains characterized.

We recently described that the binding of both the variant nar3 and the wt GST-VP5 protein is partially inhibited by monoclonal antibody 2D9, which is directed to an as yet unknown molecule on the surface of MA104 cells (*11*). Even though the behavior of MAb 2D9 is similar to that of the α2 integrin antibody, 2D9 is probably not directed to this integrin, since its pattern of staining of mouse small intestinal cells is quite different from that obtained with a MAb to α2 (unpublished data). Nevertheless, the cell structure recognized by 2D9 must be in close proximity to integrin α2β1 on the surface of MA104 cells, since MAb 2D9 displaces the binding of antibodies to α2 by flow cytometry (Isa *et al.*, unpublished results). Thus, the antigen recognized by 2D9 might serve as an alternative cell receptor for the variant nar3, since cells that lack α2β1 but are 2D9-positive, like L or CHO cells (*11*), can be infected by this virus, albeit with much lower efficiency.

We proposed a model for the early interactions of animal rotaviruses with MA104 cells, in which we suggested that RRV initially binds to an as yet unidentified SA-containing compound in the cell membrane through the VP8 domain of VP4. Subsequent to this initial interaction we proposed that the virus interacts with a second, SA-independent, cell receptor through the VP5 domain of VP4, and we also hypothesized that the NA-resistant variant nar3 interacts directly through VP5 with the second receptor (*17*). In this work we identified this second receptor as integrin α2β1.

The results reported here further support our hypothesis that the interaction of rotaviruses with their host cell is a multistep process in which sequential contacts of the viral capsid with the cell surface take place (*15*). These multiple interactions of viruses with cell receptors have been observed for different viruses, such as HIV, adenovirus, measles virus, and herpes simplex virus (*16*). In the case of rotaviruses, how many more contacts the virus has with the cell surface before it reaches the cell's cytoplasm and which cellular and viral partners are involved in these interactions remain to be determined.

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3. El extremo carboxilo de VP5 media la interacción de los rotavirus con la proteína de choque térmico hsc70

Ahora bien, durante la caracterización del dominio de unión del virus nar3 a la superficie celular encontramos que a pesar de que la proteína mutante GST-VP5^{D308A} es incapaz de bloquear la unión del virus nar3 a la célula, sigue uniéndose a la superficie celular; en este trabajo nos propusimos determinar si esta otra interacción de la proteína VP5 con la superficie de la célula es específica, e identificar al receptor. Los resultados de este trabajo se encuentran en el artículo "Molecular characterization of rotavirus interaction with its receptor hsc70", que ha sido sometido al Journal of Virology, para su publicación. Las figuras mencionadas en el texto a continuación se encuentran en el manuscrito anexo.

Como una estrategia para identificar otros dominios de VP5 que estuvieran involucrados en las interacciones iniciales del virus con su célula huésped, se mandaron sintetizar químicamente péptidos que representan distintas regiones de VP5 y se probó el efecto de estos péptidos en la infectividad de los rotavirus nar3 y RRV. En la figura 1 del artículo 3, se muestra la distribución de estos péptidos en la secuencia de VP5 (numerados del 2 al 7). En esta caracterización también se incluyó al péptido DGE, como control, cuyos efectos sobre la infectividad y la unión ya han sido caracterizados previamente (79). Encontramos que los péptidos denominados 4, 5 y DGE fueron capaces de bloquear la infectividad de los rotavirus RRV y nar3 (Fig. 2A, art. 3), indicando que la región comprendida entre los aa 594-670 podría estar involucrada en la entrada de los rotavirus a su célula huésped. Además, los péptidos 3 y 6 dis,inuyen la infectividad de nar3, pero no la de RRV. Cuando probamos el efecto de estos péptidos sobre la unión de RRV y nar3, encontramos que ninguno de los péptidos 4, 5, ó 6, afectaron la unión de estos virus (fig 2B, art. 3); lo que es consistente con nuestros resultados previos, pues habíamos encontrado que RRV se une a la célula a través de VP8 y que la variante nar3 se une a través de la región en DGE presente en la proteína VP5. En este trabajo cambiamos el protocolo de los ensayos de

unión; en vez de utilizar células en suspensión, los ensayos se hicieron directamente sobre la monocapa de células, ya que el método es más sencillo y nos permite hacer más experimentos simultaneamente; este protocolo está descrito en Materiales y Métodos.

Considerando que los péptidos sintéticos 4, 5, y 6 bloquearon la infectividad de los rotavirus, pero no su unión, evaluamos el efecto de estos péptidos, así como el del péptido DGE, en la unión, tanto de la proteína GST-VP5 silvestre, como en la unión de la mutante GST-VP5^{D308A} a la superficie celular. En la figura 3A del artículo 3, se muestra que el péptido DGE bloqueó la unión de la proteína GST-VP5, pero no la unión de GST-VP5^{D308A}; por otro lado, el péptido 5 fue capaz de bloquear la unión de ambas proteínas, lo que sugiere que en el extremo carboxilo terminal de VP5 se encuentra un dominio de unión a la superficie de la célula, que participa en un paso posterior a la unión inicial de los virus RRV y nar3.

Para caracterizar este dominio de unión a la célula, utilizamos dos AcM dirigidos contra la proteína VP5: el anticuerpo 2G4 es un AcM neutralizante de la infección viral, que reacciona en una región alrededor del aminoácido 393 de VP5, y el AcM HS2, que es un anticuerpo no neutralizante, que reconoce a la proteína VP5 en la región alrededor de los aminoácidos 540 al 593. Al evaluar el efecto de estos AcM en la unión de las proteínas GST-VP5 y GST-VP5^{D308A} a la superficie de la célula, encontramos que el AcM 2G4 fue capaz de bloquear la unión de la proteína silvestre, pero no la unión de la mutante, mientras que el AcM HS2 inhibió la unión de ambas proteínas (Fig. 3B, art. 3). Además, la mezcla de estos AcM tuvo un efecto ligeramente mayor sobre la unión de la proteína VP5 silvestre, indicando que esta proteína es capaz de utilizar ambos dominios para unirse a la superficie de la célula. Por otro lado, en el caso de la proteína mutante, la mezcla de anticuerpos da el mismo nivel de inhibición que el observado para el AcM HS2, esto confirma que el AcM 2G4 no tiene ningún efecto sobre la unión de esta proteína. Estos resultados confirmaron que la proteína VP5 posee dos dominios de unión a la superficie celular.

Para estudiar si estos dominios de unión a la célula, presentes en VP5, son independientes, se hicieron dos construcciones de VP5. La construcción NH_2-VP5 contiene la región amino de VP5 (aa 248-474), donde se encuentran el dominio DGE (de unión a la integrina $\alpha2\beta1$) y el sitio de reconocimiento del AcM 2G4. La otra construcción, llamada VP5-COOH (aa 474-776), comprende el sitio de reconocimiento del AcM HS2 y la región representada por los péptidos 4, 5 y 6 (Fig. 3C, pag. 17); ambas construcciones tienen seis histidinas en el extremo COOH terminal, lo que facilita su purificación mediante cromatografía de afinidad. En ensayos de unión encontramos que tanto la proteína NH_2-VP5, como la VP5-COOH fueron capaces de unirse a la superficie de la célula (datos no mostrados) y que el péptido DGE bloqueó solamente la unión de la proteína NH_2-VP5, mientras que el péptido 5 inhibió únicamente la unión de VP5-COOH (Fig. 4A, art. 3), el péptido S5, un control con la misma composición de aminoácidos que el péptido 5, pero con diferente secuencia, no afectó la unión de ninguna de las dos proteínas, lo que sugiere que las regiones amino y carboxilo de VP5 se unen de manera específica a dos moléculas distintas en la superficie de la célula. Además en ensayos de competencia encontramos que las proteínas NH_2-VP5 y VP5-COOH no compiten entre sí por la unión a la superficie de la célula (datos no mostrados), lo que confirma la independencia de estos dos dominios de unión presentes en la proteína VP5.

Como ya se ha mencionado, existen varias moléculas en la superficie de la célula que han sido propuestas como posibles receptores para los rotavirus. Para tratar de identificar la molécula celular con la cual interacciona el extremo carboxilo de VP5, se utilizaron anticuerpos dirigidos contra las integrinas $\alpha2\beta1$ y $\alpha4\beta1$, dado que en VP5 se encuentran los tripéptidos DGE e IDA que son capaces de mediar la interacción de ligandos con estas integrinas (59, 72); también se utilizó un anticuerpo dirigido contra la proteína de choque térmico hsc70, que interacciona con los rotavirus en un paso posterior a la unión inicial de éstos a la superficie de la célula (28).

En estos ensayos encontramos que el AcM dirigido contra la integrina $\alpha4\beta1$ no afectó la unión de ninguna de las dos proteínas recombinantes; el AcM anti $\alpha2\beta1$ integrina bloqueó la unión de la proteína NH_2-VP5 como era de esperarse (ver capítulo 2 de Resultados), pero no afectó la unión de la proteína VP5-COOH. Encontramos que el anticuerpo dirigido contra la proteína de choque térmico hsc70 fue capaz de bloquear específicamente la unión de la proteína VP5-COOH, sugiriendo que la interacción de los rotavirus con la proteína hsc70 podría ser mediada por el dominio carboxilo terminal de VP5 (figura 4B, art. 3).

En estudios previos habíamos encontrado que los rotavirus son capaces de unirse de manera específica a la proteína hsc70 inmovilizada en placas de ELISA (28). Utilizamos este mismo tipo de ensayo para determinar si las proteínas NH_2-VP5, VP5-COOH y la proteína VP5his completa son capaces de unirse a la proteína hsc70 recombinante. Encontramos que mientras la proteína VP5his y la proteína VP5-COOH se unieron a hsc70 de manera saturable, la proteína NH_2-VP5 no se unió a hsc70 (datos no mostrados); lo que sugiere que la proteína VP5 es capaz de unirse a hsc70 y que probablemente la región que media esta interacción se encuentra en la región carboxilo terminal de la proteína.

Dado que las proteínas VP5 y VP5-COOH se unen a la proteína hsc70, probamos si éstas son capaces de bloquear la unión del virus a la proteína hsc70. Como se puede observar en la figura 5 del artículo 3, las proteínas VP5-COOH y VP5his bloquearon la unión del rotavirus RRV a hsc70, mientras que la proteína NH_2-VP5 no tuvo ningún efecto sobre la unión del virus a la placa de ELISA. Estos datos sugieren que el dominio de unión a hsc70 de los rotavirus se encuentra en la proteína VP5. Además, encontramos que el péptido 5 fue capaz de bloquear la unión del virus RRV y de las proteínas VP5his y VP5-COOH a la proteína hsc70, mientras que el péptido S5, utilizado como control, no tuvo ningún efecto sobre esta interacción (Fig. 6, art. 3). Estos datos indican que la región contenida en el péptido 5 es la responsable de la interacción entre hsc70 y los rotavirus.

En conjunto, los resultados obtenidos en este trabajo nos permitieron confirmar que existen al menos dos sitios de unión en la proteína VP5 de los rotavirus, el primero corresponde al motivo DGE responsable de la interacción de los rotavirus con la integrina $\alpha 2\beta 1$ (79), y el segundo sitio, que encontramos en este trabajo, se encuentra ubicado hacia el extremo carboxilo de la proteína, e interacciona con la proteína hsc70; pensamos que muy probablemente esta segunda interacción ono está relacionada con la unión inicial de los rotavirus a su célula huésped, sino que ocurre en un momento posterior y es importante para la ifectividad de estos virus.

Molecular Characterisation of Rotavirus Interaction with its Receptor Hsc70.

Running title: Rotaviruses' hsc70 binding site

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Abstract

Rotaviruses are the leading cause of severe dehydrating gastroenteritis in children under three years of age. These viruses selectively infect the mature enterocytes located on the tip of intestinal villi. Rotavirus infection seems to be a multistep process involving at least two viral proteins and three cellular receptors which include gangliosides, integrins, and hsc70, a constitutive member of the heat shock-induced hsp70 protein family. We have shown that the virus particle interacts with this chaperone on the cell membrane, at a post-binding step, probably penetration. The blocking of this interaction inhibits viral infection. Using an in vitro enzyme immunoassay we have determined that VP4, the virus spike protein, is responsible for the interaction with hsc70. The use of fragments of VP4 expressed in E. coli, and of synthetic peptides that mimic regions of this protein, has allowed us to show that VP4 interacts with hsc70 through a region located at the carboxy terminal region of the viral protein.

Rotaviruses cell entry is a multistep process; we have characterised the binding of two rotavirus strains, which differs in their susceptibility to neuraminidase cell treatment. RRV is a simian rotavirus strain, which infectivity is sensitive to neuraminidase, and nar3 is a variant derived from RRV which is resistant to such treatment. We have previously found that both viruses bind to the cell surface through the spike protein VP4, which is cleaved by trypsin into polypeptides VP8 and VP5; RRV binds to a sialic acid containing receptor through the VP8 domain of VP4, while the variant nar3 binds to the integrin $\alpha 2\beta 1$ through VP5. Other interactions between rotaviruses and cell surface molecules take place during virus entry. In this work we have found that the interaction between rotaviruses and hsc70 protein, which is involved in the infectivity of these viruses, is mediated by VP5, since the binding of VP5 to the cell surface could be blocked with antibodies to hsc70, and VP5 is able to block the binding of rotaviruses to hsc70. The region of VP5 that mediates this interactions is located on the COOH-terminal of the protein, and it is independent of the binding domain to the integrin $\alpha 2\beta 1$, which is also present in VP5. A synthetic peptide derived from VP5 can block the infectivity of rotaviruses, the binding of VP5 to the cell surface, and the binding of rotaviruses and VP5 to hsc70. All these data implies that there are two cell surface binding domains in VP5, one of them could mediate the initial binding of rotaviruses to the cell surface,to the integrin $\alpha 2\beta 1$, while the other is involved on the post-attachment binding to hsc70.

Introduction

Rotaviruses, the leading cause of mortality and morbidity due to severe gastroenteritis in children all over the world, belong to the *Reoviridae* family (17) . The genome of these viruses is composed by eleven segments of double-stranded RNA which is surrounded by a capsid formed by three concentric layers of proteins. The outermost layer, which is responsible for the initial interactions of the virus with the cell surface, consist of two proteins; VP7, a glycoprotein that forms the smooth surface of the virion, and VP4, which forms the spikes that extend form the surface of the particle (8). This last protein has essential functions in the early interactions between the viral particle and the cell surface, including receptor binding and cell penetration (3, 6, 18, 20, 26, 32). To be infectious rotaviruses depend on a proteolytic activation by trypsin, which cleaves VP4, the viral attachment polypeptide, into subunits VP5 and VP8; this cleavage does not affect cell binding (5, 9, 16), and has been associated with the entry of the virus by direct cell membrane penetration (5, 9, 16, 22, 27). The role of VP7 during the early interactions of the virus with the cell is not clear, although it has been shown that it can modulate some of the VP4-mediated virus phenotypes, including receptor binding (21).

Rotaviruses have a specific cell tropism, infecting primarily the mature enterocytes at the tip of intestinal villi, and the susceptibility of these cells seems to be limited to a narrow age window (17). In cell culture, the infectivity of some rotavirus strains has been shown to be sensitive to the neuraminidase (NA) treatment of the cells, indicating that the presence of sialic acid (SA) on the cell surface is relevant for virus infectivity. However, it has been established that the interaction with SA is not essential for rotavirus infectivity since variants of a NA-sensitive rhesus rotavirus (RRV) which are NA resistant have been isolated (20); in addition, some animal and most human isolated rotaviruses are naturally NA-resistant (4).

It has become evident that the entry of a virus to its host cell is a multistep process where more than one interaction of the virus with the cell surface is involved (28). Rotaviruses are not the exception, and at least four different cellular molecules have been identified to play a role as receptors for this virus. The rotavirus strains that are sensitive to the NA-treatment of the cells bind in first place to a sialic acid (SA)-containing receptor, probably the ganglioside GM3 (25). After this initial interaction, a second interaction, which apparently is shared by the NA-sensitive and -resistant strains, with the integrin $\alpha 2\beta 1$ takes place (31). Besides these two interactions, we have found that the integrin $\alpha v\beta 3$ and the heat shock protein hsc70 are also involved in rotavirus cell entry, most probably at a post-attachment step (1, 10, 11).

We have previously characterized the binding of two rotavirus strains to the surface of MA104 cells: RRV, a rotavirus strain of simian origin, which is sensitive to

NA treatment, and nar3 a NA-resistant variant of RRV (20) which no longer requires SA to bind to and infect MA104 cells. We found that RRV binds to a SA-containing molecule present in the cell surface through the VP8 domain of VP4, while nar3 bound to the cell through the VP5 domain of VP4 (32). Integrin $\alpha 2\beta 1$ was found to serve as the binding receptor for the neuraminidase resistant virus nar3, while the wild type RRV interacted with this integrin after its initial interaction with a SA-containing compound. We also showed that nar3 binds to $\alpha 2\beta 1$ through the DGE integrin recognition motif located at amino acid residues 308-310 in the virus surface protein VP4 (32). These studies were performed using a recombinant VP5, expressed as a GST-fusion protein in bacteria, which was able to bind specifically to the surface of MA104 cells, and which was able to block the binding of the nar3 variant. However, a mutant GST-VP5 protein in which the integrin binding motif DGE was replaced by AGE, and was no longer able to block the binding of nar3 rotavirus, was still able to bind to the cell in a dose dependent manner, although this binding was not blocked by antibodies to the integrin $\alpha 2\beta 1$, suggesting that the recombinant protein VP5 could have a region, different from the integrin binding site, that allowed it to interact with a different cell molecule (31). In this work we found that indeed, there is another cell surface binding domain present in the VP5 protein of rotaviruses. We also found that this domain mediates the interaction of these viruses with the heat shock protein hsc70.

Materials and Methods

Cell and viruses. MA104 cells were cultured in Eagle's minimal essential medium (MEM) supplemented with 10 % fetal bovine serum. RRV was obtained from H. B. Greenberg, Stanford University, Stanford, Calif., and rotavirus variant nar3 has been described previously (20). RRV and nar3 were propagated in MA104 cells as described (7). Purified viral particles were prepared by CsCl gradients as previously described (32).

Antibodies. The monoclonal antibodies (MAbs) 2G4, specific for VP5, and 159, directed against VP7, were provided by H. B. Greenberg. Non-neutralizating MAb HS2 directed to VP5 was described by Padilla-Noriega et al. (24). MAbs to integrins $\alpha 2\beta 1$ (P1E6) and $\alpha 4\beta 1$ (P1H4) were obtained from Chemicon and were used at 10 μg/ml. The antibody to hsc70 protein used in this work is a rabbit polyclonal serum described by Guerrero et al (10), this serum was used at 80 μg/ml.

Proteins and peptides. The cloning of RRV VP5 protein, and the mutant $VP5^{D308A}$ as fusion proteins with GST has been previously described (31, 32). These fusion proteins were expressed in E. coli and were purified by affinity chromatography as previously described by Isa et al. (14). The following proteins: VP5 his (aa 247-776), NH_2-VP5 (aa 248-474), VP5-COOH (aa 474-776) and hsc70 of human origin, were cloned as fusions with a 6 histidine tail at their carboxy terminus, in the pET 28 expression vector (Novagen), and were affinity purified with the AKTA system using HiTrap chelating columns (Pharmacia). Peptides were chemicaly synthesized by Research Organics and used at the concentrations indicated in each figure legend. The aminoacid sequence of the peptides used in this work is shown in Table I.

Infectivity Assay. MA104 cells grown in 96-well plates were washed twice with MEM, and then 2000 focus-forming units (ffu) of RRV or nar3 viruses (previously activated with trypsin [10 mg/ml] for 30 min 37 °C) were adsorbed to the cell surface for 1 h at 37°C. After the adsorption period, the virus inoculum was removed, the cells were washed twice with MEM, and cultures were maintained for 14 h at 37 °C. Infected cell cultures were fixed and immunostained as previously described (2). The ffus were counted by using a Visiolab 1000 station (12).

Binding Assay. MA104 cells grown in 48-well plates were washed twice and incubated with MEM without serum for 30 min at 37 °C. After this time the MEM was removed and 500 μl of a solution of 1% of bovine albumin (BSA) in PBS were added to avoid non-specific binding, the cells were incubated with this solution for 1 h at room temperature. The cells were then washed with an ice-cold solution of 0.5 % BSA/PBS, and incubated with the indicated amount of virus or protein, diluted in ice-cold MEM, for 1 h at 4 °C. After this time the cells were washed thrice with ice-cold PBS, and finally 120 μl of lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.1 % TritonX-100)

were added. The cells were frozen and thawed twice and the amount of virus or protein present in the lysate was determined by an ELISA.

Capture ELISAs for rotavirus and rotavirus recombinant proteins. To detect the virus, goat and rabbit sera to rotavirus were used as capture (diluted 1:10,000) and detection (diluted 1:1,500) antibodies, respectively. The GST fusion proteins were captured with a goat anti-rotavirus sera and detected with a rabbit polyclonal sera to GST (diluted 1:1,500). His-tail proteins were captured with a goat anti-rotavirus sera and detected with MAb 2G4 for NH_2-VP5 or MAb HS2 for VP5 and VP5-COOH (both diluted 1:1,000). The corresponding alkaline phosphatase-conjugated anti-immunoglobulin was used (Kirkergaard and Perry, diluted 1:1,500), and finally the substrate Sigma 104 (1 mg/ml) diluted in diethanolamine buffer (100 mM diethanolamine [pH 9.4], 1 mM $MgCl_2$, 5 mM sodium azide) was added. The absorbance at 405 nm was recorded in a Microplate Autoreader EL311 (Bio-Tek Instruments).

Binding to hsc70. To study the interaction between rotavirus virion and hsc70, ELISA assays were carried out essentially as described by Guerrero et al. (10). Briefly, 96-well ELISA plates (Costar) were covered with 500 ng/well of purified recombinant hsc70 protein in PBS for 2 h at 37 °C. To avoid non-specific binding, 200 µl of a solution 1% BSA-PBS were added to the plate, and incubated for 2 h at 37 °C. Then, the plates were washed twice and incubated with the indicated amount of peptide, protein, or virus, for 1 h at 37 °C, and the presence of the recombinant protein or virus was determined by using specific antibodies. To detect RRV a rabbit polyclonal sera was used (diluted 1:1,500). The NH_2-VP5 protein was detected with MAb 2G4 (diluted 1:1,000). The VP5his and VP5-COOH proteins were detected with MAb HS2 (diluted 1:1000). The corresponding alkaline phophatase-conjugated antibody and substrate were added (see above).

Results

Synthetic peptides that mimic discrete regions of VP5 are able to block the infectivity of rotavirus. To find out if there was a region of VP5, different from the DGE integrin binding domain, that allowed the GST-VP5 recombinant protein to bind to the surface of MA104 cells, and since the number of monoclonal antibodies directed to the VP5 of animal rotaviruses is reduced, we decided to chemically synthezise a set of peptides representing different discrete regions of VP5 (Fig. 1). The effect of these peptides on the infectivity of RRV rotavirus and its variant nar3 was tested by preincubating a monolayer of MA104 cells with the peptides, and then infecting the cells with a fixed amount of virus (2000 ffus/well). Figure 2A, shows that peptides 4 and 5 were able to decrease the infectivity of both, RRV and nar3 viruses, by about 45 to 55 %, peptide 6 decreased the infectivity of nar3, but not that of RRV, while peptides 2, 3, and 7 did not have a signifficative effect on the infectivity of either virus. A peptide containing the $\alpha 2\beta 1$ integrin binding motif, DGE, was used as a control since we have already shown that this peptide is able to block the infectivity of RRV and nar3 viruses by about 50 and 40 %, respectively (31).

To determine the level effect (attachment or post-attachment) at which peptides 4, 5, and 6, affected the infectivity, we performed a binding assay in which MA104 cells were preincubated with the peptides for 1 h at 4 °C, and then 500 ng of purified virus were added and incubated for 1 h at 4°C. The amount of virus attached to the cells was determined by an ELISA (see Material and Methods). We found that none of the peptides tested had an effect on the binding of either RRV or nar3. The peptide DGE, used as control, blocked the binding of nar3 but did not affect the attachment of RRV to the cell surface, as we have previously reported (31) (Figure 2B). Since peptides 4, 5, and 6, blocked rotavirus infectivity but did not affect virus binding, these results suggest that the peptides might be inhibiting an interaction between the virus and the cell surface, that occurs at a post-attachment level.

The VP5 protein contains two independent domains of interaction with the cell surface. As previously mentioned, we have found that a recombinant GST-VP5 mutant protein in which the integrin binding site DGE was replaced by AGE, no longer displaced the binding of the variant nar3, but was still able to bind to the surface of MA104 cells, suggesting the existence of an additional binding site in this protein. To study if this additional site was represented by the synthetic peptides used in the previous assay, we performed a binding assay in which the wild type recombinant GST-VP5, and the mutant GST-$VP5_{D308A}$ proteins, were added to cells that were previously incubated with peptides 4, 5, 6 and DGE, and the amount of recombinant protein that remained attached to the cells was determined by an ELISA. We found that while peptide DGE

blocked only the binding of wt GST-VP5 by about 50%, the synthetic peptide 5 blocked the binding of both, GST-VP5 and GST-$VP5_{D308A}$ proteins (figure 3A). Peptides 4 and 6 did not have a significative effect on the binding of either fusion protein. These results suggest that there could be a cell attachment domain, different from the $\alpha 2\beta 1$ binding domain, located in the region around aminoacids 642 to 658 of VP5, represented by peptide 5.

To further confirm these results we studied the effect of two monoclonal antibodies directed to VP5 on the binding of the recombinant VP5 proteins; The MAb 2G4 is a neutralizing antibody that recognizes an epitope that has been mapped to residue 393 of the VP4 protein of rotavirus RRV (19), and that has been previously shown to block the binding of the virus nar3, and the wt GST-VP5 protein to the cell surface (32); MAb HS2, is a non-neutralizing antibody, that recognizes VP5 in a region between aa 540-593 by ELISA, and Western blot analysis (Minerva Camacho, personal communication). The effect of these antibodies on the binding of the wild type and mutant GST-VP5 proteins to the surface of MA104 cells was evaluated. We found that when the recombinant proteins were preincubated with MAb 2G4, the binding of the wild type GST-VP5 was blocked by abot 50%, while the attachment of GST-$VP5_{D308A}$ to the cell surface was unaffected. In contrast, MAb HS2 blocked the binding of both, wild type and mutant fusion proteins (figure 3B). Preincubation of the recombinant proteins with a mixture of both MAbs resulted in a more pronounced inhibition of the wild type GST-VP5, while the binding of the GST-$VP5_{D308A}$ was very similar to that obtained when preincubated with MAb HS2 alone. These results further confirm that there are two distinct cell surface binding domains present in the VP5 protein of rotaviruses.

To find out if these two domains were independent of each other, we made two constructions of VP5 to divide the protein in two separate halves. One of them, called NH_2-VP5, contains from the trypsin cleavage site (aa 248) to residue 474; this recombinant protein contains the integrin $\alpha 2\beta 1$-interaction motif DGE, and the epitope recognized by MAb 2G4. The other construction, called VP5-COOH, includes from amino acid 474 to the end of the protein (aa 776), it contains the region recognized by the MAb HS2, and the region represented by peptides 4, 5 and 6. Both proteins were constructed as fusions with a six-histidine tail, and were purified by affinity chromatography. In a binding assay, both recombinant proteins were able to bind to the surface of MA104 cells to a similar extent (data not shown). The binding of these recombinant proteins in the presence of peptide DGE, peptide 5 or peptide S5 (a scrambled version of peptide 5) was determined. We found that the DGE peptide blocked the binding of the protein NH_2-VP5 without affecting the binding of the VP5-COOH protein. Conversely, peptide 5 only blocked the binding of VP5-COOH, and did not alter the attachment of the NH_2-VP5 protein. The peptide S5, used as a control, did not affect

the binding of either protein (Figure 4A). These results suggest that the binding of each half of VP5 is specific, and that they probably bind to different molecules in the cell surface.

The carboxy-teminal domain of VP5 interacts with the heat shock protein hsc70. To identify the cell molecule to which the COOH-terminal half of VP5 binds we tested the effect of antibodies directed to cell surface molecules that have been involved as rotavirus receptors. In this assay we used antibodies to the integrins $\alpha2\beta1$ and $\alpha4\beta1$, and also an antibody to the heat shock protein hsc70, which has been recently described as a putative rotavirus receptor (10). Integrin $\alpha2\beta1$ is the cell molecule that is used as the intial receptor by rotavirus variant nar3, and this interaction has already been characterized (31). An antibody to integrin $\alpha4\beta1$ was included since it has been described that the VP5 protein contains an IDA tripeptide at amino acids 538-540 (13), that could be responsible for the interaction of the rotaviruses with integrin $\alpha4\beta1$. We also tested an Ab to the hsc70 protein since the interaction between rotavirus and hsc70, and the interaction mediated by the region represented by peptide 5, seem to take place at a post-attachment step during rotavirus entry.

In these assays, MA104 cells were preincubated with antibodies directed to integrins $\alpha2\beta1$ and $\alpha4\beta1$, and to the protein hsc70 for 1 h at 4 °C. The cells were washed twice and 200 ng of NH_2-VP5 or VP5-COOH were added and allowed to bind for 1h. The amount of protein that remained bound to the cell surface was determined by an ELISA (see Materials an Methods). We found that the MAb to integrin $\alpha4\beta1$ did not have a significative effect on the binding of either protein. As expected, a MAb to the integrin $\alpha2\beta1$ diminished the binding of NH_2-VP5, but did not affect the binding of VP5-COOH. Interestingly, we found that an antibody directed to hsc70 was able to decrease the binding of the VP5-COOH recombinat protein while it did not alter the attachment of the NH_2-VP5 (figure 4B).

We have previously demonstrated that triple layered rotaviruses, but not double layered particles, could bind to purified hsc70 protein that had been immobilized on an ELISA plate (10). We used the same kind of assay to test if the VP5 protein of rotaviruses was indeed able to bind to hsc70; for these assays the recombinant NH_2-VP5, VP5-COOH proteins, and a full length VP5 protein also expressed with a six histidine tail (VP5-his) were used. We found that while the NH_2-VP5 protein did not bind to hsc70, the complete VP5 protein and the VP5-COOH bound specifically to it; we also found that the binding of these two recombinant proteins was saturable and concentration dependent (data not shown).

Given that the VP5-COOH and VP5-his proteins were able to bind to hsc70, we tested whether their binding to this protein was able to displace the binding of RRV virus to the heat shock protein. For this, an ELISA plate covered with hsc70 was preincubated

with increasing amounts of the recombinant proteins for 1h at 37 °C; after this period the unbound protein was removed and a fixed amount of RRV was added. The virus bound to the plate was detected using a MAb directed to the VP7 protein. As shown in figure 5, proteins VP5-his and VP5-COOH blocked the binding of rotavirus RRV to hsc70 in a concentration dependent maner, while the protein NH_2-VP5 did not have any effect, as expected since this protein did not bind to hsc70.

The region contained in peptide 5 is responsible for the interaction with hsc70. The data presented above suggest that rotaviruses bind hsc70 through a region located on the COOH-terminal half of VP5. To determine if the region of interaction between the COOH- terminal portion of VP5 and hsc70 was the region contained in peptide 5, we performed an ELISA in which peptide 5, or its scrambled version (S5) were adsobed to ELISA plates precoated with hsc70, and then the VP5-his, the VP5-COOH recombinant proteins, or the virus RRV were added. The amount of virus or protein attached to the plate were determined using the appropiate antibodies (see Materials and Methods). We found that peptide 5 decreased the binding of the proteins VP5-his, VP5-COOH and the virus RRV, while the contol peptide S5 did not affect the binding of the proteins or the virus to hsc70 (fig. 6). This result indicated indirectly that the region represented by peptide 5 was able to interact with hsc70, however the direct binding of the peptide to the heat shock protein was not detected.

To show this interaction directly, recombinant M13 phages displaying in their surface protein pIII the aminoacid sequence of peptide 5 (residues 642-658) or a larger version comprising aminoacids 636-665 of VP4, were constructed. Three additional recombinant phages displaying amino acids 161-169 and 141-190 from the VP7 protein, and a phage displaying aa from the scorpion toxin, were used as a control. An ELISA assay was performed by coating the plate with recombinant hsc70, then increasing amounts of the recombiant phages were added. After the incubation period the unbound phages were washed off and the bound phages were detected using an antibody specific for the phage. Fig 7 shows that the phages displaying the aminoacid sequence of VP5 were able to bind to hsc70 in a concentration dependent manner, while the recombinant phages displaying aminoacids from VP7 or the scorpion toxin, used as controls, did not bound to this protein.

Altogether these data indicate that the VP5 protein of rotaviruses contains at least two regions that interact with the cell surface, the first is the tripeptide DGE at amino acids 308-310, which mediates the binding of rotaviruses to the integrin $\alpha 2\beta 1$ (31). In this work we found a second domain that is capable of interacting with the cell surface, this region is located toward the COOH-terminal part of VP5 at amino acids 642-658 and mediates the binding of rotaviruses with the heat shock protein hsc70. This interaction apparently takes place at a post-attachment step during rotavirus infection.

Discussion

The possibility that several cell surface molecules may be involved in the early interactions of rotaviruses with the host cell which ultimately lead to the entry of the virus particle into the cytoplasm of the cell, is not far-fetched. In fact, the need for multiple receptor binding events to achieve an efficient cell entry is becoming a frequent observation in virus-host cell interactions. Viruses from different families (e.g. adenovirus (23), herpes simplex virus (29)) utilize at least two different receptors to interact with their host cells: the attachment receptors which in general allow the virus to rapidly bind to the cell surface; and the entry receptors, also known as co-receptors, or post-attachment receptors, which facilitate the entry of virus into the cell. It has been observed that this second interaction frequently induces conformational changes of the viral surface proteins that are essential for the penetration of the virus into the cellular cytoplasm.

The use of synthetic peptides, which represent different regions of VP5 lead us to identify a region between aa 642-659 that mediates binding of VP5 to the cell surface, besides the construction of a VP5 protein which only content the last 300 aa of the protein let us to establish that this interaction was independent on the binding of the protein through the integrin $\alpha 2\beta 1$-binding site. Recently Jolly et al, reported several regions within VP4, which are able to bind to the surface of MA104 cells. These regions were identified using a phage-display approach. Three of the clones that were analyzed in that work represented a region between aa 650-657 (15); this region is also represented by the peptide 5. The identification of this cell binding domain by two completely different approaches reinforces the data obtained in this work.

In addition, the peptide which contains the sequence between aa 642- 659 (p5), block the infectivity of rotaviruses RRV and nar3, but not their binding, which implies that the interaction with the cell surface, that is blocked by the peptide, ocurrs as a postattachment step. Our group have found that the heat shock protein hsc70 was involved during a post-attachment step of rotavirus infectivity. In the present work we have found that the protein VP5 of rotavirus has a region able to mediate this interaction; this region is located between the aa 642 to 659, since the peptide 5, that contains this sequence, is able to block this interaction.

The role of hsc70 on rotavirus infection is not clear; one possibility is that hsc70 could act as a contact point of the virus in the cell surface that enable the virus to interact with other cell molecules that would be the responsibles of virus entry. However, the chaperone activity of hsc70 let us to speculate that hsc70 could play a more active role on rotavirus entry, maybe tiggering conformational changes on the particle that allows it to reach the citoplasm or to trigger the uncoating of the viral particle, which is necessary for the transcription to begin.

Recently it has been reported a secondary structure prediction of VP4 protein, based on cryomicrocopy studies of rotavirus in the presence of the MAbs 7A12 and 2G4, directed toward VP8 and VP5 proteins, respectively (30). This work shown that the head of the spike is formed by VP8, and just beneath it, is located the region recognized by the MAb 2G4 (aa 393). This mean that the region of interaccion with the integrin $\alpha 2\beta 1$ is located near the head of the spike. On the other hand, the region represented by the peptide 5 should be located at the bottom of the spike, near to the region that contacts with VP7.

In the model of consecutive interaccions with the cell surface for rotavirus entry, we propose that the initial interaction of the NA-sensitive strain RRV involves a first interaction with sialic acid, and a second one with the integrin $\alpha 2\beta 1$. Both interactions would be mediated by the top of the spike (which means by VP8 and by the tripeptide DGE present in VP5), and we specultate that this trigger a conformational change in the viral particule that allows it to interact with hsc70 by using the bootom of the spike.

It is known that the spike is not completely exposed, and a portion of it is found beneath VP7, VP4 also contact the second layer protein VP6. However, we speculate that the region represented by the peptide 5 should be exposed, since rotaviruses could interact with purified hsc70 in ELISA. This idea fits with the fact that MAb 159, directed to VP7 protein, blocks rotaviruses interaction with hsc70. This probably means that the region of interaction with hsc70 is located near the interface between VP4 and VP7. MAbs to VP8 and the NH-terminal of VP5 (7A12 and 2G4, respectively) block the binding of rotavirus to hsc70 at a lesser extent; since these antibodies map toward the top of the spike (30), it is logical to suppose that they can only partially block the interaction of the viral particle with hsc70.

Futher experiments toward understanding the role of hsc70 on rotavirus infectivity are needed. In the other hand, structural information should also be very useful in order or fully understand the data obtained in biological assays.

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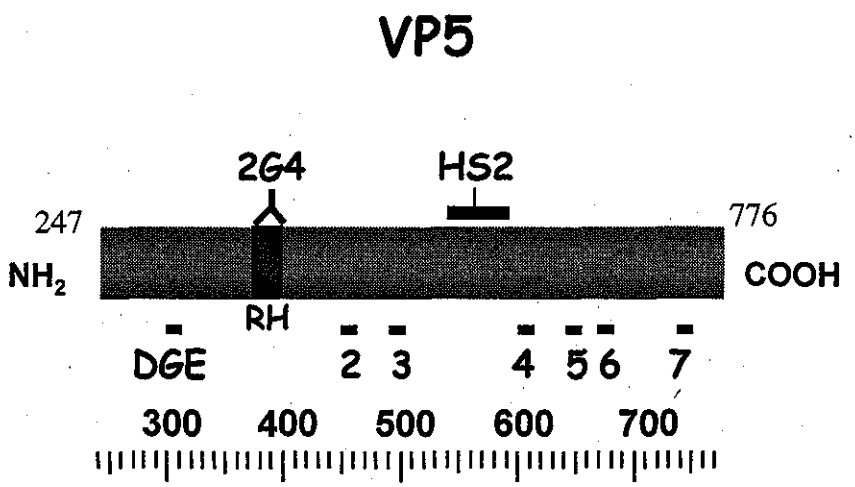

Peptido	Secuencia
DGE	^{300}KPANYAYTYTRDGEDVTAHTOC^{321}
2	^{453}AYPNNGKEYYE^{463}
3	^{490}RQDLERQLGELREEF^{504}
4	^{595}STQITDVSSSVSSISTQT^{612}
5	^{642}KTKIDRSTQISPNTLPD^{658}
C5	TIRPSITPKDKQTNSLD
6	^{676}INNDEVFEAGTDGRY^{690}
7	^{738}RQQAFNLLRSDPRVLRE^{754}

Figure 1. Distribution and aminoacid sequence of the synthetic peptides derived form the VP5 protein. The recognition sites for antibodies 2G4 and HS2 are also shown.

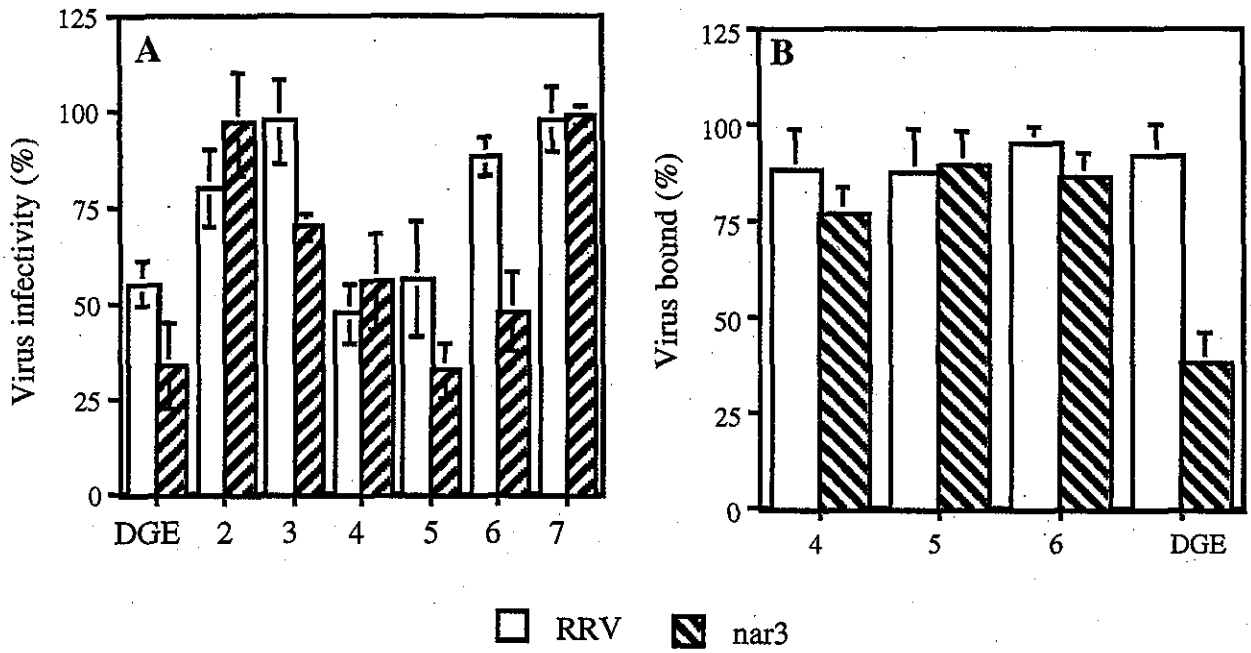

Figure 2. Effect of the peptides on the infectivity and binding of RRV and nar3 viruses. (A) MA104 cells grown in 96-well plate were preincubated with 4 mg/ml of the peptides for 1 h at 37 °C. Then, 2000 FFUs of the corresponding virus were added and adsorbed for 1 h at 37 °C. The excess of virus was removed and the infection was allowed to procced for 14 h at 37 °C. Finally, the cells were fixed and immunostained as described in Materials and Methods. Data are expressed as the percentage of the virus infectivity obtained when the cells were preincubated with PBS as a control. (B) MA104 cells grown in 48-well plates were preincubated with the peptides (4 mg/ml) for 1 h at 4°C; then, 500 ng of virus were added and the cells were futher incubated for 1 h at 4 °C. The excess of virus was removed and the amount of virus attached to the cells was determined by an ELISA. Data are expressed as the percentage of virus bound to the cells when the cells were preincubated with PBS as a control. The arithmetic means and standard deviations for at least two independent experiments performed in duplicate are shown.

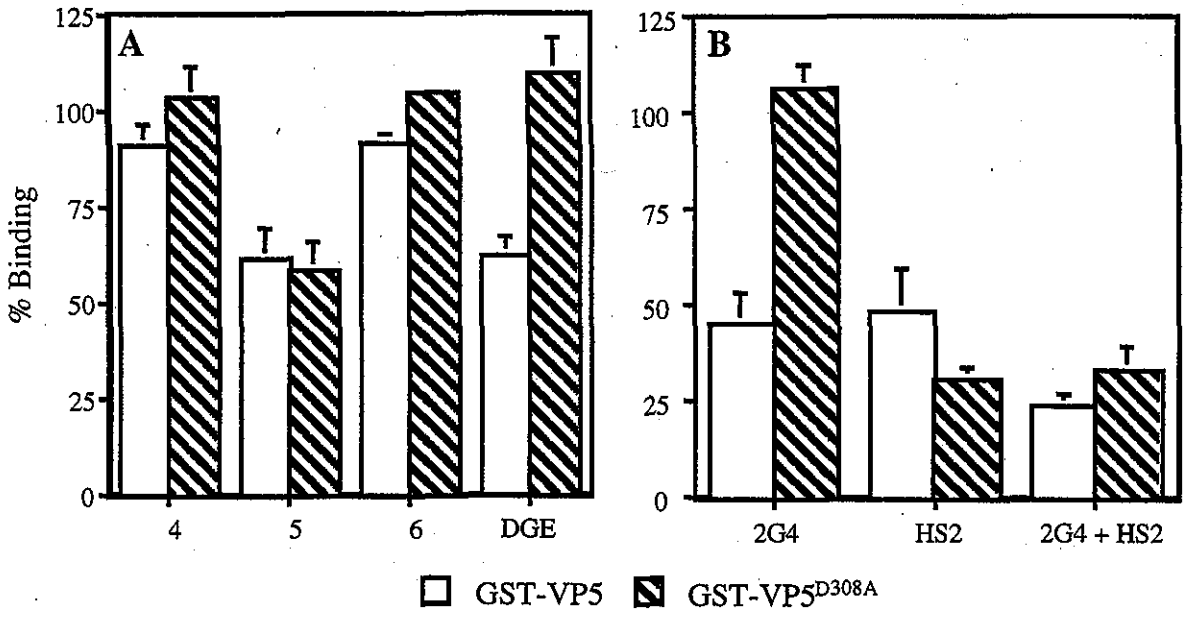

Figure 3. Binding of GST-VP5 and GST-VP5^{D308A} in the presence of synthetic peptides and antibodies to VP5. (A) MA104 cells grown in monolayer were preincubated with 4 mg/ml of the peptides for 1 h at 4°C, then 1.5 µg of the recombinant protein were added, and the cells were incubated for another hour at 4 °C. The amount of bound protein was determined by ELISA. Data are expressed as the percentage of protein bound to the cells when they were preincubated with PBS as a control. (B) Proteins (1.5 µg) were preincubated with the MAbs for 1 h at room temperature, the mix was added to MA104 cells grown in monolayer and incubated for 1 h at 4 °C. The amount of bound protein was determined by ELISA. Data are expressed as the percentage of protein bound to the cells when the proteins were preincubated with PBS as a control. The arithmetic means and standard deviations for at least two independent experiments performed in duplicate are shown.

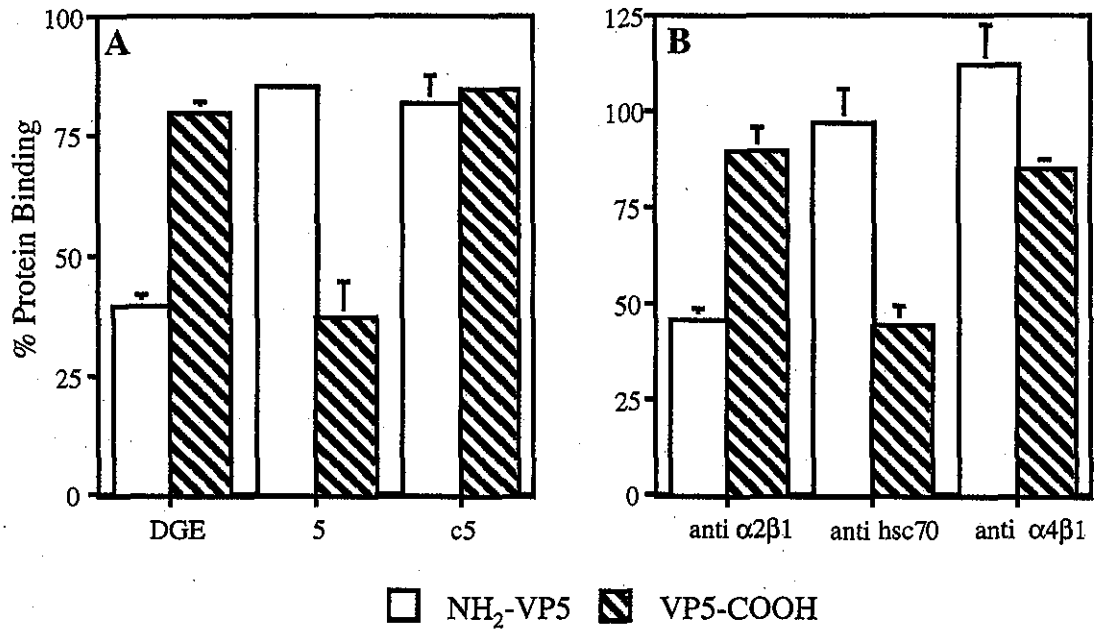

Figure 4. Effect of the peptides and antibodies directed to cell surface molecules in the binding of NH_2-VP5 and VP5-COOH proteins. MA104 monolayers were preincubated with 4 mg/ml of peptide (A) or with antibodies against integrins $\alpha 2\beta 1$ and $\alpha 4\beta 1$ or against hsc70 (B) for 1h at 4 °C. Then 200 ng of the proteins NH_2-VP5 or VP5-COOH were added, and incubated for 1 h at 4 °C. The amount of protein bound to the cells was determined by ELISA. Data are expressed as the percentage of protein bound to the cells when they were preincubated with PBS as a control. The arithmetic means and standard deviations for at least two independent experiments performed in duplicate are shown.

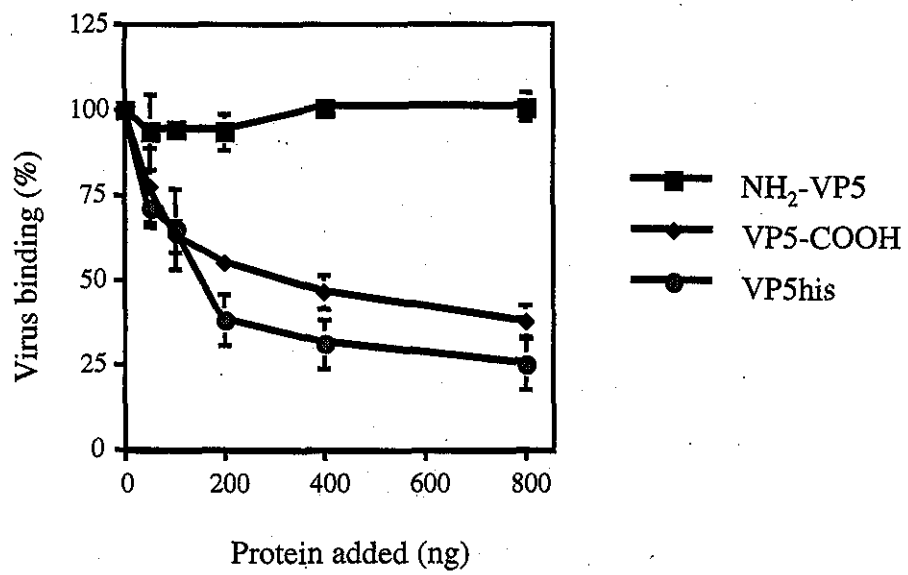

Figure 5. Effect of VP5 in the binding of rotaviruses to hsc70 protein. A 96-well ELISA plate covered with hsc70 (500 ng/well) was incubated for 1 h at 37 °C with increasing amounts of VP5his, NH_2-VP5 or VP5-COOH. After removing the protein, 300 ng/well of purified RRV were added, and the plate was futher incubated for 1 h at 37 °C. The amount of virus vound to hsc70 was determined by using an antibody to VP7 rotavirus protein (MAb 159). Data are expressed as the percentage of virus bound relative to the condition without protein.The arithmetic mean and standard deviation for at least two independent experiments performed in duplicate are shown.

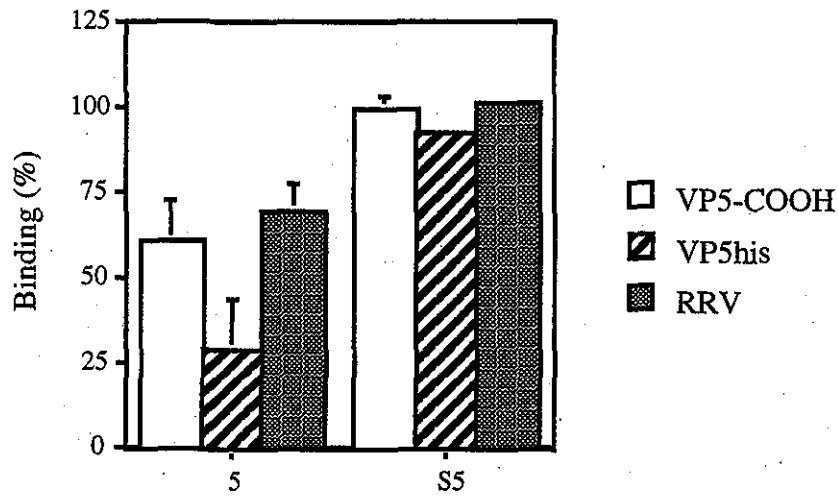

Figure 6. Effect of peptide 5 in the binding of VP5 and RRV to hsc70. A 96-well ELISA plate covered with hsc70 (500 ng/well) was preincubated with 2 mg/ml of peptide 5 or S5, as a control, for 1 h at 37 °C. Then 200 ng of VP5his or VP5-COOH, or 300 ng of RRV were added. The plate was incubated for 1 h at 37 °C. The protein or the virus bound to the plate were detected with antibodies (see Material and Methods). Data are expressed as the percentage of protein or virus bound to hsc70 relative to the condition without peptide. The arithmetic mean and standard deviation for at least two independent experiments performed in duplicate are shown.

4. La unión del rotavirus nar3 no cortado con tripsina es mediada por VP7 y su receptor es la integrina αvβ3

Como se mencionó en la introducción, se ha establecido que al tratar a los rotavirus con tripsina se incrementa la infectividad de estos virus. Además, se ha encontrado que los virus tratados con tripsina entran más rápido a las células, posiblemente por penetración directa (40, 42). Otras propiedades de los rotavirus también dependen del corte con tripsina, por ejemplo el virus tratado con tripsina tiene actividad lipofílica (61, 68) y puede inducir la fusión de células MA104 a las que se les ha aumentado el colesterol (21, 27). El mecanismo mediante el cual el corte con tripsina incrementa la infectividad de los rotavirus aún no ha sido dilucidado, pero se ha propuesto que los extremos de VP4 recién generados por el corte con tripsina podrían favorecer la entrada del virus; o bien, que el corte podría inducir un cambio conformacional en el virión que le permita exponer nuevos sititos de unión a la superficie celular, que pordrían mediar la entrada del virus al citoplasma. En este trabajo nos propusimos estudiar el efecto del corte con tripsina en la unión de los virus RRV y nar3 a las células. Los resultados de este trabajo serán incluidos en el artículo: "Identification of a novel binding motif to the integrin αvβ3 present in rotavirus capsid protein VP7", que está siendo preparado para su publicación en el Journal of Biological Chemistry.

Al evaluar la capacidad de los virus RRV y nar3 cortados o no cortados con tripsina (obtenidos según se describe en Materiales y Métodos) para unirse a la superficie celular, encontramos que independientemente del corte con tripsina, ambos virus se unen a la célula de manera saturable y dependiente de la concentración de virus añadida (datos no mostrados). Para determinar si la unión de los virus no cortados conserva las características que habíamos observado para los virus RRV y nar3 previamente, se incubaron monocapas de células MA104 con las proteínas GST-VP8 o GST-VP5; posteriormente se añadió una cantidad fija de virus cortado o no, y se cuantificó el virus unido a las células mediante un ELISA. Encontramos que la proteína recombinante GST-VP8 disminuye la unión del virus RRV cortado o no con tripsina en un 75 %, y no afecta en ningún caso la unión de la variante nar3.

Por otro lado, la proteína recombinante GST-VP5 sólo compite la unión del virus nar3 cuando está cortado con tripsina (disminuyendo su unión en un 75 %), mientras que no afectó la unión del virus nar3 no cortado, ni la unión de RRV (Fig. 5). Estos resultados sugieren que el virus nar3 se une a la superficie de la célula a una molécula distinta dependiendo de si está o no cortado con tripsina, mientras que la unión de RRV (dependiente de AS) no se ve afectada por este tratamiento proteolítico.

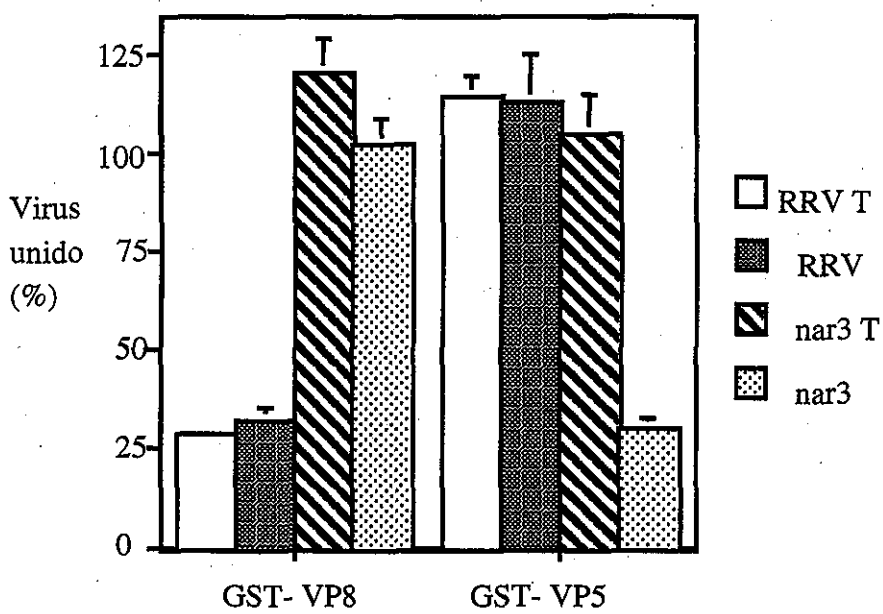

Figura 5. Unión de los virus RRV y nar3 cortados o no con tripsina en presencia de las proteínas recombinantes GST-VP8 y GST-VP5. Monocapas de células MA104 se preincubaron con 1.5 µg de las proteínas recombinantes GST-VP8 y GST-VP5 durante 1 h a 4 °C. Posteriormente se retiró el exceso de proteína y se añadieron 500 ng de virus cortado, o no, con tripsina. Las células se incubaron por 1 h a 4 °C. La cantidad de virus unido a la células se determinó mediante un ELISA, utilizando para la detección el AcM 159 (diluido 1:1500), dirigido contra la proteína VP7. Los datos se expresan como el porcentaje de virus unido a la célula, con respecto a la condición control en que las células se preincubaron con PBS. Se muestran los promedios y las desviaciones estándar de al menos dos experimentos independientes realizados por duplicado. El código para la leyenda de la figura es el siguiente: RRVT= RRV cortado; RRV= RRV no cortado; nar3T= nar3 cortado; nar3= nar3 no cortado.

Con el fin de determinar qué proteína viral es responsable de la unión del virus nar3 no cortado a la célula, preincubamos los virus con AcM dirigidos contra las diferentes proteínas de superficie de los rotavirus y posteriormente probamos la capacidad de estos virus para unirse a la superficie celuar. En la figura 5, se muestra que la unión de RRV se bloquea en un 45% cuando utilizamos un AcM dirigido contra la proteína VP8 (7A12), sin importar si el virus estaba o no cortado con tripsina. La unión del virus nar3 cortado se bloquea cuando este virus se preincubó con un AcM dirigido contra la proteína VP5 (2G4), como ya habíamos reportado previamente (80). Sin embargo, la unión del virus nar3 sin cortar no se afectó por el AcM 2G4. Cabe destacar que la unión del virus nar3 no cortado sólo disminuyó entre un 30 y un 45 % cuando el virus se preincubó con los AcM neutralizantes 159 y 57-8, dirigidos contra la proteína viral VP7.

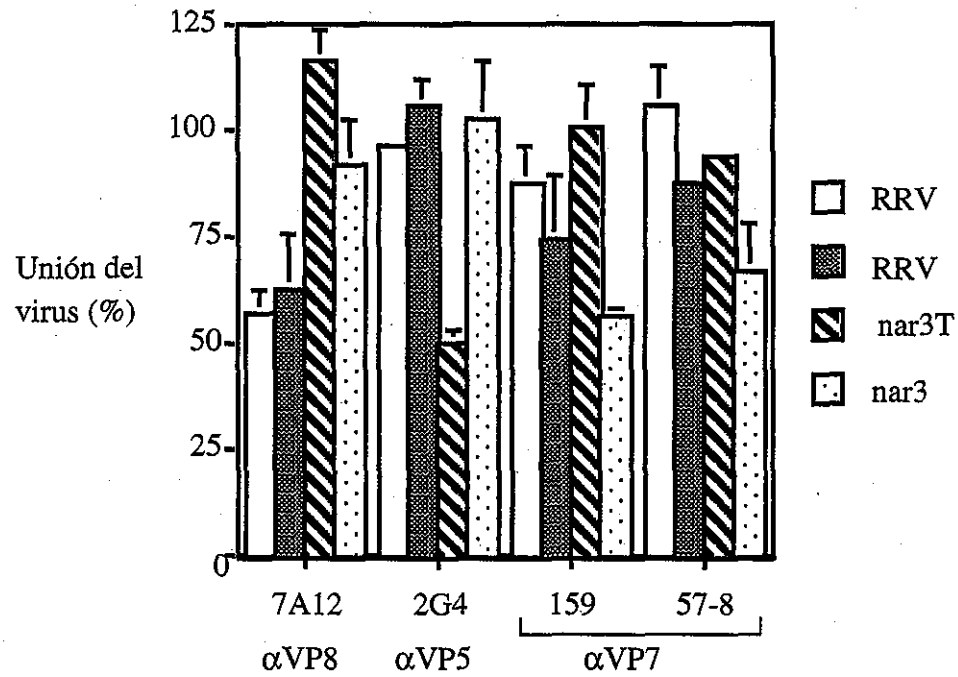

Figure 6. Unión de los virus RRV y nar3 cortados y no cortados con tripsina. Partículas virales purificadas (500ng) se preincubaron con AcM neutralizantes dirigidos contra diferentes proteínas virales (diluídos 1:100 de ascitis) durante 1 h a TA. Posteriormente, esta mezcla se añadió a una monocapa de células MA104 y se incubó durante 1 h a 4 °C. La cantidad de virus unido a las células se determino utilizando un ELISA (ver Materiales y Métodos). Los datos se expresan como el porcentaje de virus unido a las células con respecto a la condición control en la que el virus se preincubo con PBS. Se muestra el promedio y la desviación estándar de al menos dos experimentos independientes realizados por duplicado.

Los resultados anteriores indican que el virus nar3 no cortado no se une a la superficie celular a través de la proteína VP5, y parece utilizar a VP7 como proteína de unión. Para confirmar estos datos, probamos otros AcM dirigidos contra VP7, un AcM neutralizante (4F8) y dos AcM no neutralizantes (60 y 129). Encontramos que el AcM 4F8 bloqueó la unión del virus nar3 no cortado, mientras que los anticuerpos no neutralizantes no tuvieron ningún efecto sobre la unión de este virus (datos no mostrados). Para descartar la posibilidad de que los AcM utilizados tuvieran una reactividad diferente con los virus cortados y no cortados con tripsina, realizamos un ELISA, utillizando los AcM como anticuerpos de captura y comparando la reactividad de los virus cortados, o no, con tripsina; en este ensayo no encontramos diferencias significativas en el reconocimiento de los virus por estos anticuerpos (datos no mostrados). El conjunto de los resultados anteriores sugiere fuertemente que el virus nar 3 no cortado se une a la célula a través de la proteína VP7; sin embargo, es necesario recalcar que el virus no cortado no es infeccioso, pero puede ser utilizado como modelo para estudiar las interacciones de VP7 con la superficie celular.

Para identificar la molécula celular con la cual interacciona el virus nar3 no cortado, probamos el efecto que pudieran tener anticuerpos dirigidos contra las integrinas $\alpha 2$, $\beta 1$, $\beta 3$ y $\alpha v \beta 3$ sobre la unión de los virus no cortados (Fig. 7). Encontramos que ninguno de estos anticuerpos afecta la unión del virus RRV, esté cortado o no con tripsina, este resultado se puede atribuir a que en ambos casos el virus RRV se une a AS. Como habíamos mostrado previamente, el AcM dirigido contra la integrina $\alpha 2$ bloqueó en un 50 % la unión del virus nar3 cortado (79), pero no afectó la unión de este virus cuando no está cortado. En estos ensayos se probaron dos anticuerpos dirigidos contra la integrina $\beta 3$, uno de ellos se une al sitio de reconocimiento RGD, que se localiza entre las subunidades α y β de la integrina $\alpha v \beta 3$, mientras que el otro reconoce solamente a la subunidad $\beta 3$; encontramos que el anticuerpo dirigido contra la subunidad $\beta 3$ bloqueó la unión del virus nar3 no cortado en un

35 %, mientras que el otro anticuerpo (dirigido contra αvβ3) no tuvo ningún efecto. El AcM dirigido contra la integrina β1, que fue utilizado como control negativo, no afectó la unión de ningún virus.

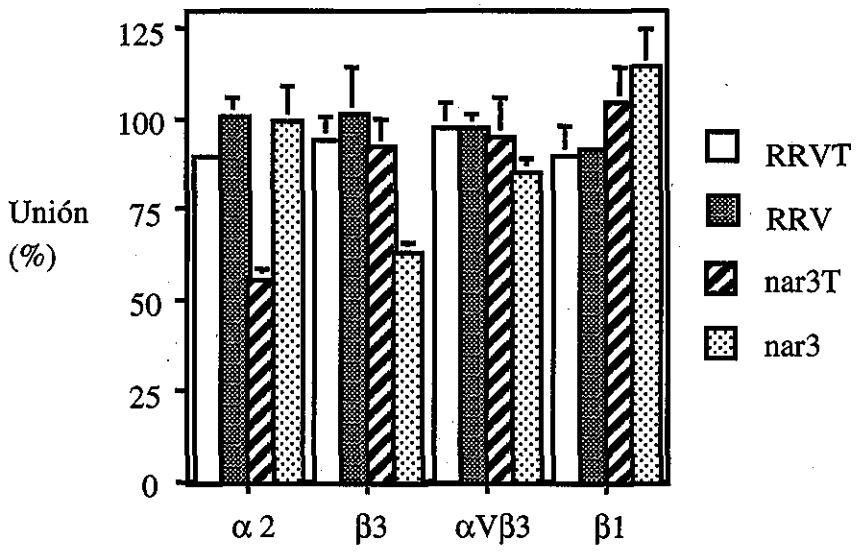

Figure 7. Efecto de anticuerpos dirigidos contra diferentes integrinas en la unión de virus cortados y no cortados con tripsina. Monocapas de células MA104 se preincubaron con anticuerpos dirigidos contra diferentes integrinas por 1 h 4 °C, se lavó el exceso de anticuerpo y se añadió una cantidad fija de virus (500 ng) a las células y se incubaron por 1 h at 4 °C. La cantidad de virus unida se determinó mediante un ELISA. Los datos se expresan como el porcentaje de virus unido respecto a la condición en que las células se preincubaron con PBS como control. Se muestra el promedio y la desviación estándar de al menos dos experimentos independientes realizados por duplicado. Los anticuerpos utilizados fueron: suero policlonal de cabra contra la integrina β3 (20 μg/ml); AcM P4G11 contra la integrina β1 (10 μg/ml); AcM LM609 contra la integrina αvβ3 (20 μg/ml); y AcM P1E6 contra la integrina α2 (10μg/ml).

En conjunto, estos resultados sugieren que la unión del virus nar3 no cortado con tripsina es independiente de la proteína VP5, y utiliza un receptor distinto al del virus nar3 cortado. También encontramos que la integrina β3 pudiera ser el receptor celular para la unión de la variante nar3 no cortada y que esta interacción pudiera estar mediada por la glicoproteína VP7.

En un trabajo previo de nuestro laboratorio, Guerrero y col mostraron que la interacción de los rotavirus con la integrina αvβ3 ocurre en un paso posterior a la unión inicial y que esta interacción es independiente de la secuencia canónica de unión a estas integrinas (RGD) (29). Un punto interesante de nuestros reulsutados es que mientras el anticuerpo dirigido únicamente contra la subunidad β3 bloquea la unión del rotavirus nar3 no cortado, un anticuerpo que está dirigido contra el sitio de unión del tripéptido RGD (anti αvβ3) no tiene el mismo efecto. Lo anterior sugiere la posibilidad de que la interacción de los rotavirus con esta integrina ocurra en una región distinta a la utilizada por los ligandos conocidos de la integrina αvβ3.

Los hantavirus también utilizan a la integrina αvβ3 como receptor y se ha observado que esta interacción también es independiente del sitio canónico RGD (26). Dado que ambos virus dependen de este tipo de interacción con la integrina αvβ3, comparamos la secuencia de la proteína de superficie de hantavirus G1G2 con la secuencia de la proteína VP7 de los rotavirus y encontramos una región de nueve aminoácidos en la que las proteínas tienen un 66.7 % de identidad (Figura 8).

```
759 NSWACNPPD G1G2 Hantavirus L99
    * * *** *
161 NEWLCNPMD VP7  Rotavirus RRV
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Figura 8. Alineamiento de la proteína VP7 de los rotavirus (aa 161-169) y la proteína G1G2 de los hantavirus (aa 759-767). En esta región las secuencias tienen una identidad de 66.7 %.

Para estudiar si esta región de 9 aminoácidos es relevante para la interacción de los rotavirus con la integrina αvβ3, se mandó sintetizar un péptido que contiene esta secuencia (NEWLCNPMD), al que hemos llamado CNP, y como control se sintetizó un péptido con la misma composición de aminoácidos pero con una secuencia disitnta, que llamamos sCNP.

También se sintetizó un péptido que contiene la secuencia derivada de la proteína G1G2 de hantavirus, al que denominamos HANTA. Se probó el efecto de preincubar estos péptidos con las células, sobre la infectividad de los rotavirus. En la figura 9A, se observa que el péptido CNP bloquea la infectividad de RRV y de nar3 en un 40 y 50 %, respectivamente, mientras que el péptido control sCNP no afecta la infectividad de ninguno de los virus; el péptido HANTA también es capaz de inhibir la infectividad de ambos virus. Estos resultados sugieren que la región de VP7 representada en el péptido CNP está involucrada en la infectividad de los rotavirus.

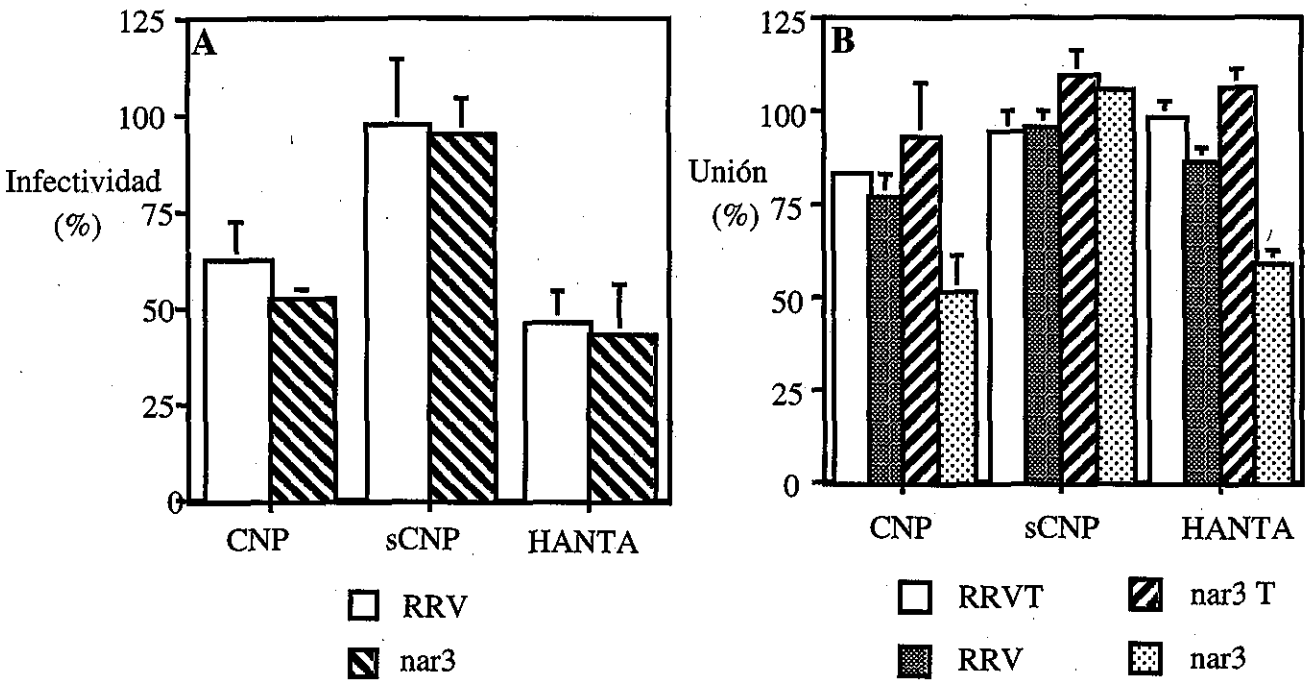

Figura 9. Efecto de los péptidos sintéticos en la infectividad y la unión de los rotavirus RRV y nar3. (A) Células MA104 crecidas en monocapa se preincubaron con 2 mg/ml de los péptidos CNP, sCNP y HANTA por 1 h a 37 °C; se removió el exceso de péptido y se adsorbió el virus RRV o nar3 (2000 FFUs por pozo) por 1h at 37 °C; después del período de adsorción se removió el inóculo y se dejó proseguir la infección por 1h at 37 °C. Las células se fijaron e inmunotiñeron como se describe en Materiales y Métodos. Los datos se expresan como el porcentaje de virus unido respecto a la condición en que las células se preincubaron con PBS como control. (B) Monocapas de células MA104, se preincubaron con 4 mg/ml de cada péptido durante 1 h at 4 °C, después de la incubación se removió el exceso de péptido y se añadió una cantidad fija de virus (500 ng), y se incubó por 1 h a 4 °C. La cantidad de virus unido se determinó utilizando un ELISA. Los datos se expresan como el porcentaje de virus unido respecto a las células control preincubadas con PBS. Se muestra el promedio y la desviación estándar de al menos dos experimentos independientes realizados por duplicado.

Para definir a que nivel el péptido CNP bloquea la infectividad, probamos el efecto de estos péptidos en la unión de los rotavirus. Encontramos que ninguno de los péptidos afecta la unión de los virus cortados con tripsina, así como tampoco afectan la unión del virus RRV no cortado, como se muestra en la figura 9B. Sin embargo tanto el péptido CNP, como el péptido HANTA, bloquean en aproximadamente un 50 % la unión del virus nar3 no cortado. Dado que los péptidos no bloquearon la unión de ninguno de los virus cortados, pero sí disminuyen su infectividad, suponemos que la interacción que bloquean los péptidos CNP y HANTA ocurre en un momento posterior a la unión inicial del virus, y que esta interacción se hace evidente en la unión del virus nar3 no cortado.

Con el objeto de determinar si la región representada en el péptido CNP es capaz de unirse directamente a la integrina αvβ3, se mandaron sintetizar péptidos CNP y sCNP que contuvieran los aminoácidos KYG en uno de sus extremos, para facilitar su biotinilación. Los péptidos biotinilados se utilizaron en ensayos de ELISA, en los cuales placas de 96 pozos para ELISA se cubrieron con las integrinas αvβ3 o α5β1, obtenidas comercialemente, y posteriormente se añadieron los péptidos CNP o sCNP marcados con biotina. Ninguno de los dos péptidos fue capaz de unirse a la integrina α5β1, mientras que el péptido CNP se unió a la integrina αvβ3 de manera saturable y dependiente de la concentración de péptido añadida (Fig 10A). Además, la unión del péptido CNP a la integrina αvβ3 se desplaza cuando se utilizan cantidades crecientes del mismo péptido no marcado (fig 10B), lo que indica que la unión del péptido a esta integrina es específica.

Para establecer si los rotavirus se pueden unir directamente a la integrina αvβ3 se cubrieron placas de 96 pozos para ELISA con las integrinas αvβ3 o α5β1, y posteriormene se añadió el virus nar3 cortado o no con tripsina. En este experimento encontramos que el virus nar3 cortado con tripsina no fue capaz de unirse a ninguna de las dos integrinas integrina, mientras que el virus nar3 no cortado se une a la integrina αvβ3 de manera saturable y

dependiente de la concentración de virus añadida, pero no se une a la integrina α5β1 (Fig 11 A).

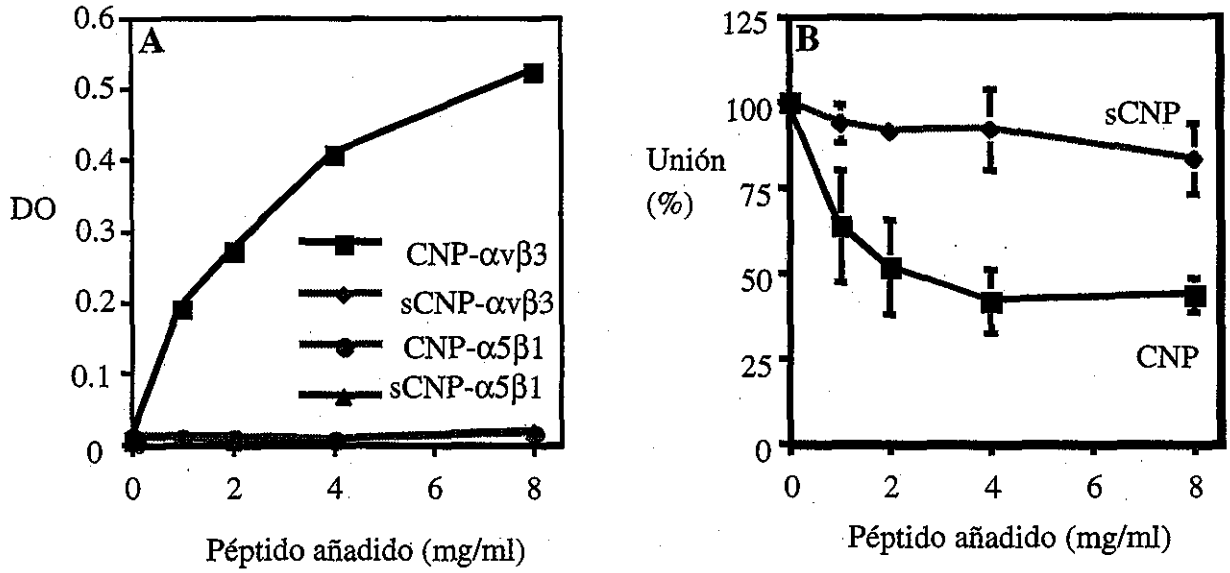

Figura 10. Unión directa del péptido sintético CNP a la integrina αvβ3 purifcada. (A) Una placa de 96 pozos para ELISA, se incubó toda la noche a 4 °C con 100 ng/pozo de las integrinas αvβ3 o α5β1, obtenidas comercialmente. Posteriormente se añadieron cantidades crecientes de los péptidos CNP o sCNP biotinilados, y la placa se incubó por 1 h a 37 °C. La cantidad de péptido unido a la integrina se determinó utilizando estreptavidina conjugada con peroxidasa (ver Materiales y Métodos). Los datos se expresan como la densidad óptica a 490 nm obtenida, contra la cantidad de péptido añadida. Se muestra un resultado representativo de dos experimentos independientes realizados por duplicado. (B) Se añadieron cantidades crecientes de los péptidos CNP y sCNP no biotinilados a una placa de 96 pozos para ELISA, previamente cubierta con 100 ng/pozo de la integrina αvβ3, durante 1h a 37 °C. Posteriormente se añadieron 2 mg/ml de péptido CNP biotinilado, y la placa se incubó por 1h a 37 °C. La cantidad de péptido biotinilado unida a la placa se determinó utilizando estreptavidina peroxidasa (ver arriba). Los datos se expresan como el porcentaje de péptido biotinilado unido con respecto a la condición en que la integrina se preincubó con PBS como control. Se muestra el promedio y la desviación estándar de al menos dos experimentos independientes realizados por duplicado.

Finalmente, probamos si el péptido CNP era capaz de bloquear la interacción del virus nar3 no cortado con tripsina con la integrina αvβ3. Hicimos un ELISA preincubando la

integrina αvβ3 inmovilzada en la placa con concentraciones crecientes de los péptidos CNP o sCNP; eliminamos el exceso de péptido y añadimos una cantidad fija de virus nar3 no cortado. Encontramos que el péptido CNP bloqueó la unión de la integrina con el virus nar3 no cortado, mientras que el péptido control sCNP no tuvo ningún efecto sobre la unión del virus (Fig. 11B).

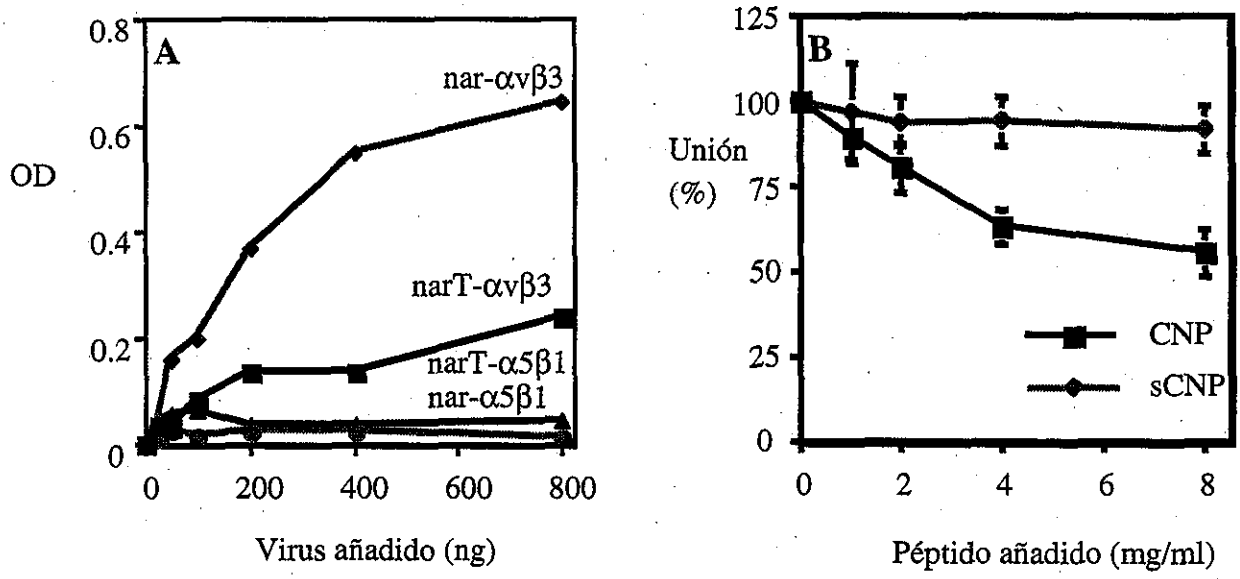

Figura 11. Unión del rotavirus nar3 no cortado a la integrina αvβ3 purificada. (A) Cantidades crecientes de virus nar3 cortado o no con tripsina se añadieron a una placa de ELISA de 96 pozos cubierta con 100 ng/pozo de integrina αvβ3 o α5β1, y se incubó por 1 h a 37 °C. La cantidad de virus unida se determinó con anticuerpos específicos contra rotavirus (ver Materiales y Métodos). Los datos se muestran como la densidad óptica obtenida, contra la cantidad de virus añadida en cada caso. El resultado mostrado es representativo de dos experimentos independientes realizados por duplicado. (B) Una placa para ELISA cubierta con integrina αvβ3 se preincubó con cantidades crecientes de los péptidos CNP o sCNP durante 1 h a 37 °C. Posteriormente, se eliminó el exceso de péptido, se añadió una concentración fija de virus (300 ng) y se incubó la placa por 1 h a 37 °C. Los datos se expresan como el porcentaje de virus unido, con respecto a la condición en la que la integrina se preincubó con PBS como control. Se muestra el promedio y la desviación estándar de al menos dos experimentos independientes realizados por duplicado.

Como se ha mencionado previamente, los ligandos naturales de la integrina αvβ3 contienen en su secuencia un motivo RGD, este tripéptido es el responsable de la unión a esta integrina (34). Para explorar si el sitio de unión del péptido CNP era independiente del que

utiliza el motivo RGD en la integrina αvβ3, preincubamos la integrina αvβ3, inmovilizada en placas de ELISA, con los péptidos RGD o HANTA, y posteriormente añadimos el péptido CNP biotinilado y se determinó la cantidad de éste que se unía a la integrina αvβ3. Encontramos que el péptido HANTA fue capaz de bloquear la unión del péptido CNP a la integrina, y que el péptido RGD no tuvo ningún efecto sobre la unión del péptido CNP biotinilado (Fig 12A); esto sugiere que tanto el péptido CNP, como el HANTA se unen a la misma región de la integrina, y que esta región es distinta de la utilizada por el péptido RGD. Para confrimar este resultado, se probó la unión del péptido RGD biotinilado en presencia del péptido CNP o del péptido RGD no marcado. Encontramos que la presencia del péptido CNP no afectó la unión del péptido RGD, mientras que cuando se añadió el péptido RGD no marcado se desplazó el pegado del péptido RGD biotinilado (Fig 12B).

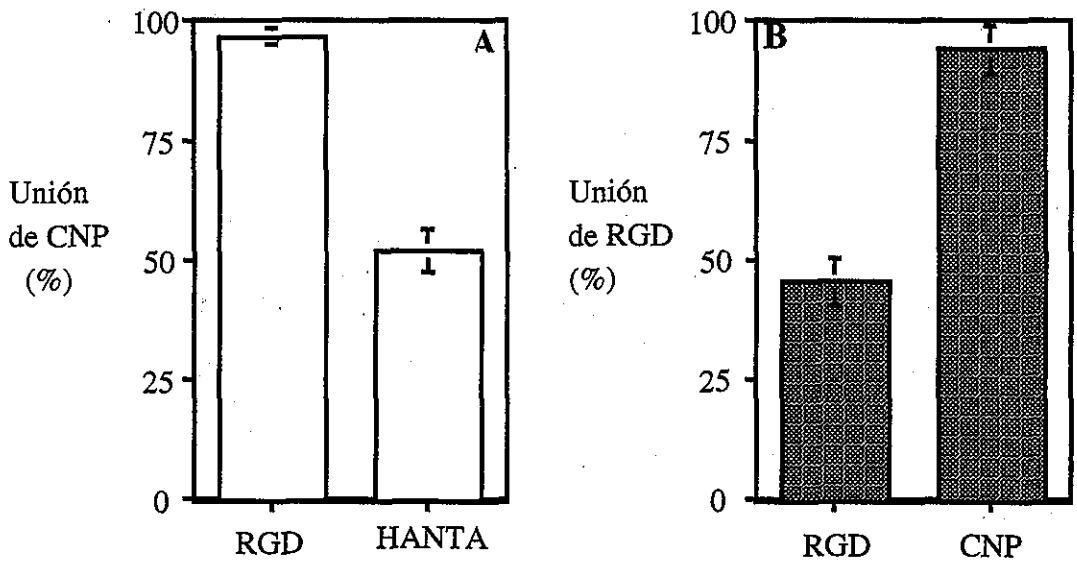

Figure 11. La unión del péptido CNP a la integrina αvβ3 es independiente del sitio de unión del péptido RGD. (A) Se añadió 1 mg/ml de los peptidos RGD o HANTA a una placa cubierta con integrina αvβ3 y se incubó por 1 h 37 °C. Posteriormente, se agregaron 0.5 mg/ml de péptido CNP biotinilado y se incubó la placa por 1 h a 37 °C. La cantidad de péptido biotinilado se determina utilizando estreptavidina peroxidasa. Los datos se expresan como el porcentaje de péptido unido, respecto a la condición control en la que la integrina se preincubó con PBS. (B) Una placa cubierta con integrina αvβ3 se preincubó con 1 mg/ml de péptido CNP o RGD, por 1 h 37 °C. Posteriormente se agregaron 0.5 mg/ml de péptido RGD biotinilado y la cantidad de éste unido a la placa después de incubarla por 1 h 37 °C, se determinó utilizando estreptavidina peroxidasa. Los datos se expresan como el porcentaje de péptido RGD unido con respecto al control sin péptido. Se muestra el promedio y la desviación estándar de al menos dos experimentos independientes realizados por duplicado.

Estos datos nos permiten afirmar que hemos encontrado una nueva región de interacción con integrinas del tipo αvβ3, que es utilizada por los rotavirus y que es independiente del sitio de unión utilizado por el motivo RGD. Es posible que esta secuencia sea también utilizada por los hantavirus en su interacción con la integrina αvβ3, sin embargo esta posibilidad debe ser confirmada. Además este nuevo motivo de unión a integrinas de tipo αvβ3 podría ser utilizado por otras proteínas virales o celulares; una primera búsqueda en bancos de secuencias, dio como resultado un conjunto de proteínas tanto celulares como bacterianas y virales que poseen regiones similares al péptido CNP, sin embargo es necesario evaluar la relevancia de esta región en cada caso. Tomando en cuenta solamente las proteínas virales obtenidas en esta búsqueda encontramos regiones parecidas al péptido CNP en proteínas de distintos virus, la comparación de ellas se muestra en la figura 13. Si bien en muchos de los casos no se ha demostrado que estos virus interaccionen con la integrina αvβ3, sería interesante evaluar el papel que pudiera tener esta integrina en el ciclo replicativo de los distintos virus, ya sea como receptor, o en la modulación de procesos celulares que afecten el proceso de infección.

Hantavirus G1G2 (L99)	**NSWACNPPD**
Coronavirus 229E antireceptor	**SITPCNPPD**
Hepatitis C poliprotein	**ARPDCNPPL**
Ranid herpes virus	**YWWQCNPPV**
Hantavirus G1G2(NY)	**TSWGCNPPD**
Rabbit fibroma virus	**ITRLCNPMK**
Gibbon ape leukemia virus envelope	**QTGWCNPLK**
Rotavirus G195C VP7	**REWLCNPMD**
RRV VP7	**NEWLCNPMD**
consensus	**w CNPpd**

Figure 13. Alineamiento múltiple de las proteínas virales que contiene regiones similares a la región CNP de la proteína VP7 de los rotavirus. La secuencia consenso se determinó utilizando los programas Clustawl y Box Shade.

RESULTADOS ADICIONALES

5. Caracterización de la unión de los rotavirus

Además de los resultados presentados anteriormente, durante mi trabajo de doctorado participé en otros proyectos relacionados con el estudio de los primeros eventos de la infección por rotavirus. A continuación se describen brevemente estos trabajos.

A. Caracterizción de un AcM dirigido contra la superficie de células MA104 que bloquea la infectividad de los rotavirus.

En el laboratorio se aisló el AcM 2D9, este anticuerpo se obtuvo utilizando células MA104 tratadas con NA como inmunógeno y se seleccionó por su capacidad de bloquear la infectividad del virus nar3. En este trabajo se utilizaron las cepas de rotavirus RRV y nar3, así como la cepa de origen humano Wa, cuya infectividad es resistente al tratamiento de las células con NA. El AcM 2D9 es una inmunoglobulina de tipo M, que disminuye la infectividad de las cepas de rotavirus RRV y nar3; además este anticuerpo se une a la superficie de la línea celular MA104, indicando que el antígeno al cual reconoce se encuentra presente en la superficie de estas células, aunque la molécula específica no ha sido identificada. Cuando probamos el efecto del AcM 2D9 en la unión de los virus RRV y nar3, encontramos que al preincubar las células con este anticuerpo, la unión del virus nar3 se bloqueó en un 80 %, pero la unión de RRV no se afectó. Además, se probó el efecto del AcM 2D9 en la unión de las proteínas recombinantes GST-VP8 y GST-VP5, y consistentemente con los datos encontrados para nar3 y RRV, el AcM 2D9 no afectó la unión de la proteína GST-VP8, pero fue capaz de bloquear la unión de GST-VP5. Estos resultados, así como la caracterización del AcM 2D9 fueron publicados en el artículo "Characterization of a monoclonal antibody directed to the surface of MA104 cells that blocks the infectivity of rotaviruses" (49), que se presenta a continuación .

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Characterization of a Monoclonal Antibody Directed to the Surface of MA104 Cells That Blocks the Infectivity of Rotaviruses

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Rhesus rotavirus (RRV) binds to sialic acid residues on the surface of target cells, and treatment of these cells with neuraminidase greatly reduces virus binding with the consequent reduction of infectivity. Variants that can efficiently infect neuraminidase-treated cells have been isolated, indicating that attachment to sialic acid is not an essential step for animal rotaviruses to infect cells. To identify and characterize the neuraminidase-resistant receptor for rotaviruses, we have isolated a hybridoma that secrets a monoclonal antibody (MAb) (2D9) that specifically blocks the infectivity of wild-type (wt) RRV and of its sialic acid-independent variant nar3, in untreated as well as in neuraminidase-treated cells. The infectivity of a human rotavirus was also inhibited, although to a lesser extent. MAb 2D9 blocks the binding of the variant to MA104 cells, while not affecting the binding of wt RRV; in addition, this MAb blocked the attachment of a recombinant glutathione *S*-transferase (GST)–VP5 fusion protein, but did not affect the binding of GST–VP8. Altogether these results suggest that MAb 2D9 is directed to the neuraminidase-resistant receptor. This receptor seems to mediate the direct attachment of the variant to the cell, through VP5, while the receptor is used by wt RRV for a secondary interaction, after its initial binding to sialic acid, through VP8. MAb 2D9 interacts specifically with the cell surface by indirect immunofluorescence, immunoelectron microscopy, and FACS. By a solid-phase immunoisolation technique, MAb 2D9 was found to react with three proteins of ca. 47, 55, and 220 kDa, which might form a complex.

INTRODUCTION

The initial event in the interaction of a virus with the host cell is the attachment of the virus to receptors in the cell membrane. Attachment is mediated by a variety of moieties on host cells ranging from the general, such as sialic acid (SA), to specific extracellular integral membrane proteins. Any cell surface entity that mediates this attachment is defined as a viral receptor; consequently the expression of the receptor on specific cells or tissues in the whole host is a major determinant of the route of virus entry into the host, the pattern of virus spread, and the resulting pathogenesis (Haywood, 1994). An understanding of the mechanisms of viral cell attachment may provide insight into the tissue tropism of a particular virus, as well as potential treatments for viral diseases.

Rotaviruses are the leading cause of morbidity and mortality, due to acute gastroenteritis, in children under 2 years of age (Kapikian and Chanock, 1996). These viruses, members of the family *Reoviridae*, are nonenveloped and possess a genome of 11 segments of dsRNA contained in a triple-layered protein capsid. The outermost layer is composed of two proteins, VP4 and VP7. The smooth external surface of the virus is made up of 780 copies of the glycoprotein VP7, while 60 spike-like structures, formed by dimers of VP4, extend about 12 nm from the VP7 surface (Estes, 1996; Prasad *et al.*, 1990).

VP4 has essential functions in the virus life cycle, including receptor binding and cell penetration (Estes and Cohen, 1989). The properties of this protein are therefore important determinants of host range, virulence, and induction of protective immunity. The infectivity of rotaviruses is dependent on trypsin treatment of the virus, and this proteolytic treatment results in the specific cleavage of VP4 to polypeptides VP8 and VP5 (Arias *et al.*, 1996; Espejo *et al.*, 1981; Estes *et al.*, 1981). The cleavage of VP4 does not affect cell binding (Clark *et al.*, 1981; Fukuhara *et al.*, 1988; Kaljot *et al.*, 1988) and has been associated with the entry of the virus by direct plasma membrane penetration (Kaljot *et al.*, 1988; Nandi *et al.*, 1992; Ruiz *et al.*, 1994). Recently, it was shown that the virus uses both VP8 and VP5 proteins to bind to the surface of MA104 cells in a SA-dependent and -independent manner, respectively (Zárate *et al.*, 2000). The role of VP7 during the early interactions of the virus with the cell is not clear, although it has been shown that it can modulate some

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of the VP4-mediated virus phenotypes, including receptor binding (Méndez *et al.*, 1996).

Rotavirus infection is highly restricted *in vivo* to the mature villus tip cells of the small intestine. The infection *in vitro* is also restricted, being most permissive in a variety of epithelial cell lines of renal and intestinal origin (Kapikian and Chanock, 1996). The high degree of selectivity of these viruses suggests the presence of specific receptors in the surface of susceptible cells, which might be at least one of the factors responsible for determining the virus tropism.

Some rotaviruses of animal origin bind to the cell surface through a SA-containing cell receptor (Ciarlet and Estes, 1999; Fukudome *et al.*, 1989; Keljo and Smith, 1988; Méndez *et al.*, 1993). We have isolated variants from a SA-dependent rhesus rotavirus (RRV) that no longer depend on the presence of SA to bind and thus to infect the cell. The characterization of these variants showed that binding to SA is not an essential step for infection of cells by SA-dependent animal rotaviruses; it also showed that the initial interaction with SA can be superseded by an interaction with a secondary receptor [neuraminidase (NA)-resistant] that might be responsible, at least in part, for the tropism of these viruses (Méndez *et al.*, 1993). In contrast to animal rotaviruses, most, if not all, human rotaviruses do not require SA to infect the cells (Ciarlet and Estes, 1999; Keljo and Smith, 1988).

Based on competition experiments among strains of human and animal origin, together with a SA-independent RRV variant, it was established that the interaction of rotavirus with its host cell is a multistep process in which a SA-containing component, a SA-independent molecule, and probably a third unidentified molecule are involved (Méndez *et al.*, 1999).

Recently, it was reported that VP4 and VP7 contain integrin ligand sequences, and peptides containing these ligand motifs and monoclonal antibodies (MAbs) to the respective integrins inhibited the infectivity of the simian rotavirus strain SA11 and of the human rotavirus strain RV5, implicating $\alpha 2\beta 1$ and $\alpha 4\beta 1$ integrins in the cell entry of these viruses (Coulson *et al.*, 1997; Hewish *et al.*, 2000).

To identify other possible cell surface molecules involved in the process of rotavirus entry we have generated MAbs directed to MA104 cells. In this work we report the isolation and characterization of a MAb directed to the surface of these cells that is able to specifically prevent the infectivity of the SA-dependent RRV and of its NA-resistant variant nar3.

RESULTS

Isolation and screening of hybridomas. We were interested in isolating a MAb directed to the NA-resistant rotavirus receptor. Thus, hybridomas were prepared from mice immunized with whole NA-treated MA104 cells. Approximately 2500 hybridoma culture supernatants were screened for their ability to block the infectivity of the NA-resistant rotavirus variant nar3 in an immunoperoxidase focus reduction assay (Arias *et al.*, 1987). One hybridoma that efficiently inhibited nar3 infection was subcloned three times by limiting dilution until a stable antibody-producing clone was isolated. The MAb secreted by this clone, 2D9, was isotyped as IgM. In the experiments described next, MAb 2D9 was used as a purified fraction from ascites fluid.

Virus specificity of monoclonal antibody 2D9. The specificity of MAb 2D9 was determined by its ability to block the infectivity of the wt virus RRV, its SA-independent variant nar3, and the human rotavirus strain Wa. Preincubation of MA104 cells with different dilutions of MAb 2D9 resulted in the reduction of the focus-forming units (FFU) by 75% for nar3, by 60% for RRV, and by 25% for Wa. This reduction in infectivity was dependent on the concentration of MAb added to the cell monolayer. Preincubation of the cells with a commercial, control mouse IgM did not affect the infectivity of any of these viruses (Fig. 1).

Given that the hybridoma secreting 2D9 was obtained by immunizing mice with MA104 cells treated with NA, we tested the ability of the MAb to prevent the infection of the same three virus strains on cells treated with this enzyme. Given the low level of infectivity of RRV under these conditions (Méndez *et al.*, 1993), the amount of RRV used in this experiment was increased sixfold to maintain a similar number of FFU per well. The blocking activity of MAb 2D9 in NA-treated cells for the three virus strains was essentially that observed in untreated cells (Fig. 1).

To rule out the possibility that MAb 2D9 protected MA104 cells by nonspecific masking of the cell surface, the ability of this antibody to protect MA104 cells against the infection of two other nonenveloped viruses was evaluated. Figure 2 shows that 2D9 did not affect the infectivity of either poliovirus type 3 or reovirus type 1 in MA104 cells.

Altogether these results indicate that MAb 2D9 is able to specifically block the infectivity of rotaviruses RRV and nar3, and to a lesser extent that of the human strain Wa, and that, most likely, the epitope recognized by this MAb does not involve SA.

MAb 2D9 prevents the binding of nar3 but not that of RRV. Since MAb 2D9 was able to significantly block the infectivity of both RRV and its SA-independent variant nar3 in MA104 cells, we next asked whether the inhibition of infectivity was due to the prevention of binding of the viruses to the cell surface. For this, we used a nonradioactive binding assay in which the cell-bound virus was detected by a specific enzyme-linked immunosorbent assay (ELISA) (Zárate *et al.*, 2000). In this experiment, a suspension of MA104 cells was preincu-

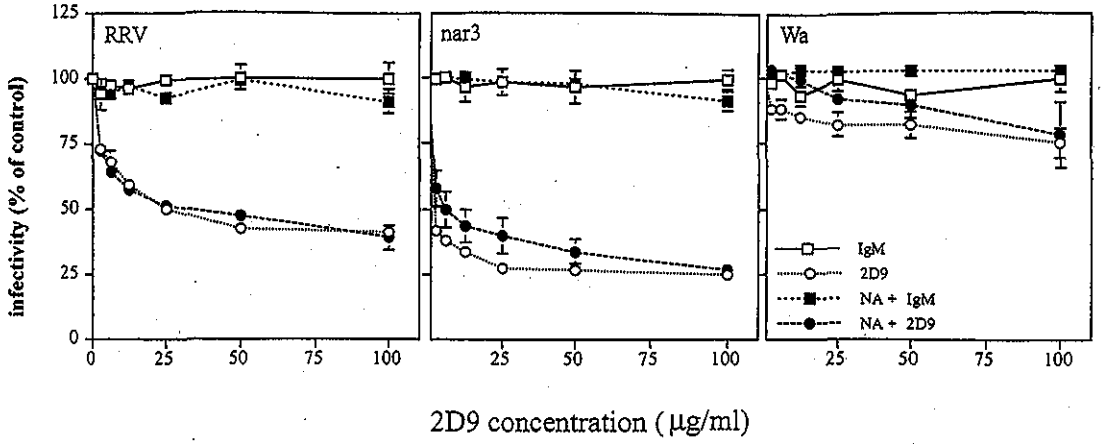

FIG. 1. MAb 2D9 blocks rotavirus infectivity in neuraminidase-treated and untreated MA104 cells. The indicated concentrations of purified 2D9 or a control IgM were added to monolayers of MA104 in 96-well plates that had been either treated (solid symbols) or not (open symbols) with NA for 1 h at 37°C. After incubation with the antibodies, the cells were washed twice with PBS and then 2×10^3 FFU of RRV, nar3, or Wa viruses was added per well to both treated and untreated cells, with the exception of RRV in NA-treated cells, where 1.2×10^4 FFU was used, since the infectivity of RRV under these conditions is reduced five- to six-fold. After 1 h of adsorption at 37°C, the viral inoculum was removed, and the infection was left to proceed for 16 h at 37°C, at which time the cells were fixed and immunostained, as described under Materials and Methods. Data are expressed as a percentage of the virus infectivity obtained when the cells were preincubated with PBS as a control. The arithmetic mean ± standard error from five independent experiments performed in duplicate is shown.

bated with different dilutions of either 2D9 or a control mouse IgM for 1 h at 4°C; after removal of the excess antibody the cells were incubated with a fixed amount of either RRV or nar3. We found that while the preincubation of the cells with 2D9 did not affect the binding of wt RRV, the binding of nar3 was reduced in a concentration-dependent manner up to about 25% of that of the control cells (Fig. 3). The binding of the human strain Wa, in the presence of either 2D9 or IgM, was not significantly affected (not shown).

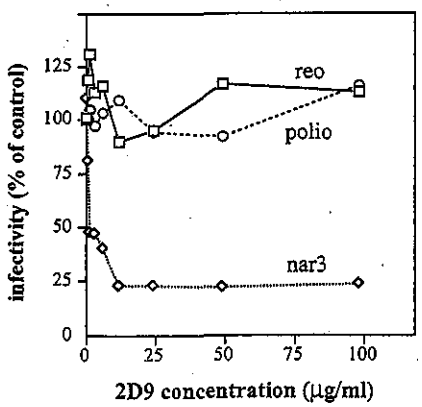

FIG. 2. Blocking specificity of MAb 2D9. The indicated concentrations of purified 2D9 were added to monolayers of MA104 in 96-well plates for 1 h at 37°C and washed twice with PBS and then, approximately 2×10^3 FFU of nar3, reovirus type 1, or poliovirus type 3 was added per well. After 1 h of adsorption at 37°C, the viral inoculum was removed, and the infection was left to proceed for 16 h at 37°C, at which time the cells were fixed and immunostained, as described under Materials and Methods. Data are expressed as percentage of the virus infectivity obtained when the cells were preincubated with PBS as a control. The arithmetic mean ± standard error from two independent experiments is shown.

We have recently shown that RRV initially binds to the cell surface through the VP8 domain of VP4, while nar3 binds through VP5 (Zárate *et al.*, 2000). Since 2D9 is able to inhibit the binding of nar3 but not that of RRV, we next asked whether this MAb was able to differentially prevent the binding of recombinant VP5 and VP8 proteins, which have been previously shown to bind specifically to the cell surface (Zárate *et al.*, 2000). For this, a suspension of MA104 cells was preincubated with MAb 2D9, or a mouse IgM as control, and then a constant amount of either glutathione *S*-transferase (GST)–VP5 or GST–VP8 fusion proteins produced in bacteria was added. The cell-bound recombinant proteins were detected by ELISA as previously described (Zárate *et al.*, 2000). Figure 4 shows that while the binding of GST–VP8 was not affected by preincubation of the cells with either 2D9 or the IgM control, the binding of GST–VP5 was reduced to about 30% of the control value with no antibody. Altogether these results suggest that MAb 2D9 might be recognizing the same molecule used by the NA-resistant variant nar3 to interact with the cell, through its VP5 protein.

The epitope recognized by 2D9 localizes to the surface of MA104 cells. The cellular localization of the epitope recognized by MAb 2D9 was investigated by immunoelectron microscopy of MA104 cells. Figure 5A shows that MAb 2D9 labeled primarily the surface of the cells as judged by the deposition of the peroxidase substrate, DAB, in the plasma membrane, whereas in the control cells, incubated with a mouse IgM, this pattern was not observed (Fig. 5B). The DAB substrate stained only one face of the cells due to the fact that fixation and immunostaining were done while the cells were still bound to the surface of the flask.

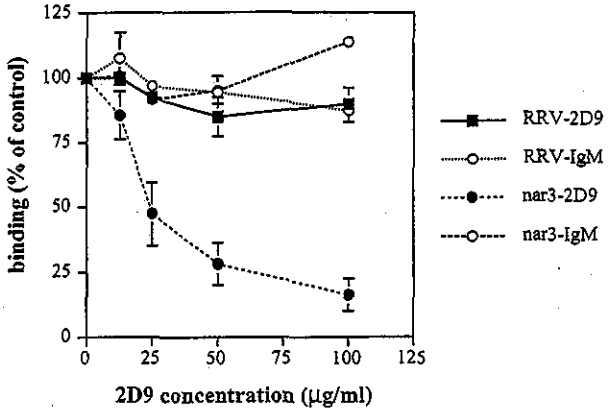

FIG. 3. Binding of RRV and nar3 viruses to cells in the presence of MAb 2D9. The indicated amounts of MAb 2D9 or a control IgM were preincubated with 5 × 10^4 MA104 cells in suspension for 1 h at 4°C. The excess, unbound antibody was removed, and then 300 ng of either RRV or nar3 purified virus particles was added, and the mixture was further incubated for 1 h at 4°C. The amount of virus bound to cells was determined by an ELISA as described under Materials and Methods. Data are expressed as percentage of the virus binding obtained when the virus particles were preincubated with PBS as a control. The arithmetic mean ± standard error from two independent experiments performed in duplicate is shown.

The presence of the epitope recognized by 2D9 on the cell surface was further confirmed by indirect immunofluorescence of unpermeabilized MA104 cells. MAb 2D9 recognized an epitope that was distributed in a patched pattern on the surface of the cells, which was more clearly observed in the junction between cells (Fig. 5C). Control cells stained with an IgM did not show this pattern (Fig. 5D). Also, using flow cytometric analysis, we found that 2D9 was able to bind to the surface of MA104 cells (Fig. 5E), as predicted if directed to a virus receptor structure.

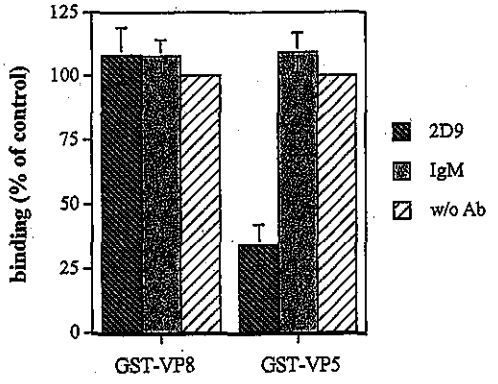

FIG. 4. Binding of recombinant proteins GST–VP8 and GST–VP5 to cells in the presence of MAb 2D9. MAb 2D9 or a control IgM (100 μg/ml) was preincubated with 5 × 10^4 MA104 cells in suspension for 1 h at 4°C. The excess, unbound antibody was removed, and then 1.5 μg of affinity-purified GST–VP5 or GST–VP8 was added, and the mixture was further incubated for 1 h at 4°C. The amount of cell-bound protein was determined by ELISA as described under Materials and Methods. Data are expressed as percentage of the recombinant protein binding obtained when the fusion proteins were preincubated with PBS as a control (w/o MAb). The arithmetic mean ± standard error from two independent experiments performed in duplicate is shown.

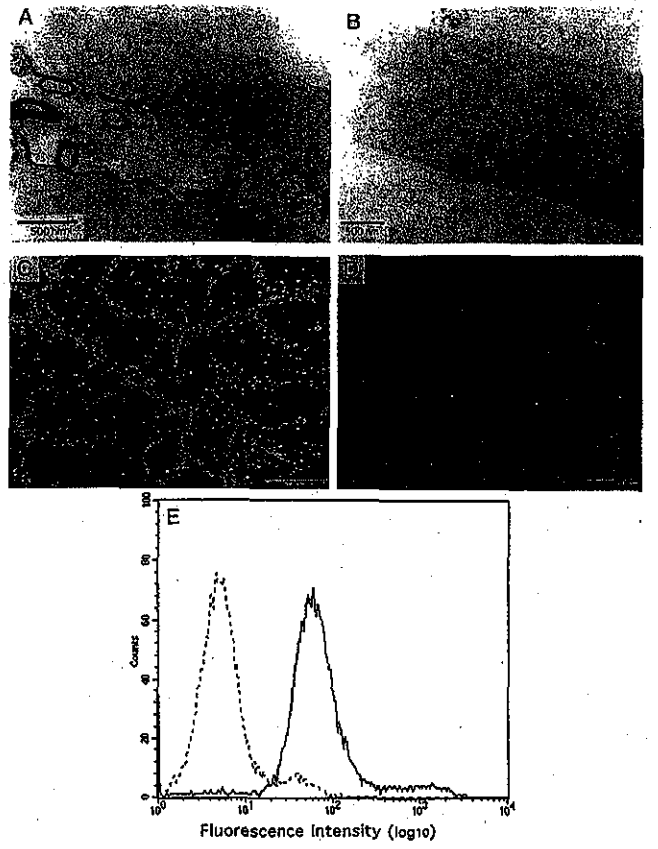

FIG. 5. Monoclonal antibody 2D9 interacts with the surface of MA104 cells. (A and B) Electron micrographs of immunoperoxidase staining of MA104 cells. Immunomarking was performed directly on MA104 cell monolayers, which were incubated with (A) MAb 2D9 (10 μg/ml) or with (B) a control IgM (10 μg/ml) and then stained with peroxidase-conjugated goat anti-mouse IgM and DAB as substrate. The cells were then detached from the flasks, postfixed with 1% OsO_4, and processed for electron microscopy as detailed under Materials and Methods. (C and D) Indirect immunofluorescence of MA104 cells. Cells were incubated with MAb 2D9 (10 μg/ml; C) or with a control IgM (10 μg/ml; D) and then stained with affinity-purified goat anti-mouse IgM antibodies conjugated to fluorescein isothiocyanate. (E) Binding of MAb 2D9 to MA104 cells as determined by flow cytometry. Cells were incubated either with MAb 2D9 (20 μg/ml, solid line) or with IgM (20 μg/ml, dashed line) and stained with affinity-purified goat anti-mouse IgM antibodies conjugated to fluorescein isothiocyanate. The amount of IgM bound was assayed by flow cytometry as described under Materials and Methods.

Solid-phase immunoisolation of MA104 cell proteins by MAb 2D9. Attempts to immunoprecipitate proteins from a cell lysate with MAb 2D9 were unsuccessful. Thus, to determine the cell surface protein to which the MAb was directed, we performed a solid-phase immunoisolation technique (SPIT) assay, in which either MAb 2D9 or a control IgM were adsorbed to wells of an ELISA plate. Total MA104 cell lysates labeled metabolically with ^{35}S or with sulfo-NHS-biotin, which labels proteins exposed in the cell surface (since it is a membrane-impermeable

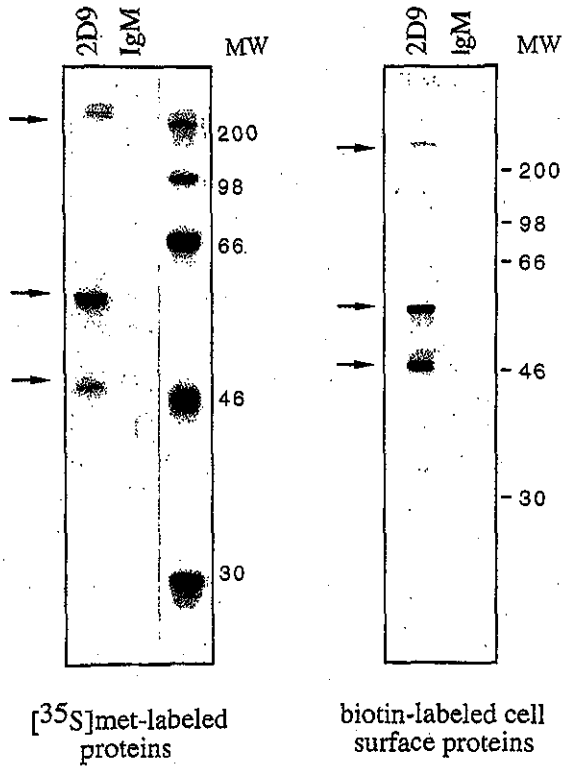

FIG. 6. Solid-phase immunoisolation of MA104 cell proteins by MAb 2D9. Cell lysates metabolically labeled with ^{35}S ([^{35}S]Met-labeled proteins) or with sulfo-NHS-biotin (biotin-labeled proteins) were added to antibody (2D9 or IgM)-coated wells. After incubation, the wells were washed, and the proteins that remained bound to the wells were released with Laemmli sample buffer. The samples were analyzed by SDS–PAGE and fluorography (for ^{35}S-labeled proteins) or by Western blot (for the biotin-labeled proteins) staining with streptavidin coupled to peroxidase, as indicated under Materials and Methods. MW, molecular weight markers.

agent), were added to the antibody-coated wells. The cells were later extensively washed, and the proteins that remained bound were released with Laemmli sample buffer and analyzed by SDS–PAGE and fluorography (for ^{35}S-labeled proteins) or transferred to nitrocellulose (for the biotin-labeled proteins) and stained with streptavidin coupled to peroxidase (Fig. 6). It can be observed that MAb 2D9 captured from the total protein cell lysate a group of three proteins of ca. 47, 55, and 220 kDa, which were not recognized by the control IgM. These three proteins must be exposed on the surface of the cell, since the SPIT assay performed with the biotin-labeled polypeptides showed three proteins with the same molecular masses. Again, when the biotin-labeled lysate was incubated in a well coated with the control IgM, no biotinylated proteins were observed. These results suggest that MAb 2D9 interacts with a cell surface protein that might be forming a complex of at least three proteins or that the three proteins that are captured by 2D9 share a common epitope recognized by the antibody.

DISCUSSION

It has been known for some time that rotaviruses can interact with the surface of susceptible cells by at least two different mechanisms; some rotaviruses of animal origin attach to a sialic acid-containing molecule, while rotaviruses of human origin bind through a neuraminidase-resistant compound (Ciarlet and Estes, 1999; Mendez *et al.*, 1993). However, it has been recently shown that the initial interaction of animal rotaviruses with SA is not essential, since variants that bind and infect cells in a SA-independent manner have been isolated from the SA-dependent RRV (Ciarlet and Estes, 1999; Ludert *et al.*, 1996; Mendez *et al.*, 1993).

One of the strategies that has been successfully used to identify the cell receptors for viruses belonging to several different families has been the isolation of hybridomas that secrete MAbs directed to the surface of susceptible cells, which are able to block virus entry. The putative receptors for measles virus (Dunster *et al.*, 1994), Sindbis virus (Wang *et al.*, 1992), enterovirus 70 (Karnauchow *et al.*, 1996), echoviruses (Bergelson *et al.*, 1994), and vaccinia virus (Chang *et al.*, 1995), among other examples, have been identified following this strategy.

Following this approach, we isolated a hybridoma that secretes a MAb directed against the surface of NA-treated MA104 cells, a highly permissive cell line for rotavirus. This MAb (2D9), of IgM isotype, is able to specifically block the infectivity of both wt RRV and its variant nar3, and to a lesser extent, the infectivity of the human strain Wa. MAb 2D9 also blocks the infectivity of these viruses in NA-treated cells.

The antigen recognized by 2D9 is present on the surface of MA104 cells as judged by fluorescence-activated cell sorting (FACS) analysis, immunofluorescence, and immunoelectron microscopy. Using the first two methods we screened several cell lines for the presence of this antigen. We found that MAb 2D9 specifically recognized the surface of CV-1, CaCo2, and COS7 cells, all susceptible to rotavirus infection. However, this MAb also reacted with the surface of Hep-2, L, CHO, and BHK cells, which are much less susceptible to infection by rotaviruses (Espinosa *et al.*, unpublished results), indicating that the antigen recognized by 2D9 is not the only factor that determines the tropism of these viruses.

A number of glycoconjugates have been shown to bind to, and to block the infectivity of, SA-dependent animal rotavirus strains, and some of them have been suggested to play a role as possible receptors, like GM3 gangliosides in newborn piglet intestine (Rolsma *et al.*, 1998), GM1 in LLC-MK2 cells (Superti and Donelli, 1991), and 300 to 330-kDa glycoproteins in murine enterocytes (Bass *et al.*, 1991). More recently, it was reported that integrins $\alpha 2\beta 1$ and $\alpha 4\beta 1$ are involved in the entry of rotaviruses (Coulson *et al.*, 1997). The fact that the

epitope recognized by MAb 2D9 is present on the surface of CHO and L cells, which do not express $\alpha 2$ or $\alpha 4$ integrins (Hewish *et al.*, 2000; Zhang and Racaniello, 1997), and the fact that the reported molecular weight for these integrins does not coincide with that of the proteins detected by 2D9 suggest that this MAb recognizes a molecule different from integrins $\alpha 2\beta 1$ and $\alpha 4\beta 1$.

We have recently reported that wt RRV interacts initially with SA residues on the surface of MA104 cells, through the VP8 domain of its VP4 protein. We proposed that this initial interaction is subsequently followed by a second interaction of the VP5 domain of VP4 with a NA-resistant cell receptor. We also showed that the SA-independent variant nar3 is able to interact directly with the cell surface through VP5, obviating the first VP8–SA interaction (Zárate *et al.*, 2000). In this work we have found that MAb 2D9, despite inhibiting the infectivity of both wt RRV and nar3 viruses, competes only the attachment of the variant, suggesting that it blocks the infectivity of the wt virus at a postattachment step, in agreement with our previous observations. In accordance with these results, we found that 2D9 blocks the binding of the GST–VP5 fusion protein, while it does not affect the attachment of the GST–VP8 recombinant polypeptide (Fig. 4). Taken together, these results suggest that the epitope recognized by 2D9 is independent of sialic acid and is probably present in the cell receptor that interacts with VP5 or in a molecule closely associated with it.

Based on an infection competition assay, designed to detect competition for cell surface molecules at both attachment and postattachment steps (Mendez *et al.*, 1999), we found that the human strain Wa efficiently competed the infectivity of the variant nar3 both in untreated and in NA-treated cells. This competition was nonreciprocal since nar3 did not compete the infectivity of Wa. The fact that the competition between the two NA-resistant strains, nar3 and Wa, was not reciprocal indicates that they bind to different molecules. In addition, the SA-dependence phenotype clearly differentiates RRV from nar3 and Wa, suggesting the existence of at least three cellular structures involved in rotavirus cell infection, with at least one being shared by human, SA-dependent animal, and NA-resistant variant strains. The antigen recognized by 2D9 on the surface of the cell might represent one of these interactions.

The list of viruses that have more than one interaction with the cell surface during cell entry is growing (Haywood, 1994; Norkin, 1995), indicating that our initial view that the virus–receptor interactions resembled those of simple ligands with their receptors was not completely true. It is becoming more and more apparent that the interaction of a virus with the surface of its host cell, which ultimately leads to the entry of the virus into the cell's cytoplasm, is a dynamic process in which more than one virus–cell interaction often takes place and in which conformational changes of both viral and host cell proteins might occur (Haywood, 1994; Norkin, 1995; Olson *et al.*, 1999). Furthermore, it has become apparent that some viruses may use more than one receptor to gain access into their host cells (Baranowski *et al.*, 2000; Thoulouze *et al.*, 1998; Tufano, 1997).

Despite our efforts, it was not possible either to immunoprecipitate or to detect by Western blots the molecule(s) recognized by MAb 2D9. However, by a solid-phase immunoisolation technique, 2D9 specifically interacted with three proteins that were present on the surface of the cell, as judged by the fact that they could be labeled with a reagent that is impermeable to the cell membrane. Although it is possible that these three proteins contain a common epitope recognized by MAb 2D9, it seems more likely that the proteins might be forming a complex, which remains as such under the conditions used to lyse the cells, with only one of them being recognized by 2D9. The identity of these proteins is currently under investigation.

The fact that neither 2D9 nor any of the anti-integrin antibodies that have been assayed so far are able to completely block the infectivity of rotaviruses [this work and Coulson *et al.*, (1997)], together with the finding that MAb 2D9 reacts with the surface of cells that are poorly susceptible to rotavirus, supports our idea that the interaction of rotavirus with its host cell is a multistep process (Mendez *et al.*, 1999) that involves interactions with several different molecules on the cell surface. The exquisite tropism of rotaviruses, which *in vivo* infect only a very narrow set of cells in the intestine, might be explained if only this type of cell possesses on its surface the appropriate combination of the required receptor molecules, which might be present individually in many different cell types. It remains to be determined how many cell surface molecules are involved in the complex process of rotavirus cell attachment and penetration and the role they may play.

MATERIALS AND METHODS

Cells, viruses, and monoclonal antibodies. MA104 and L929 cells (L cells) were cultured in Eagle's minimal essential medium (MEM) supplemented with 10% fetal bovine serum. Rotavirus strains RRV and Wa were originally obtained from H. B. Greenberg (Stanford University, Stanford, CA), and rotavirus variant nar3 has been described previously (Méndez *et al.*, 1993). RRV, Wa, and nar3 viruses were propagated in MA104 cells as previously described (Espejo *et al.*, 1980). Reovirus serotype 1 was obtained from C. Ramos (Instituto Nacional de Salud Pública, Cuernavaca, Morelos, México) and was grown in L cells as described (Cuadras *et al.*, 1997). Poliovirus type 3 was obtained from R. M. del Angel (CINVESTAV, Mexico D.F., Mexico) and was grown in MA104 cells. Rabbit polyclonal antibody against reovirus type 1 was

kindly provided by T. Dermody (Vanderbilt Medical School, Nashville, TN).

To prepare purified virus, virus-infected cells were harvested after complete cytopathic effect was attained, the cell lysate was frozen–thawed twice, and the virus was pelleted by centrifugation for 60 min at 25,000 rpm at 4°C in the SW28 rotor (Beckman). The virus pellet was resuspended in TNC buffer [10 mM Tris–HCl (pH 7.5), 140 mM NaCl, 10 mM $CaCl_2$], extracted with Freon, and subjected to isopicnic centrifugation in CsCl as previously described (Espejo *et al.,* 1981). The protein content of the purified triple-layered particles was determined by the Bradford protein assay (Bio-Rad).

The infectious titer of the viral preparations was obtained by an immunoperoxidase focus assay in MA104 cells grown in 96-well tissue culture plates, as described (Arias *et al.,* 1987). Titers are expressed as focus-forming units per milliliter. When indicated, cells were treated with 20 mU/ml of NA from *Arthrobacter ureafaciens* (Sigma Chemical Co.) for 1 h at 37°C. After two washes with PBS [0.2 M NaCl, 2.7 mM KCl, 1.4 mM KH_2PO_4, 0.8 mM Na_2HPO_4 (pH 7.4)], the cells were infected as described (Méndez *et al.,* 1993).

Production of monoclonal antibodies. Confluent monolayers of MA104 cells were washed and brought into a single-cell suspension by incubation with 5 mM EDTA in PBS for 10 min at 37°C and dispersed by gentle pipetting. The cell suspensions were centrifuged at 1000 rpm for 1 min at 4°C, washed, resuspended into PBS, and treated with 20 mU of *A. ureafaciens* neuraminidase (Sigma Chemical Co.) for 1 h at 37°C with gentle rocking. The cells were then washed twice with PBS and resuspended in PBS and their concentration was determined with a hemocytometer. Inbred female BALB/c mice (8 weeks old) were immunized intraperitoneally with 7.5×10^6 whole MA104 cells, pretreated with NA, at 2-week intervals (four times total). Antibody production was monitored by measuring the ability of the sera to block the infectivity of nar3 and RRV viruses (see above). Two days prior to cell fusion, mice were primed by tail vein injection of 1×10^7 whole MA104 cells in PBS, pretreated with NA. Spleens from immunized mice were fused with FOX myeloma cells essentially as described by Padilla-Noriega *et al.,* (1993). Supernatants from viable hybridoma cultures were screened for the presence of antibodies that blocked the infectivity of nar3 by an immunoperoxidase focus reduction assay, in MA104 cells grown in 96-well tissue culture plates, as previously described (Arias *et al.,* 1987). Hybridomas producing blocking antibodies were cloned three times by limiting dilution and their supernatants were retested.

The blocking activity of one hybridoma, named 2D9, was confirmed after repeated tests. This MAb was shown to be of the IgM class by double immunodiffusion using isotype-specific antibodies (ICN). For production of ascites fluid containing MAb 2D9, 8-week BALB/c female mice, primed intraperitoneally with 200 μl of Pristane, were inoculated with the 2D9 hybridoma. The ascitic fluid was collected 2 weeks after the hybridoma immunization, and the IgM fraction was obtained by dialysis of the ascitic fluid in distilled water as described (Andrew *et al.,* 1997). The protein content of the purified IgM fraction was determined by the Bradford protein assay (Bio-Rad) and by OD_{280} reading.

Binding assays. The binding of purified rotavirus particles and of affinity-purified GST–fusion proteins to MA104 cells in suspension was performed by a nonradioactive binding assay essentially as described by Zárate *et al.,* (2000). Briefly, a suspension of 5×10^4 cells preincubated with the appropriate dilution of MAb 2D9 or control IgM for 1 h at 4°C was mixed either with purified virus or with recombinant proteins (previously sonicated and centrifuged for 2 min in the Eppendorf centrifuge) in MEM–1% bovine serum albumin (BSA) in a final volume of 200 μl and incubated for 1 h at 4°C with gentle mixing. The cell–virus or cell–protein complexes were washed three times with ice-cold PBS containing 0.5% BSA and then treated with 50 μl of lysis buffer (LyB) [50 mM Tris(pH 7.5), 150 mM NaCl, 0.1% Triton X-100]. In the last wash, the cells were transferred to a fresh tube. The virus and recombinant proteins present in the lysates were quantified by ELISAs. In all binding assays of either virus or recombinant proteins, a binding control with no cells was performed.

Capture ELISAs for rotavirus particles and GST–fusion viral proteins. To detect the virus particles, goat and rabbit polyclonal sera to rotavirus were used as capture (diluted 1:10,000) and detection (diluted 1:1500) antibodies, respectively. The rotavirus proteins fused to GST were captured with the goat anti-rotavirus serum and detected with a rabbit serum to GST (diluted 1:1500). In general, the ELISA was performed as follows: polystyrene 96-well plates were coated with 100 μl of capture antibody diluted in PBS, for 2 h at 37°C. Residual free protein-binding sites were blocked by incubation with 200 μl of 1% (w/v) BSA in PBS for 2 h at 37°C. Incubation with 50 μl per well of viral or protein antigen sample in lysis buffer for 1 h at 37°C was followed by incubation with 50 μl per well of the appropriate detection antibody (see above) diluted in 1% BSA in PBS. Finally, 50 μl per well of the respective alkaline phosphatase-conjugated anti-immunoglobulin serum [goat anti-rabbit IgG or goat anti-mouse IgG (diluted 1:1500), Kirkergaard and Perry] was incubated for 1 h at 37°C, and then Sigma 104 phosphatase substrate diluted in diethanolamine buffer [100 mM diethanolamine (pH 9.4), 1 mM $MgCl_2$, 5 mM sodium azide] was added. The absorbance at 405 nm was recorded with a Microplate Autoreader EL311 (Bio-Tek Instruments).

Cloning, expression, and purification of GST–fusion proteins. The cloning, expression, and purification of RRV GST–VP8 and GST–VP5 proteins have been described (Iša *et al.,* 1997; Zárate *et al.,* 2000).

Flow cytometry (FACS). MA104 cells grown to 80% confluence were washed and brought into a single-cell

suspension by incubation with 0.5 mM EDTA in PBS at 37°C and dispersed by gentle pipetting. Cells were collected by low-speed centrifugation (200 *g*) and resuspended in ice-cold MEM without serum, and the cell concentration was determined with a hemocytometer. In each experiment, 5×10^5 cells were incubated with either MAb 2D9 or IgM control antibody (20 μg/ml) for 1 h at 4°C, washed twice with 2% fetal calf serum in PBS, and then incubated with fluorescein-conjugated anti-mouse IgM antibodies (15 μg/ml; Biosourse, USA) for 1 h at 4°C. Fluorescence-activated cell sorting analysis was done using a FACScan flow cytometer and Cellquest software (Becton Dickinson) with appropriate gating parameters.

Biotinylation of cell surface proteins. A single-cell suspension of MA104 cells was prepared as described above. Cells (1×10^7) were incubated for 30 min at 4°C with water-soluble sulfo-NHS-biotin (2 mg/ml; Pierce) in PBS, with occasional gentle mixing. Unreacted sulfo-NHS-biotin was blocked by incubation with an equal volume of 10 mM glycine in PBS for 30 min at 4°C, and then the cells were washed twice with PBS and solubilized with LyB supplemented with 1 mM PMSF, 20 μg/ml aprotinin, and 20 μg/ml leupeptin for 10 min at 4°C. After lysis the cells were centrifuged for 10 min at 3500 rpm in the Eppendorf microfuge and the cell lysate was used for the SPIT assay (see below).

Radiolabeling of MA104 cell proteins. MA104 cells were grown in 6-well plates (Costar) to approximately 70% confluence. Cell monolayers were washed twice with methionine-free MEM and incubated for 16 h in radiolabeling medium [MEM with 1/20 the regular concentration of methionine, 10% dialyzed serum, and 100 μCi/ml Easy Tag Express-^{35}S protein labeling mix (NEN)]. Labeled cells were washed twice with PBS and solubilized with 1 ml of LyB as described above for the biotin-labeled cells.

Solid-phase immunoisolation technique. Direct SPIT was carried out essentially as described by Burns *et al.*, (1988). ELISA 96-well plates were coated with 50 μl of the antibody of interest (10 μg/ml) in PBS overnight at 4°C. The plates were washed four times with PBS and blocked with 0.5% gelatin in PBS for 1 h at 37°C, followed by addition of the radiolabeled or biotinylated cell lysates. After overnight incubation at 4°C, unbound lysate was removed and the plates were washed four times with wash buffer (PBS containing 0.01% SDS, 0.1% sodium deoxycholate, 1% Nonidet-P40). The bound proteins were solubilized by addition of gel sample buffer [1% SDS, 5% 2-mercaptoethanol, 0.5 M urea, 50 mM Tris–HCl (pH 6.8), 10% glycerol, 0.0025% phenol red] and boiling for 3 min. The samples were analyzed by SDS–PAGE and fluorography in the case of the ^{35}S-labeled proteins or by Western blot with streptavidin-peroxidase and enhanced chemiluminiscence (ECL, Amersham), in the case of the biotin-labeled proteins.

Indirect immunofluorescence. Semiconfluent MA104 cells grown in coverslips were fixed with 2% formaldehyde, 0.25% glutaraldehyde in PBS, for 15 min at room temperature, washed three times with PBS, and blocked with 1 M glycine in PBS (w/v), for 1 h at room temperature. After three further washes, the cells were incubated with 10 μg/ml of either MAb 2D9 or a control IgM (Sigma Chemical Co.) for 1 h at room temperature. Finally, following two more washes, the cells were incubated with a 1:20 dilution of a FITC-conjugated goat anti-mouse IgM (Sigma Chemical Co.) for 1 h at room temperature. The cells were washed three times and mounted on glass slides in 80% glycerol in PBS. The slides were analyzed by using a Bio-Rad MRC-600 confocal microscope and CoMOS MPL-1000 software.

Immunoelectron microscopy. Confluent MA104 cells in 25-cm^2 flasks were fixed with 2% formaldehyde, 0.25% glutaraldehyde in PBS, for 30 min at 4°C, washed three times with PBS, and blocked with 1% BSA in PBS for 15 min at room temperature. After three washes, the cells were incubated with 10 μg/ml of either MAb 2D9 or a control IgM (Sigma Chemical Co.) for 1 h at room temperature. Finally, following two washes with PBS, the cells were incubated with a 1:50 dilution of a peroxidase-conjugated goat anti-mouse IgM (Sigma Chemical Co.) for 1 h at room temperature. After three washes with PBS, the cells were incubated with the peroxidase substrate [0.5 mg/ml of DAB (Sigma Chemical Co.) in PBS with 0.1% H_2O_2], for 15 min at 37°C, and the reaction was stopped by removing the substrate and washing with ice-cold PBS. The cells were then detached from the flask with the aid of a rubber policeman, postfixed in 1% osmium tetroxide, dehydrated, and embedded in Poly/Bed 812/DMP30 (Polysciences, Warrington, PA); 30-μm sections were obtained from selected areas of trimmed blocks, floated onto formvar-coated nickel grids, and counterstained for 5 min with a 2.5% uranyl acetate solution in 40% ethanol. Sections were examined and photographed in a Jeol JEM 1200 EXII electron microscope.

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B. Caracterización bioquímica de los receptores de los rotavirus en células MA104

Como una aproximación para encontrar al receptor de los rotavirus se probó el efecto de inhibidores metabólicos, de la reducción de la cantidad de colesterol presente en la membrana citoplasmática y de la extracción de moléculas de la superficie celular, sobre la susceptibilidad de células MA104 a la infección por rotavirus . Estos tratamientos mostraron que el bloqueo de la N-glicosilación, la inhibición de la biosíntesis de glicolípidos, el secuestro del colesterol de la membrana y la extracción de moléculas celulares con el detergente octil-β-glucósido (OG) en condiciones no líticas, reducen la infectividad de los rotavirus en las células tratadas; de manera consistente, al preincubar a los rotavirus RRV, nar3 y Wa con el extracto celular de OG, la infectividad disminuyó con respecto al virus control incubado sólo con OG. Estos resultados invoucran N-glicoproteínas, glicolípidos y colesterol en los primeros eventos de la infección por rotavirus, sugiriendo que el receptor (o más probablemente receptores) de los rotavirus se encuentra formando parte de los microdominios lipídicos que se conocen como rafts.

Con el objeto de determinar cuales son los componentes del extracto de OG responsables del bloqueo de la infectividad se analizó la naturaleza bioquímica de éste. Se encontró que al tratar al extracto celular con proteasas, el extracto pierde la capacidad de bloquear la infectividad de los rotavirus, suigiriendo que las moléculas inhibidoras son de naturaleza protéica. Posteriormente se fraccionó el extracto y mediante electroforesis preparativa se encontraron cinco bandas que conservan la capacidad de bloquear la infectividad de los rotavirus; los pesos de esas bandas fueron de aproximadamente 30, 45, 57, 75 y 100 kDa.

Además se probó el efecto del extracto de OG en la unión de las cepas de rotavirus RRV, nar3 y Wa. Encontramos que al preincubar a los virus con el extrtacto se bloqueó la unión de las tres cepas de rotavirus en un 40 %, y que un suero policlonal evocado contra el extracto de OG bloqueó la unión de las cepas nar3 y Wa. Este trabajo fue publicado con el título "Biochemical characterization of rotavirus receptors in MA104 cells" (30).

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Biochemical Characterization of Rotavirus Receptors in MA104 Cells

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We have tested the effect of metabolic inhibitors, membrane cholesterol depletion, and detergent extraction of cell surface molecules on the susceptibility of MA104 cells to infection by rotaviruses. Treatment of cells with tunicamycin, an inhibitor of protein N glycosylation, blocked the infectivity of the SA-dependent rotavirus RRV and its SA-independent variant nar3 by about 50%, while the inhibition of O glycosylation had no effect. The inhibitor of glycolipid biosynthesis *d,l*-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) blocked the infectivity of RRV, nar3, and the human rotavirus strain Wa by about 70%. Sequestration of cholesterol from the cell membrane with β-cyclodextrin reduced the infectivity of the three viruses by more than 90%. The involvement of N-glycoproteins, glycolipids, and cholesterol in rotavirus infection suggests that the virus receptor(s) might be forming part of lipid microdomains in the cell membrane. MA104 cells incubated with the nonionic detergent octyl-β-glucoside (OG) showed a ca. 60% reduction in their ability to bind rotaviruses, the same degree to which they became refractory to infection, suggesting that OG extracts the potential virus receptor(s) from the cell surface. Accordingly, when preincubated with the viruses, the OG extract inhibited the virus infectivity by more than 95%. This inhibition was abolished when the extract was treated with either proteases or heat but not when it was treated with neuraminidase, indicating the protein nature of the inhibitor. Two protein fractions of around 57 and 75 kDa were isolated from the extract, and these fractions were shown to have rotavirus-blocking activity. Also, antibodies to these fractions efficiently inhibited the infectivity of the viruses in untreated as well as in neuraminidase-treated cells. Five individual protein bands of 30, 45, 57, 75, and 110 kDa, which exhibited virus-blocking activity, were finally isolated from the OG extract. These proteins are good candidates to function as rotavirus receptors.

Rotaviruses, the leading cause of severe dehydrating diarrhea in infants and young children worldwide, are nonenveloped viruses that possess a genome of 11 segments of double-stranded RNA contained in a triple-layered protein capsid. The outermost layer is composed of two proteins, VP4 and VP7. VP4 forms spikes that extend from the surface of the virus and has been associated with a variety of functions, including the initial attachment of the virus to the cell membrane and the penetration of the cell by the virion (14).

Rotaviruses have a very specific cell tropism, infecting only enterocytes on the tips of intestinal villi (26), which suggests that specific host receptors must exist. In vitro, they also display a strict tropism, binding to a variety of cell lines but infecting efficiently only those of renal or intestinal epithelium origin (15). Despite the advances in the molecular and structural biology of the virus, little is known about the rotavirus cell receptors. Some animal rotavirus strains interact with sialic acid (SA) on the cell surface, and this interaction is a requirement for the efficient attachment and infection of the virus to susceptible cells (9, 17, 27, 34, 39, 57). Accordingly, a number of glycoconjugates bind to and block the infectivity of animal rotaviruses in vitro and in vivo (3, 4, 6, 17, 32, 46, 52–54, 56, 57). Some of these glycoconjugates may play a role as possible receptors, like GM3 gangliosides in newborn piglet intestine (47), GM1 in LLC-MK2 cells (52), and 300- to 330-kDa glycoproteins in murine enterocytes (3). More recently, it has also been suggested that α2β1, αxβ2, and α4β1 integrins may be involved in rotavirus cell entry (11, 24).

The binding of animal rotaviruses RRV and SA11 to an SA-containing cell receptor is nonessential since variants whose infectivity is no longer dependent on the binding to these acid sugars have been isolated (35, 39). The secondary importance of SA as an attachment site for rotaviruses, at least under laboratory conditions of infection, is also reflected by the fact that the infectivity of most, if not all, human rotavirus (HRV) strains is not affected by neuraminidase treatment of cells (9, 17, 19, 41). Recently, through competition infection assays using the SA-dependent RRV, its SA-independent variant nar3, and the naturally neuraminidase-resistant HRV strain Wa, the existence of at least three cell surface sites involved in the interaction of rotaviruses with MA104 cells during the early steps of infection was determined (41).

In this study we used two approaches to characterize the cell surface structures that could serve as rotavirus receptors. In the first approach, MA104 cells were treated with metabolic inhibitors of glycosylation as well as of glycolipid synthesis to determine the effects on the infectivity of rotaviruses RRV, nar3, and Wa. In the second approach, the putative receptors for rotaviruses were extracted with the nonionic detergent octyl-β-glucoside (OG) under noncytolytic conditions. The molecules present in the extract, which were shown to inhibit rotavirus infectivity when incubated with the viruses in solution, were biochemically characterized and partially purified.

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MATERIALS AND METHODS

Cells and viruses. The human rotavirus strain Wa and the rhesus strain RRV were obtained from Harry B. Greenberg, Stanford University, Stanford, Calif. The SA-independent rotavirus RRV variant nar3 has been previously described (39, 40). All rotavirus strains were propagated in MA104 cells as described previously (13). The rhesus monkey epithelial cell line MA104 was grown in Eagle's minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and was used for all experiments carried out in this work. BHK-21, CHO, and L929 cells were grown in MEM containing 10% FBS. Reovirus type 1 and poliovirus type 3, Leon strain, were kindly provided by C. Ramos (CISEI, National Institute of Public Health, Cuernavaca, Mexico) and R. M. del Angel (CINVESTAV-IPN, Mexico City, Mexico).

Infectivity assay. MA104 cells in 96-well plates were washed twice with phosphate-buffered saline (PBS) and then about 1,000 focus-forming units (FFUs) of a trypsin-activated cell lysate containing rotaviruses RRV, nar3, Wa, or control viruses, reovirus and poliovirus, was adsorbed to the cells for 45 min at 4°C. After the adsorption period, the virus inoculum was removed, the cells were washed once with PBS, MEM was added, and the infection was left to proceed for 14 h at 37°C. The infected cells were detected by an immunoperoxidase focus detection assay, using as the detecting antibody a rabbit polyclonal hyperimmune serum to porcine rotavirus YM, as described previously (33). The FFUs were counted with the help of a Visiolab 1000 station (Biocom). This station, which was used for both image acquisition and analysis, is configured with a Matrox Meteor RGB frame grabber and a 8295 Cohu RGB CCD color TV camera. Motorized stages (Marzhauser) were adapted to an inverted Nikon Diaphot 300 microscope. The stage control unit was a Marzhauser Multicontrol MC2000, piloted by Explo (Biocom). Macro command files for Explo were developed to perform a semiautomated counting of the infected cells. In this manner, an accurate positioning in the center of each well was achieved automatically for later predefined scanning and visual counting of infected cells within a selected well area.

Treatment of MA104 cells with metabolic inhibitors. Monolayers of MA104 cells in 96-well plates were grown to confluence; either 2 μg of tunicamycin (Boehringer) per ml or 2 mM benzyl *N*-acetyl-α-D-galactosamide (benzylGal NAc) (Oxford Glyco Systems) in MEM was added, and the cells were further incubated for either 24 h (tunicamycin) or 3 days (benzylGalNAc). To inhibit the synthesis of glycolipids, 60% confluent MA104 cells were treated with 25 μM *d,l*-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) (Matreya, Inc.) in MEM–10% FBS for 3 days, replacing the medium with fresh drug daily. After treatment with the respective drug, the cells were washed twice with PBS and then infected with rotaviruses, reovirus, or poliovirus, as described above. To determine the reconstitution of the susceptibility of the cells to virus infection after drug treatment, the cells were washed twice with PBS at time zero, MEM was added, and the cell monolayers were kept at 37°C. At the indicated times, the cells were washed once with PBS and infected as described above. Cell viability was determined by exclusion of trypan blue (22).

The effect of tunicamycin and benzylGalNAc on the cellular synthesis of N- and O-glycans, respectively, was evaluated by detection of sugars on immunoblots, using a digoxigenin glycan detection kit (Boehringer Mannheim; no. 1500-783). Under the treatment conditions described above, glycoconjugates were reduced by about 50% in both tunicamycin- and benzylGalNAc-treated cells compared to untreated cells (data not shown).

Extraction and immunochemical analyses of lipids from MA104 cells. PDMP-treated or untreated MA104 cells were harvested by centrifugation and washed twice with PBS. Total lipids were extracted essentially as described by Guo et al. (19). Thin-layer chromatography was carried out in a solvent system of chloroform-methanol-water (5:4:1) containing 12 mM $MgCl_2$. The plastic plate was dried for 2 h at 37°C and then soaked by capillarity in *n*-hexane containing 10% poly(isobutyl methacrylate) (Aldrich). The glycolipids were then detected immunochemically on the thin-layer chromatograms, as reported previously (30), employing the same carbohydrate detection kit described above. After treatment of MA104 cells with PDMP, as described above, the content of mono- and disialogangliosides was about 30 to 40% of that found in untreated cells (data not shown).

Cholesterol depletion of MA104 cell monolayers. Confluent MA104 cell monolayers in 96-well plates were washed twice with PBS and then incubated with 10 mM methyl-β-cyclodextrin (Aldrich) in PBS for 1 h at 37°C with occasional shaking. After this time the cells were washed twice with PBS and infected as described above.

To replenish the cells with cholesterol after methyl-β-cyclodextrin treatment, the drug was removed and the cells in 96-well plates were washed twice with PBS and then underwent essentially the same treatment as that described by Falconer et al. (16). Briefly, 200 μl of MEM–7%FBS with or without 0.1 mM cholesterol (5-cholesten-3β-ol-3β-hydroxy-5-cholestene) (Sigma), which was freshly made in 100% ethanol, was added per well and left for the indicated periods. At the end of the incubation time the cells were washed twice with MEM and infected as described above.

To determine the cholesterol content of untreated or cyclodextrin-treated MA104 cells, the cells in suspension were pelleted, the pellet was suspended in 0.8% OG by extensive vortexing, and the suspension was cleared by centrifugation for 5 min at 2,000 × *g* in an Eppendorf centrifuge. The cholesterol present in the supernatant or in the cyclodextrin extract of cells was assayed spectrophotometrically using the Boehringer Mannheim diagnostic kit (no. 139050). All cholesterol determinations were made in the presence of 0.2% OG.

OG treatment of MA104 cell monolayers. Confluent MA104 cell monolayers in 96-well plates were washed twice with PBS. The cells were then incubated with 0.2% OG (Pierce) in MEM for 90 min at room temperature with occasional shaking. After this time, the cells were washed twice with PBS and infected as described above. To determine the cell viability and the degree of cell membrane permeabilization that may have been caused by the detergent, we evaluated the ability of the cells to exclude the vital dye trypan blue and the level of the cytoplasmic enzyme lactate dehydrogenase in the OG extract (25). Treatment of cells with 0.2% OG was shown to release less than 5% of the total lactate dehydrogenase activity. A 100% lysis was determined by homogenization of the cells in 0.2% OG.

Binding assay. Rotavirus binding was determined by a nonradioactive assay, essentially as described by Zárate et al. (59). Briefly, a suspension of 5×10^4 MA104 cells either untreated, previously treated with PDMP or tunicamycin, or extracted with OG or cyclodextrin, as described above, was incubated for 1 h at 4°C with 300 ng of trypsin-activated purified viruses in MEM–1% bovine serum albumin. The cell-virus complexes were washed three times with ice-cold PBS containing 0.5% bovine serum albumin. During the last wash, the cells were transferred to a fresh Eppendorf tube and then lysed in 50 mM Tris (pH 7.5)–150 mM NaCl–0.1% Triton X-100. The viruses present in the lysates were quantified by an enzyme-linked immunosorbent assay (59). In all assays, a binding control with no cells was performed.

To assay the binding-blocking activity of the OG extract, 300 ng of purified virus particles was incubated with 20 μg of OG-extracted proteins per ml for 90 min at 37°C. The virus-OG extract mixture was then added to MA104 cells in suspension, and the assay was performed as described above. The blocking activity of the hyperimmune sera to the 57- and 75-kDa protein fractions (see below) was assayed by preincubating the MA104 cells with a 1:5 dilution of the corresponding preimmune or hyperimmune sera for 1 h at 4°C. After the cells were washed with PBS, the viruses were added and the assay was carried out as described above.

Effect of the OG extract on rotavirus infectivity. Confluent MA104 cell monolayers in T-flasks were washed twice with PBS–0.5 mM EDTA and left to detach in this buffer for 30 min at 37°C. The cells were counted, pelleted at 85 × *g* for 5 min at 4°C, resuspended at a concentration of 2.2×10^7 to 2.5×10^7 cells/ml in MEM–0.2% OG, and incubated with gentle shaking for 90 min at room temperature. After this time the cells were pelleted, and the concentration of extracted proteins in the supernatant was determined by the method of Lowry (Bio-Rad); a typical concentration was approximately 5 μg of protein/10^6 cells. The inhibitory activity of this extract on the infectivity of rotaviruses was measured by incubating dilutions of the extract in MEM with the virus for 90 min at 37°C. As a control, the viruses were incubated with 0.2% OG in MEM. To test for the specificity of inhibition, reovirus and poliovirus were assayed in the same manner as were rotaviruses. The biochemical nature of the inhibitory factor present in the OG extract from untreated cells was determined by boiling (95°C) for 15 min or by incubation of the extract (50 μg of protein/ml) with 2 mg of tosyl phenylalanine chloromethyl ketone (TPCK)-treated trypsin (Sigma) per ml for 1 h at 37°C or with 36 mU of neuraminidase per ml for 2 h at 37°C.

Preparative gel electrophoresis. The proteins extracted from about 5×10^7 cells (in 2 ml of 0.2% OG) were adjusted with nonreducing Laemmli sample buffer to give the following final concentrations: 62.5 mM Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS), 10% glycerol, and 0.025% bromophenol blue. These proteins were immediately loaded, without heating, in a single lane of a 3-mm-thick, 14-cm-wide preparative SDS–11% polyacrylamide gel. The gel was run at 8 mA until the bromophenol blue ran out of the gel. After electrophoresis, the gel was stained in 1% Coomassie blue R-250 in water (22) and slices about 3 mm wide were cut and minced in PBS; the proteins in the gel pieces were eluted into PBS by mild shaking for 48 h at room temperature. The eluted proteins were split into several aliquots, precipitated with 5 volumes of acetone, washed twice with 80% cold acetone, and dried for 1 min in a Savant evaporator. To analyze the precipitated proteins, one protein aliquot was resuspended in reducing (5% β-mercaptoethanol) Laemmli sample buffer, boiled for 3 min, and run in an SDS–11% polyacrylamide gel. The ability of the eluted proteins to block rotavirus infectivity was tested, as described above, using a second protein aliquot resuspended in MEM with 1 mM β-mercaptoethanol. After the first round of gel purification, the protein fractions with inhibitory activity were run in a second preparative 7% polyacrylamide gel and all the Coomassie blue-stained bands were cut out again, eluted, and assayed for inhibitory activity. After three rounds of preparative gel electrophoresis, five protein bands, all of which blocked rotavirus infectivity, were isolated (see Fig. 8). In each case, after the bands had been cut out, the proteins were eluted, acetone precipitated, and resuspended in MEM–1 mM β-mercaptoethanol, as described above. Starting from the second preparative gel, the proteins were recovered by electroelution: the gel slices were immersed in sample buffer (2% SDS, 19.2 mM glycine, 2.5 mM Tris base) and electroeluted in an ISCO chamber for 45 min (3 W) using 0.1% SDS–192 mM glycine–25 mM Tris base as a running buffer.

Preparation of polyclonal antibodies. The proteins eluted from fractions 6 (~57 kDa) and 10 (~75 kDa) (see Fig. 5A), which were shown to have the maximal inhibitory activity for rotavirus infection, were used to immunize rab-

TABLE 1. Effect of metabolic inhibitors, cell membrane cholesterol depletion, and OG on the infectivity of rotaviruses in MA104 cells

Inhibitor[a]	% Infectivity (SE)[b] of:				
	RRV	nar3	Wa	Reovirus	Poliovirus
None	100	100	100	100	100
PDMP (25 μg/ml)	20 (2)	40 (9.4)	23 (3.8)	95 (3)	114 (0)
Tunicamycin (2 μg/ml)	56 (2.5)	48 (2.8)	—[c]	91 (5.5)	192 (15)
BenzylGalNAc (2 mM)	101 (0.5)	150 (4.8)	147 (7.2)	110 (4.5)	108 (4.5)
OG (0.2%)	41 (5.4)	41 (2.4)	39 (4.8)	89 (2)	199 (29)
β-Cyclodextrin (1 mM)	9 (1.8)	6 (2.3)	5 (1.8)	96 (0)	95 (3)

[a] MA104 cell monolayers were incubated with the indicated concentration of inhibitor for 1 h (β-cyclodextrin), 24 h (tunicamycin), or 72 h (PDMP and benzylGalNAc) at 37°C or for 90 min (OG) at room temperature before virus infection.

[b] SE, one standard error of the mean of at least three independent experiments carried out in duplicate.

[c] The infectivity of Wa was inhibited by about 50% regardless of whether tunicamycin was added to the cells 24 h before or immediately after the virus adsorption; thus, this inhibition was considered nonspecific.

bits, as described previously (22). Briefly, New Zealand White rabbits (3 to 4 kg) were immunized subcutaneously with 500 μg of protein in Freund's complete adjuvant. Two booster injections were given subcutaneously at 2-week intervals with the same amount of protein emulsified in Freund's incomplete adjuvant. The rabbits were bled after the third immunization. A sample of serum was obtained from each animal before immunization.

The ability of the sera to block rotavirus infectivity was assayed by incubating dilutions of the sera with monolayers of MA104 cells in 96-well plates for 90 min at 37°C. The cells were washed twice with PBS and then infected as described above. The preimmune sera were used as negative controls. The hyperimmune sera were tested for their ability to recognize viruses RRV, nar3, and Wa by an enzyme-linked immunosorbent assay, as described by Menchaca et al. (37); at the lowest dilution tested (1:100), no reactivity was found (data not shown). These antisera did not inhibit the hemagglutination of RRV and nar3 (data not shown).

Western immunoblotting. The proteins present in the 0.2% OG extract were separated in an 11% polyacrylamide gel and transferred to nitrocellulose. The transferred proteins were incubated with the sera to the 57- and 75-kDa fractions, diluted 1:1,000 in PBS. The bound antibodies were developed by incubation with protein A-peroxidase, and 3-amino-9-ethyl-carbazole (Sigma) was added as a substrate, as previously described by Arias et al. (1).

Immunofluorescence. MA104 cells grown on glass coverslips to approximately 80% confluence were fixed with 4% paraformaldehyde in PBS for 20 min at 37°C. After this time the cells were washed twice with PBS, either permeabilized or not by incubation with PBS–0.5% Triton X-100 for 5 min at room temperature, and then washed twice with PBS with gentle swirling. The fixed cells were blocked with 1 M glycine for 1 h at 37°C, washed twice with PBS, and then incubated with a 1:1,000 (anti-57-kDa fraction) or 1:1,500 (anti-75 kDa fraction) dilution of the sera for 90 min at 37°C. The cells were washed four times with PBS and then incubated in the dark for 1 h at 4°C with a goat anti-rabbit immunoglobulin G coupled to fluorescein isothiocyanate (Dako Co.), diluted 1:100 in PBS. The cells were washed four times with PBS and mounted on glass slides on 10% glycerol in PBS. The slides were analyzed using a Bio-Rad MRC-600 microscope. The preimmune sera were used as negative controls.

RESULTS

Inhibitors of N glycosylation and glycolipid synthesis block rotavirus infection. To assess the biochemical nature of the cellular receptor for rotaviruses, MA104 cells were treated with specific inhibitors of glycosylation prior to infection. Two inhibitors were used: tunicamycin, which blocks an early step in the N-glycosylation pathway involving transfer between UDP-GlcNAc and dolichol-1-phosphate (12), and benzylGalNAc, which is a competitive inhibitor of the transferase (*N*-acetyl-α-D-galactosaminyltransferase) involved in the first step of the biosynthesis of most types of O-linked carbohydrates (5). In addition, we used the synthetic analog of ceramide, PDMP, to inhibit the biosynthesis of the glycosphingolipid precursor glucosylceramide (45). The cells pretreated with the inhibitors were then infected with either wild-type RRV, the neuraminidase-resistant RRV variant nar3, or the HRV strain Wa.

Treatment of cells with 2 μg of tunicamycin per ml for 24 h before infection inhibited the infectivity of rotaviruses RRV and nar3 by about 50%, while preincubation of the cells for 3 days with PDMP, the inhibitor of glycolipids synthesis, blocked the infectivity of the viruses by about 80% (RRV and Wa) or 60% (nar3) (Table 1). On the other hand, inhibition of O glycosylation by benzylGalNAc had no effect on the infectivity of RRV but increased the infectivity of nar3 and Wa by about 50%, indicating that under conditions where the levels of cell surface O-linked carbohydrates are decreased, these viruses infect the cell more efficiently. The total cell content of N- and O-glycoproteins was reduced by at least 50% by the corresponding inhibitory drug (data not shown). The infectivity of reovirus and poliovirus, which were used as controls, was not inhibited by any of these three drugs, with poliovirus actually showing a twofold increase in infectivity in the cells treated with tunicamycin (Table 1), as has been reported for other viruses like human immunodeficiency virus type 2 and B-lymphotropic papovavirus (28, 43).

Under the conditions employed, the inhibitors did not have a significant effect on cell protein synthesis, as judged by electrophoresis of ^{35}S-labeled proteins, or on the viability of cells, as judged by trypan blue exclusion (data not shown). To control for a possible nonspecific, toxic effect of the drugs on the replication of rotaviruses, in a separate experiment we added the inhibitors immediately after the virus had been adsorbed for 45 min at 4°C. Under these conditions the drugs did not affect rotavirus infectivity, with the exception of rotavirus Wa, whose infectivity was decreased about 50% by tunicamycin; for this reason, this inhibition was considered to be nonspecific. The effect of the inhibitors was reversible since the cells became fully susceptible to rotavirus infection by about 20 and 24 h after removing tunicamycin and PDMP, respectively (data not shown). Taken together these results suggest that glycolipids and N-glycosylated but not O-glycosylated proteins are important for rotavirus infection. To determine if the treatment of cells with tunicamycin and PDMP inhibited the attachment of the virus to the cell surface or if the inhibition of infectivity occurred at a postattachment step, we performed binding assays using cells treated with the different drugs. We found that treatment of cells with tunicamycin did not affect the binding of either of the three viruses tested while treatment of cells with PDMP did not affect the attachment of RRV and Wa but decreased the binding of nar3 by 54% (Table 2). This level of inhibition in the attachment of the virus to cells is very similar to the 60% inhibition in the infectivity of nar3 caused by PDMP (Table 1), which suggests that most if not all of the blockage in the infectivity of nar3 in PDMP-treated cells is due to an inhibition of the binding of this variant to the cell surface.

Infection of octyl-β-glucoside-extracted cells. As a different approach to characterize the rotavirus receptor, we used the nonionic detergent OG to extract the receptor from the cell membrane under noncytolytic conditions, as has been described for other virus receptors (2, 10, 23, 36, 49, 55). MA104

TABLE 2. Effect of metabolic inhibitors, cell membrane cholesterol depletion, and OG on the binding of rotaviruses to MA104 cells

Inhibitor[a]	% Binding (SE)[b] of virus strain:		
	RRV	nar3	Wa
None	100	100	100
PDMP (25 μg/ml)	110 (19)	46 (20)	104 (12.5)
Tunicamycin (2 μg/ml)	111 (14)	101 (12.5)	94 (21)
Octyl-β-glucoside (0.2%)	32 (4.5)	40 (7.5)	33 (0.5)
β-cyclodextrin (1 mM)	112 (6.5)	109 (16)	116 (4.5)

[a] MA104 cell monolayers were incubated with the indicated concentration of inhibitor for 1 h (β-cyclodextrin), 24 h (tunicamycin), or 72 h (PDMP) at 37°C or for 90 min (OG) at room temperature before the assay.

[b] SE, one standard error of the mean of at least three independent experiments carried out in duplicate.

cells were incubated with 0.2% OG for 90 min at room temperature; under these conditions the cells maintained their viability and integrity, as judged by trypan blue exclusion and the low levels of lactate dehydrogenase activity, a cytosolic marker detected in the OG extract (less than 5% of total enzyme activity). The cells extracted with the detergent were found to be about 60% refractory to infection by the three viruses tested (Table 1). As described above for the metabolic inhibitors, this effect was also found to be reversible; if the detergent was washed away after the treatment period, the cells fully regained their susceptibility for infection at about 8 h posttreatment (Fig. 1A), which most probably accounts for the time of synthesis, transport, and accumulation of the receptor in the cell membrane at the levels needed for the virus to efficiently infect the cell. Of interest, the attachment of all three viruses to OG-extracted cells was inhibited by 60 to 70% (Table 2), indicating that the reduced infectivity of the viruses in OG-treated cells might be due to a decreased ability of the virus particles to bind to the cell surface.

The OG extract inhibits rotavirus infection. Since treatment of MA104 cells with OG diminished the ability of the viruses to attach to and thus to infect cells, it is likely that the detergent was extracting cell surface molecules involved in the initial interaction of rotaviruses with the cell, possibly the rotavirus receptor(s). If this were the case, the molecule(s) present in the OG extract could interact with the virus in solution, preventing the binding of the virus to the cell membrane and thus blocking its infectivity. We found that incubation of the OG extract with either of the three rotavirus strains did block their infectivity in a concentration-dependent manner (Fig. 1B). At the maximum concentration tested, 400 μg of protein per ml, the infectivity of the viruses was inhibited by about 95%; 50% inhibition was achieved at about 40 μg of protein per ml. In contrast, the infectivity of poliovirus and reovirus was not affected by the extract (Fig. 1B). These results strongly suggest a specific interaction of the viruses with the OG-solubilized cell surface molecules. Preincubation of the viruses with a solution of 0.2% OG did not affect their infectivity. The infectivity in the presence of OG was taken as the 100% value for each virus.

The inhibition of rotavirus infectivity caused by the OG extract seems to be due to a blockage in cell attachment since preincubation of the viruses with 20 μg of the OG-extracted protein per ml decreased RRV binding to the cell by 40%, nar3 binding by 41%, and Wa binding by 43% (Table 3). These percentages are in close agreement with the degree of inhibition of infectivity achieved with this concentration of extract (Fig. 1B).

The inhibitory capacity of OG extracts obtained from BHK, CHO, and L cells, which are about 1,000-fold less susceptible to rotavirus infection than MA104 cells, was determined. As can be seen in Fig. 2, the OG extracts from the three poorly permissive cells showed some inhibitory activity, although in all cases this activity was less pronounced than that observed with the extract from MA104 cells.

Biochemical nature of the inhibitory component present in the MA104 OG cell extract. To determine the biochemical nature of the inhibitory component present in the OG cell extract, we tested the effect of heat inactivation, neuraminidase, and proteolytic treatment on the inhibitory activity of the extract. We found that either boiling for 15 min or treatment with trypsin completely abolished the inhibitory activity while treatment with neuraminidase had no effect on the blocking capacity of the extract (Fig. 3). These results indicate that the inhibitory component of the extract is a protein.

The profile of proteins extracted with OG is shown in Fig. 4A, lane 5. Treatment with tunicamycin, PDMP, or neuramin-

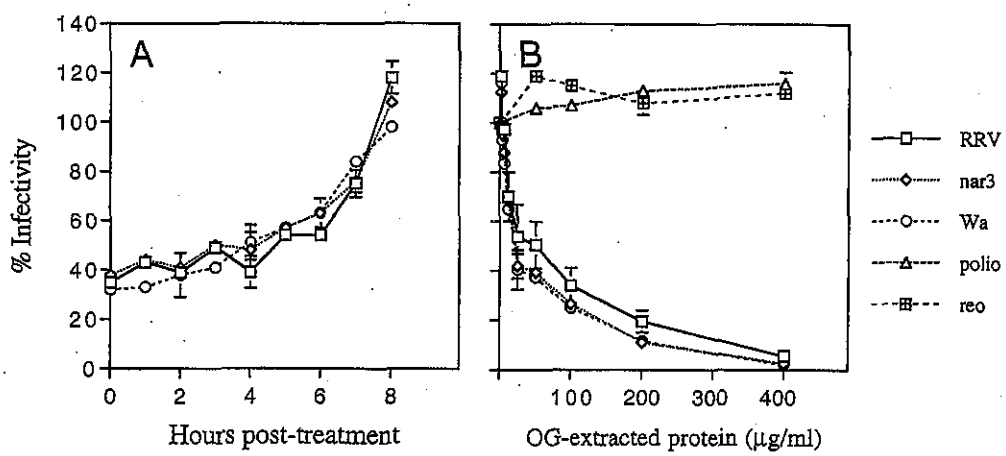

FIG. 1. (A) Recovery of the susceptibility of MA104 cells to rotavirus infection after extraction with OG. Cell monolayers in 96-well plates were extracted with 0.2% OG and allowed to recover in MEM at 37°C. At the indicated times, the monolayers were washed with PBS and infected with rotaviruses. (B) Inhibition of rotavirus infectivity by the OG extract from MA104 cells. The indicated concentrations of OG-extracted protein were incubated with the viruses for 90 min at 37°C. The virus-protein mixtures were used to infect MA104 cell monolayers in 96-well plates. In both panels, the percent infectivity is relative to the infectivity of the viruses incubated in 0.2% OG. Error bars represent 1 standard error of the mean of three or more experiments carried out in duplicate.

TABLE 3. Effect of the OG extract, and antibodies to 75 kDa OG protein fraction, on the binding of rotaviruses to MA104 cells[a]

Inhibitor	% Binding (SE)[b] of virus strain:		
	RRV	nar3	Wa
0.2% OG (control)	100	100	100
OG extract (20 μg/ml)	60 (2.5)	59 (4)	57 (1.5)
No serum (control)	100	100	100
Polyclonal antibodies to the 75-kDa OG fraction			
Preimmune serum	102 (10)	97 (8)	105 (5.5)
Hyperimmune serum	*92 (6.5)*	*68 (2.5)*	*28 (3.5)*

[a] Rotaviruses were incubated with the indicated concentration of OG-extracted proteins for 90 min at 37°C. The virus-OG extract mixture was then added to MA104 cells in suspension, and the assay was performed as described in Materials and Methods. The blocking activity of the hyperimmune sera to the 75-kDa protein fractions was assayed by preincubating the MA104 cells with a 1:5 dilution of the preimmune or hyperimmune sera for 1 h at 4°C. After the cells were washed, the viruses were added and the assay was carried out as described in Materials and Methods.

[b] SE, one standard error of the mean of at least three independent experiments carried out in duplicate.

idase modifies this profile (lanes 2 to 4), reflecting the modification in the carbohydrate content of glycoproteins caused by tunicamycin and neuraminidase. In the case of PDMP, this result suggests that the impaired synthesis of glycolipids alters either the transport of proteins to the plasma membrane or their extractability from the cell surface by OG. In this regard, it is of interest that the OG extract from PDMP-treated cells failed to block rotavirus infectivity (data not shown), suggesting that the inhibitory proteins could not be extracted from these cells.

Cholesterol depletion of MA104 cells inhibits rotavirus infectivity. It has been proposed that glycosphingolipids, cholesterol, and proteins can interact specifically in cell membranes to form microdomains termed rafts (46). Given the involvement of glycolipids and N-glycosylated proteins on rotavirus infectivity, we tested if depletion of the cell cholesterol would have any effect on virus infectivity. To do this, we incubated the cells with 10 mM β-cyclodextrin for 1 h at 37°C; this treatment

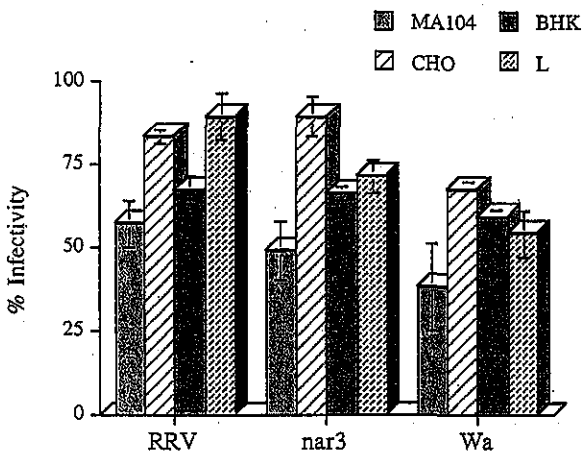

FIG. 2. Inhibition of rotavirus infectivity by OG extracts from cells poorly permissive to rotavirus infection. OG-extracted proteins (20 μg/ml) from CHO, BHK, L, or MA104 cells (as indicated) were incubated with the viruses for 90 min at 37°C. The virus-protein mixtures were used to infect MA104 cell monolayers in 96-well plates. The percent infectivity is relative to the infectivity of the viruses incubated in 0.2% OG. Error bars represent 1 standard error of the mean of three experiments carried out in duplicate.

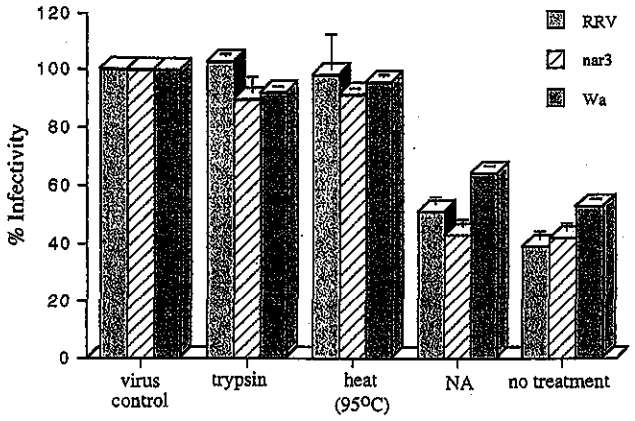

FIG. 3. Biochemical nature of the inhibitory factor present in the OG extract. A 0.2% OG extract was obtained from cells in suspension. Just prior to the incubation with the virus, the extract was either boiled (95°C) for 15 min (heat), incubated with 2 mg of trypsin per ml of extract for 1 h at 37°C (trypsin), or incubated with 36 mU of neuraminidase per ml (NA). The untreated extract (no treatment) was used as a positive control. Viruses and extract (100 μg of protein extract per ml of virus) were mixed and incubated for 90 min at 37°C, and then MA104 cells in 96-well plates were infected with the virus-protein mixtures. The percent infectivity is relative to the infectivity of viruses incubated with a solution of 0.2% OG in MEM (virus control). Error bars represent 1 standard error of the mean of three or more experiments carried out in duplicate.

has been shown to selectively extract cholesterol from the plasma membrane in preference to other membrane lipids (28). Under these conditions, about two-thirds (65%) of the cell cholesterol was removed (see Materials and Methods). The treatment of cells with β-cyclodextrin inhibited the infectivity of RRV, nar3, and Wa rotavirus strains by more than 90% but had no effect on the infectivity of reovirus and poliovirus (Table 1). It is noteworthy that the binding of the three rotavirus strains was not affected (Table 2), indicating that the

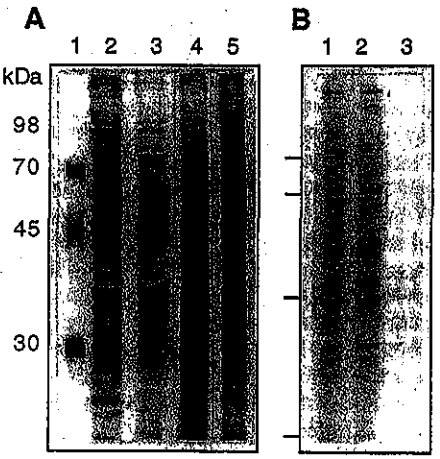

FIG. 4. Analysis of the proteins extracted from MA104 cells. Cell monolayers were treated with either neuraminidase, tunicamycin, or PDMP, as described in Materials and Methods, and the cells were then extracted with 0.2% OG for 90 min at room temperature. (A) The extracted proteins were separated by electrophoresis under reducing conditions in an SDS–11% polyacrylamide gel and silver stained. OG-extracted proteins from MA104 cells treated with neuraminidase (lane 2), tunicamycin (lane 3), or PDMP (lane 4) or left untreated (lane 5) are shown. Lane 1 contains molecular mass markers. (B) Cells in suspension were extracted with 10 mM β-cyclodextrin for 1 h at 37°C, as described in Materials and Methods. Proteins in untreated cells (lane 1), extracted cells (lane 2), and the cyclodextrin extract (lane 3) were analyzed by gel electrophoresis.

decrease in the cholesterol content of the cell affects virus infectivity at a postattachment step.

The protein profile of cells treated with β-cyclodextrin was not very different from that of untreated cells (Fig. 4B, lanes 1 and 2), even though this antibiotic extracted a small amount of protein from the cells (lane 3).

To demonstrate that the depletion of cholesterol was the cause of the reduction of virus infectivity after the β-cyclodextrin treatment, cells in 96-well plates were washed twice with MEM, and then either MEM alone, MEM–7% FBS, or MEM–7% FBS containing 0.1 mM cholesterol was added for different times. At the end of the incubation period, the cells were washed twice and infected with rotaviruses. At 8 h posttreatment, the cells incubated in the presence of cholesterol had fully recovered their susceptibility to rotaviruses while the cells incubated with MEM alone or MEM–7% FBS were still about 80 and 50% refractory to rotavirus infection, respectively (data not shown).

Fractionation of the inhibitory components present in the OG extract from MA104 cells. To characterize the proteins that block rotavirus infection, we fractionated the OG extract obtained from MA104 cells by preparative SDS-polyacrylamide gel electrophoresis. After the gel electrophoresis, slices of a single-lane gel were cutout, and the proteins were eluted in PBS, concentrated by precipitation with acetone, and resuspended in PBS with 1 mM β-mercaptoethanol. This method has been successful for recovering proteins with enzymatic activity (20, 48). The proteins obtained from the different fractions (Fig. 5B) were tested for their ability to block rotavirus infection. Proteins eluted from two well-defined regions of the gel, around 57- and 75 kDa, had the ability to efficiently inhibit the infectivity of all three rotaviruses tested (Fig. 5A). The pattern of inhibition observed in Fig. 5A was found to be consistent in independent gel fractionation experiments.

Antibodies to the OG extract protein fractions inhibit rotavirus infection. Protein fractions 6 and 10 in Fig. 5A, which represent the peak of inhibitory activity, were used to immunize rabbits. The hyperimmune sera obtained against these two fractions were found to block the infectivity of all three strains of rotavirus when preincubated with the cells for 90 min at 37°C prior to addition of the virus, while the preimmune sera had no effect (shown for the serum to the 75-kDa protein fraction in Fig. 6A). The inhibitory effect of the two antisera was not additive since a mixture of the two inhibited rotavirus infectivity by about 70% at a dilution of 1/100 (data not shown). Of interest, both sera blocked the infectivity of HRV Wa and that of the SA-independent variant nar3 in cells treated with neuraminidase (shown for the serum to the 75-kDa fraction in Fig. 6B), suggesting that they contain antibodies to an SA-independent rotavirus receptor. In a binding inhibition assay, the serum to the 75-kDa fraction did not inhibit the attachment of rotavirus RRV to MA104 cells but inhibited 32% of the binding of nar3 and 72% of that of Wa (Table 3). The blocking specificity of these antisera was confirmed by the following assays: they did not recognize any of the three viruses by enzyme-linked immunosorbent assay, and they did not inhibit the hemagglutination activity of RRV and nar3. Furthermore, the sera were shown not to inhibit the infectivity of poliovirus or that of reovirus in an FFU reduction assay as described in Materials and Methods for rotavirus (data not shown).

By Western blotting, the sera to the 75-kDa fraction recognized a protein of about 73 kDa and, to a lesser extent, a protein of about 57 kDa in the 0.2% OG cell extract (Fig. 6C, lane 3). Of interest, the serum to the 57-kDa protein fraction also recognized proteins of 73 and 57 kDa, although the latter

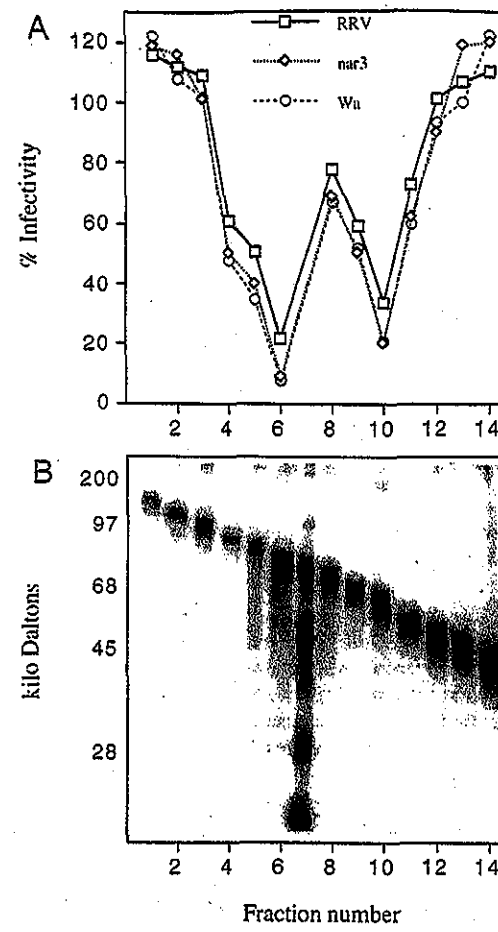

FIG. 5. Inhibition of rotavirus infectivity by OG-extracted proteins fractionated by gel electrophoresis. About 250 μg of proteins extracted with 0.2% OG from MA104 cells was separated by preparative SDS-polyacrylamide gel electrophoresis under nonreducing conditions. After electrophoresis, the gel was stained with Coomassie blue in water, gel slices were cut out, and the proteins were eluted. (A) Inhibitory activity of the eluted proteins present in the fractions shown in panel B. (B) Gel electrophoresis of the eluted protein fractions. Only the portion of the gel where inhibitory activity was found is shown; the remaining higher- and lower-molecular-mass protein fractions had no inhibitory activity.

protein was recognized more efficiently by this serum (lane 4). The preimmune sera did not recognize any of these proteins (lanes 1 and 2).

The hyperimmune sera were shown to recognize proteins on the surface of the MA104 cells, as judged by their reactivity with nonpermeabilized cells by flow cytometry (data not shown) and by indirect immunofluorescence (shown for the anti-75-kDa serum in Fig. 7). The pattern of immunofluorescence (for both anti-57- and anti-75-kDa sera) was patchy over the surface of the cell, but there was a higher concentration of the fluorescent signal on the intercellular junctions (Fig. 7A). In permeabilized cells, a weak signal associated mainly with the nuclei was found (Fig. 7B). No fluorescent signal was detected when the preimmune sera were used to stain either permeabilized or nonpermeabilized cells (Fig. 7C and D).

Purification of the cellular proteins which block rotavirus infectivity. The proteins with rotavirus blocking activity were purified by SDS-polyacrylamide gel electrophoresis from an OG extract obtained from MA104 cells. After three rounds of purification by gel electrophoresis, using the inhibitory activity of the proteins as marker, we were able to isolate five bands

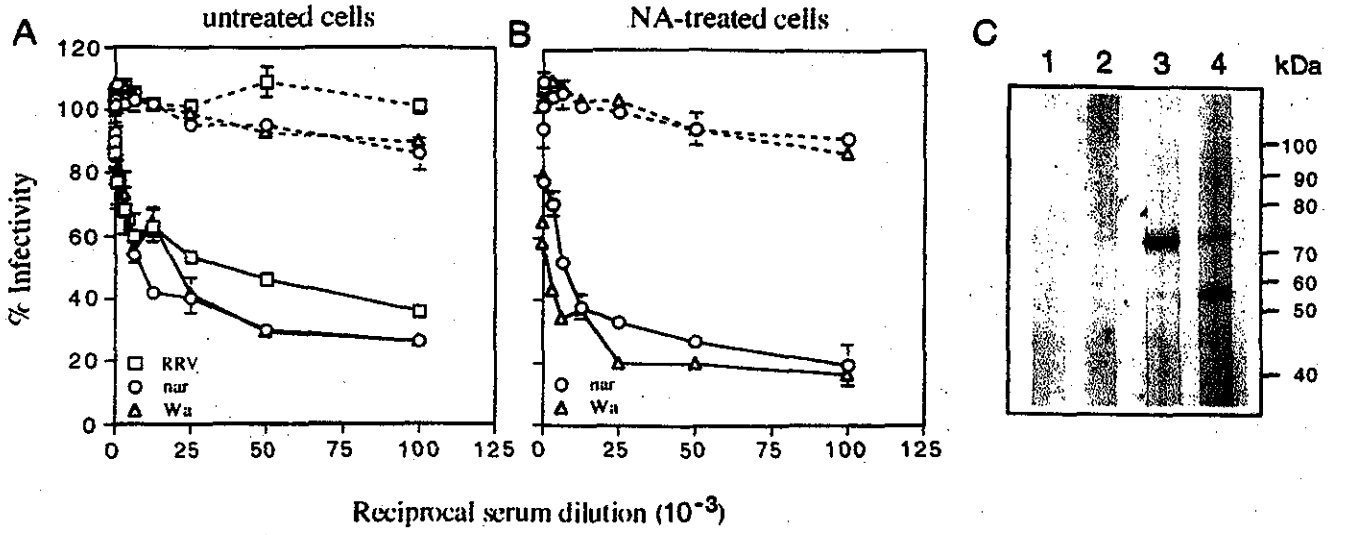

FIG. 6. Inhibitory activity of hyperimmune sera to OG-extracted proteins. (A and B) OG protein fractions 6 and 10 shown in Fig. 5B, containing polypeptides of around 57 and 75 kDa, respectively, were used to raise antibodies in rabbits. Serial dilutions of the preimmune (dashed lines) and hyperimmune (continuous lines) sera to the 75-kDa protein fraction were incubated with untreated (A) or neuraminidase (NA)-treated (B) MA104 cells for 90 min at 37°C before addition of the virus. Similar inhibition results were obtained with the serum to the 57-kDa protein fraction (data not shown). Error bars represent 1 standard error of the mean of three or more experiments carried out in duplicate. (C) Immunoblot analysis of the OG-extracted proteins. The proteins extracted from MA104 cells with 0.2% OG were separated in an SDS–11% polyacrylamide gel under reducing conditions and transferred to nitrocellulose. The transferred proteins were incubated with a 1,000-fold dilution of the preimmune (lanes 1 and 2) or hyperimmune (lanes 3 and 4) sera to the 57-kDa (lanes 2 and 4) or 75-kDa (lanes 1 and 3) protein fractions. The bound antibodies were developed by incubation with protein A-peroxidase and a chromogenic substrate.

with molecular masses of approximately 110, 75, 57, 45, and 30 kDa (Fig. 8) which were able to inhibit the infectivity of all three rotavirus strains tested. Although these proteins consistently inhibited rotavirus infectivity through the rounds of purification carried out, the final amount of protein recovered was small, which prevented us from determining the precise specific inhibitory activity for each protein and testing if they were recognized by the hyperimmune sera. Table 4 shows the results of a blocking infectivity assay with the purified proteins; in this blocking assay, the same amount of protein shown in the gel in Fig. 8 was used. The relative inhibitory activity of each protein for all three viruses was found to be 75 kDa > 110 kDa > 45 kDa = 30 kDa > 57 kDa. Given the fractionation method employed, it is quite possible that each of these bands may represent more than one protein species.

DISCUSSION

The entry of rotaviruses into MA104 cells seems to be a multistep process involving interactions of the virus surface protein VP4 and maybe of VP7 with more than one cell surface site present in either the same or a different cellular structure(s) (11, 41).

In the present study we employed two approaches to characterize the biochemical nature of the rotavirus receptor(s). In the first approach we used metabolic inhibitors of glycosylation and synthesis of glycolipids to study their effect on the infectivity of three different rotavirus strains. We found that tunicamycin, an inhibitor of protein N glycosylation, diminished the infectivity of rotaviruses RRV and nar3 despite their differential dependence on SA for infectivity, implying that these viruses interact with N-linked glycoproteins at some point during cell entry. The fact that the treatment of cells with this drug did not affect the binding of the viruses suggests that the blockage occurs after the initial attachment of the virion to the cell surface. Tunicamycin has been successfully used to specifically analyze the role of N-glycans as receptors for several viruses (7, 28, 43, 44).

Treatment of MA104 cells with PDMP, an inhibitor of glycolipid biosynthesis, resulted in the more pronounced inhibition of infectivity observed for all three rotavirus strains (Table 1). Interaction of rotaviruses with gangliosides GM1 and GM3 has been reported (47, 52), and this interaction has been shown to be SA dependent. In this case, however, the inhibition caused by PDMP seems not to be the result of a deficient attachment of the SA-dependent rotavirus RRV to the cell surface since it was not significantly affected (Table 2). This observation suggests that RRV does not interact, or at least does not interact exclusively, with the SA present on PDMP-sensitive gangliosides. On the other hand, the binding of the SA-independent variant nar3 was decreased in PDMP-treated cells, suggesting that either glycosphingolipids or, more probably, a protein whose correct transport or conformation depends on their presence might be used by nar3 to attach to the cell. Of interest, the binding of the HRV strain Wa was not affected by PDMP, in agreement with the suggestion that nar3 and Wa, despite having an infectivity resistant to neuraminidase treatment of cells, bind to different cell surface sites (41). Finally, the fact that PDMP, but not tunicamycin, affected the attachment of nar3 suggests that the inhibition caused by the N-glycosylation inhibitor is not due to its reported ability to inhibit ganglioside biosynthesis (50, 58).

In addition to the involvement of N-glycosylated proteins and glycolipids in rotavirus entry, we found that cholesterol depletion inhibited the infectivity of rotaviruses by more than 90% (Table 1). These findings are of interest with regard to the recent description of functional lipid microdomains, or rafts, in the cell membrane (51). These rafts have been proposed to be composed of cholesterol, glycosphingolipids (gangliosides among others), and a specific set of associated proteins. They are thought to function as specialized platforms for apical cell sorting of proteins and signal transduction. For some proteins

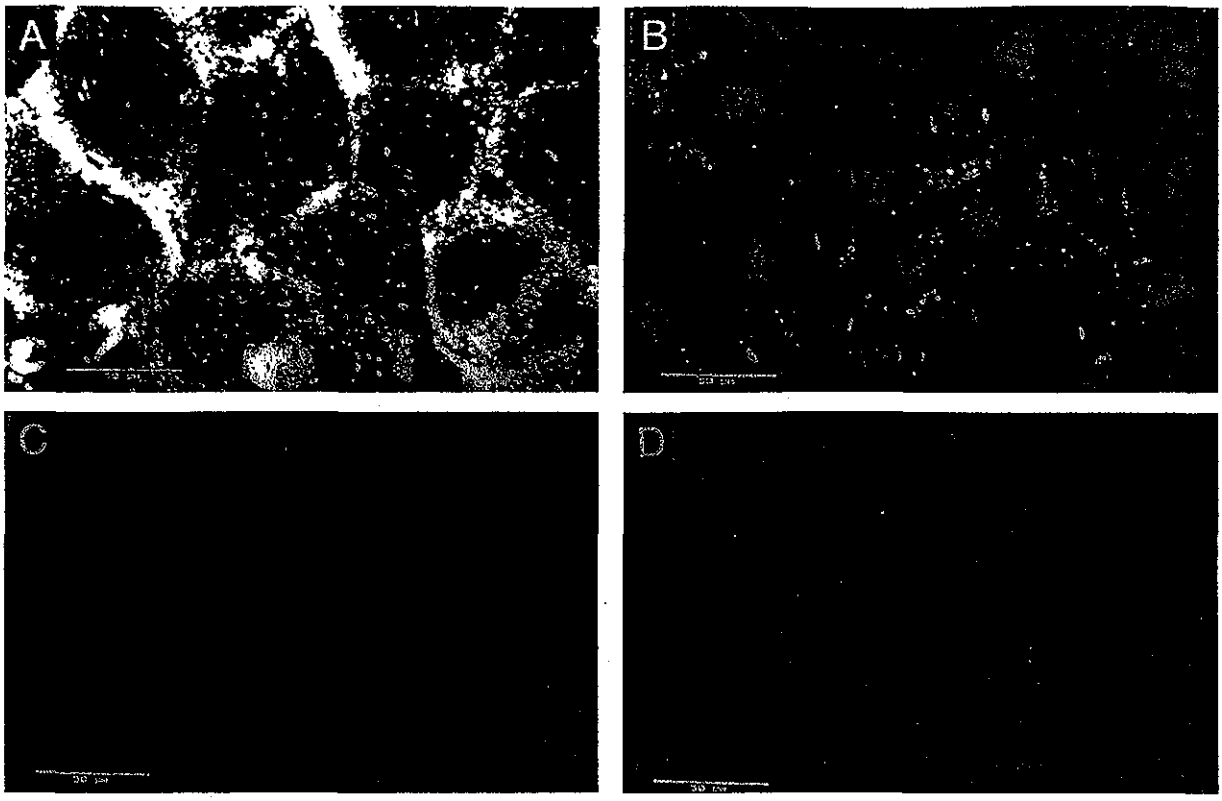

FIG. 7. Immunofluorescence of cells incubated with the serum to the 75-kDa OG protein fraction. MA104 cells were fixed with paraformaldehyde and permeabilized with Triton X-100 (B and D) or not permeabilized (A and C). The cells were incubated with a 1:1,500 dilution of the preimmune (C and D) or hyperimmune (A and B) sera to the 75-kDa protein fraction for 90 min at 37°C and stained with a goat anti-rabbit immunoglobulin G coupled to fluorescein isothiocyanate.

which form part of these lipid microdomains (oxytocin receptor, placental alkaline phosphatase, gD1 decay-accelerating factor), the disassembly of the rafts by cholesterol depletion disrupts or modifies the receptor activity, even though the receptor might be present in the same abundance on the cell membrane (21, 29). In this regard, the finding that the attachment of RRV, nar3, and Wa to cholesterol-depleted cells is not affected while their infectivity is severely impaired is consistent with the possibility that the rotavirus receptor(s) might be forming part of some of these lipid microdomains. It is tempting to hypothesize that in cholesterol-depleted cells, the receptor(s) retains its ability to bind rotavirus particles but in order to fully promote virus entry it must be organized in a lipid microdomain. In addition, the fact that the OG extract from PDMP-treated cells failed to show inhibitory activity suggests that PDMP treatment may have disrupted the lipid raft organization such than one or more of the active proteins in Fig. 8 never became associated with or localized within these membrane microdomains and as result are not extracted with OG. Experiments are under way to test this hypothesis.

The infectivity of the two nonenveloped viruses that were

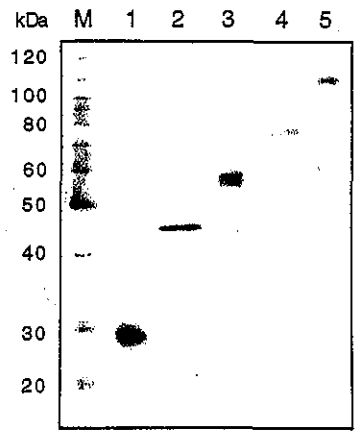

FIG. 8. Isolated proteins with inhibitory activity for rotavirus infectivity. The protein bands that were shown to block rotavirus infectivity after three rounds of purification by preparative gel electrophoresis (see Table 4) were analyzed in an 11% polyacrylamide gel under reducing conditions. The protein bands were detected by silver staining.

TABLE 4. Blocking of rotavirus infectivity by purified OG-extracted proteins from MA104 cells

Protein (kDa)	Relative amt[a] of protein	% Infectivity[b] of virus strain:		
		RRV	nar3	Wa
110	2	50	51	38
75	1	41	17	29
57	5	62	50	68
45	10	55	23	31
30	15	44	25	23

[a] The amount of each protein incubated with the rotavirus strains is the same as that shown in the gel of Fig. 8. The 75-kDa band was the least abundant and was set at 1 (about 10 ng) in relative terms. The concentration of this protein during the infectivity assay was about 100 ng/ml.

[b] The infectivity-blocking assay was carried out only once due to the small amount of material available.

used as controls, poliovirus and reovirus, was not inhibited by the described drugs, showing that the effect observed on the infectivity of rotaviruses was specific. The human poliovirus receptor is an integral membrane protein with the conserved amino acids and domain structure characteristic of members of the immunoglobulin superfamily (31, 38). The nature of the reovirus receptor is less well defined; most of the available evidence suggest that reovirus binds to multiple sialoglycoproteins rather to a single homogeneous species on the cell surface (8, 18, 42).

In a second approach to characterize the rotavirus receptor, MA104 cells were incubated with a solution of 0.2% OG. It has been shown that at low concentrations, like the one used in this work, OG is able to extract proteins from the cell surface without impairing the viability of the cells (see Results) (23, 36). This nonionic detergent has been useful in experiments to obtain the receptors for Semliki Forest virus, parvovirus, vesicular stomatitis virus, polyomavirus, simian virus 40, and rabies virus from intact cell monolayers (2, 10, 23, 36, 49, 55). MA104 cells extracted with OG lost their ability to bind rotaviruses by about the same extent (60%) to which they became refractory to infection, suggesting that OG extracts from the cell surface the receptor molecules needed by all three strains of rotavirus to attach to and thus infect the cell. In agreement with this finding is the fact that the OG extract, when preincubated with these viruses, inhibited both their binding to and infection of MA104 cells. This suggests that the putative OG-solubilized cell receptors are able to interact with the viruses in solution. The inhibitory activity of the OG extract was lost by treatment with proteases and heat but not by treatment with neuraminidase, indicating that the active component is a protein.

To test for a correlation between the susceptibility of the cell line and the ability of the OG extract to inhibit rotavirus infection, we obtained OG extracts from BHK, CHO, and L cells, which are about 1,000-fold less susceptible to rotavirus infection than are MA104 cells. The extracts from these three cell lines inhibited the infectivity of rotaviruses to different degrees but in general to a lesser extent than that achieved with the MA104 cell extract (Fig. 2). As suggested in this work and by others (11, 41), these results might be explained if more than one cell surface molecule were implicated in rotavirus infection, which would make possible the absence of one of the receptor molecules in the less susceptible cell lines while other surface components, which could be extracted with OG and block rotavirus infectivity, would still be present.

Two protein fractions with blocking activity for rotavirus infectivity were obtained by gel fractionation of the OG extract of the MA104 cells. The hyperimmune sera prepared against these two fractions were shown to react primarily with two polypeptides of 73 and 57 kDa. Although it is not possible to be certain if the more immunogenic proteins are the active inhibitory components of the extract, it seems at least that the inhibitory antibodies present in both hyperimmune sera recognize the same cell surface molecule or different molecules in a protein complex since the blocking efficiency of the individual sera was not additive and since the cell surface recognition patterns obtained with the two antisera were strikingly similar.

Five individual protein bands with inhibitory activity for rotavirus infectivity were isolated from the OG extract. These proteins need to be assayed to test the specificity of their inhibitory activity and to investigate if they are somehow related to each other. However, the fact that all of these proteins block the infectivity of RRV, nar3, and Wa rotaviruses suggest that at least one of them, or a complex formed by more than one, could be a common cellular receptor for rotaviruses. The determination of the identity of these proteins should help to define the cell surface molecules involved in the interactions that seem to occur between rotaviruses and the cell surface during infection.

As a working hypothesis, we propose that the rotavirus receptor is likely to be a complex of several cell components including gangliosides, N-linked glycoproteins, and probably other proteins which might all associate in lipid rafts and need the lipid microdomain organization to function efficiently in the binding and internalization of rotavirus particles. The protein components of this proposed complex could include the integrin molecules that have been reported recently (11, 24).

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C. La integrina αvβ3 media la entrada de los rotavirus a la célula huésped

Como resultado de la caracterización del extracto de OG arriba mencionado se identificó a la integrina αvβ3 como un posible receptor para rotavirus. En este trabajo se encontró que anticuerpos dirigidos contra esta integrina son capaces de bloquear la infectividad de los rotavirus. También se mostró que la interacción de los rotavirus es independiente del motivo RGD presente en los ligandos naturales de esta integrina. La transfección de la línea celular CHO con los genes de la integrina αvβ3, incrementó significativamente la suceptibillidad de estas células a la infección con rotavirus, y este incremento pudo bloquearse con anticuerpos dirigidos contra esta integrina.

Como parte de la caracterización de la integrina αvβ3 como posible receptor para rotavirus, hicimos experimentos para probar si esta integrina está involucrada en la unión inicial de los rotavirus a la superficie celular. En este trabajo se utilizaron las cepas de rotavirus RRV, nar3 y Wa. Encontramos que los anticuerpos dirigidos contra esta integrina no afectaron la unión de ninguno de los tres virus, indicando que la interacción de los rotavirus con la integrina αvβ3 podría ocurrir como un paso posterior a la unión inicial. Estos resultados fueron publicados en el artículo "Integrin αvβ3 mediates rotavirus cell entry" (29) .

Integrin $\alpha_V\beta_3$ mediates rotavirus cell entry

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Rotavirus strains differ in their need for sialic acid (SA) for initial binding to the cell surface; however, the existence of a postattachment cell receptor, common to most, if not all, rotavirus strains, has been proposed. In the present study, antibodies to the α_V and β_3 integrin subunits, and the $\alpha_V\beta_3$ ligand, vitronectin, efficiently blocked the infectivity of the SA-dependent rhesus rotavirus RRV, its SA-independent variant nar3, and the neuraminidase-resistant human rotavirus strain Wa. Vitronectin and anti-β_3 antibodies, however, did not block the binding of virus to cells, indicating that rotaviruses interact with $\alpha_V\beta_3$ at a postbinding step, probably penetration. This interaction was shown to be independent of the tripeptide motif arginine-glycine-aspartic acid present in the natural ligands of this integrin. Transfection of CHO cells with $\alpha_V\beta_3$ genes significantly increased their permissiveness to all three rotavirus strains, and the increment of virus infectivity was reverted by incubation of these cells either with antibodies to β_3 or with vitronectin. These findings implicate $\alpha_V\beta_3$ integrin as a cellular receptor common to neuraminidase-sensitive and neuraminidase-resistant rotaviruses, and support the hypothesis that this integrin could determine, at least in part, the cellular susceptibility to rotaviruses.

Rotaviruses, the leading cause of severe dehydrating diarrhea in infants and young children worldwide, are nonenveloped viruses that posses a genome of 11 segments of double-stranded RNA contained in a triple-layered protein capsid. The outermost layer is composed of two proteins, VP4 and VP7. VP4 forms spikes that extend from the surface of the virus, and it has been associated with a variety of functions, including initial attachment of the virus to the cell membrane and the penetration of the virion into the cell (1).

Rotaviruses have very specific cell tropism, infecting only enterocytes on the tip of intestinal villi (2), which suggests that specific host receptors must exist. *In vitro*, they also display restricted tropism, binding to a variety of cell lines, but efficiently infecting only those of renal or intestinal epithelium origin (3). Despite advances in knowledge regarding the molecular and structural biology of the virus, little is known about rotavirus cell receptors. It is known that some animal rotavirus strains attach to sialic acid (SA) on cell surfaces, and this interaction has been shown to be required for the efficient infection of virus to susceptible cells, both *in vitro* and *in vivo* (4). However, the binding of animal rotaviruses to an SA-containing cell receptor has been shown to be nonessential, because variants whose infectivity is no longer dependent on the binding to these acid sugars have been isolated (5). The secondary importance of SA as the attachment site for rotaviruses is also demonstrated by the fact that the infectivity of most, if not all, human rotavirus (HRV) strains is not affected by neuraminidase (NA) treatment of cells (6–8).

Integrins are a family of α/β heterodimers of cell adhesion receptors that mediate cell–extracellular matrix and cell–cell interactions, and are known to function as signaling receptors for a variety of cellular processes, including spreading, migration, proliferation, differentiation, and survival (9–11). These cell molecules are commonly used as receptors for many different viruses, including echoviruses 1, 8, 9, and 22 (12–15), coxsackievirus A9 (16), foot-and-mouth disease virus (17, 18), papillomavirus (19), adenovirus (20), adeno-associated virus type 2 (21), and hantaviruses (22), with integrin $\alpha_v\beta_3$ being, so far, the most frequently used as virus receptor (14, 16, 17, 20, 22).

Recently, it was found that rotavirus surface proteins contain sequence binding motifs for $\alpha_2\beta_1$, $\alpha_4\beta_1$, and $\alpha_x\beta_2$ integrins. Antibodies to these integrins, and peptides containing these sequence motifs, were shown to block the infectivity of simian rotavirus strain SA11 and the HRV strain RV5 (23). In addition, $\alpha_2\beta_1$ and $\alpha_4\beta_1$ integrins have been shown to mediate the attachment and entry of rotavirus SA11 into the human myelogenous leukemic cell line K562 (24).

We recently reported that proteins from MA104 cells, extracted with the nonionic detergent octyl β-glucoside under noncytolytic conditions, have the capacity to inhibit the infectivity of rotaviruses when preincubated with the virus before cell infection (25). In the present study, we have identified one of these proteins as the β_3 integrin subunit, and we demonstrate that $\alpha_V\beta_3$ integrin interacts with NA-sensitive and -resistant strains at a postattachment step and is capable of promoting rotavirus infection of the poorly permissive CHO (Chinese hamster ovary) cells.

Materials and Methods

Cells and Viruses. MA104 cells were cultured in Eagle's minimal essential medium (MEM) supplemented with 10% (vol/vol) FCS. CHO cells were grown in DMEM with 10% (vol/vol) FCS. CHO cells transfected with $\alpha_{IIb}\beta_3$ (CHO-A5) and $\alpha_v\beta_3$ (CHO-VNRC) integrins (26) were grown in DMEM/10% FCS, in the presence of 400 μg/ml G418 (GIBCO). Rotavirus strains RRV, Wa, and nar3 (5, 8) were propagated in MA104 cells (8). Reovirus serotype 1 was obtained from C. Ramos (Instituto Nacional de Salud Pública, Cuernavaca, Morelos, Mexico) and was grown in L929 cells as previously described (27). Poliovirus type 3 was obtained from R. M. del Angel (Centro de Investigación y Estudios Avanzados del Instituto Politécnico Nacional, Mexico D.F.) and grown in MA104 cells. Rabbit polyclonal antibody against reovirus type 1 was kindly provided by P. Lee (Univ. of Calgary, Alberta, Canada).

Ligands, Peptides, and Antibodies. Laminin, glycophorin A, chondroitin sulfate A, BSA, and collagen type I were obtained from Sigma, fibronectin was obtained from GIBCO, and vitronectin was either purchased from Sigma or purified from human plasma as described previously (28). All proteins were used at 10 μg/ml, unless otherwise indicated. Peptides GRGDSP and GRGESP (hereafter called RGD and RGE, respectively) were obtained from GIBCO and used at 400 μg/ml. Polyclonal goat IgG

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Abbreviations: SA, sialic acid; NA, neuraminidase; HRV, human rotavirus; RRV, rhesus rotavirus.

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antibodies directed against an epitope located at the amino terminus of integrin subunits α_2, α_3, α_4, α_v, α_x, α_{IIb}, β_2, and β_3, and mAb 4B7R to subunit β_1, were obtained from Santa Cruz Biotechnology and used at 20 μg/ml. mAbs to integrins α_1 (FB12), α_2 (P1E6), α_3 (P1B5), α_4 (P1H4), α_5 (P1D6), α_6 (NK1-GoH3), α_v (P3G8), α_{IIb} (CA3), $\alpha_v\beta_3$ (LM609), β_1 (P4G11), β_2 (P4H9), β_3 (25E11), and β_4 (ASC-9), purchased from Chemicon, were used at 10 μg/ml. mAbs to integrins α_2 (P1E6, 3.2 μg/ml), β_2 (MHM23, 41 μg/ml), and α_4 (P4G9, 8.1 μg/ml) were purchased from Dako and used at the concentrations indicated. mAb B5-IVF2 to β_5 (Upstate Biotechnology) was used at 10 μg/ml. mAb 26 to β_3 (Transduction Laboratories) was used at 5 μg/ml.

Infectivity Assay. MA104 or CHO cells in 96-well plates were washed twice with MEM, and then about 1,000 focus-forming units (ffu) of RRV, nar3, or Wa rotavirus, or of control virus, reovirus or poliovirus, were adsorbed to the cells for 45 min at 4°C (for 1 h at 37°C in the case of CHO cells). After the adsorption period, the virus inoculum was removed, the cells were washed twice with MEM, and cultures were maintained for 14 h at 37°C. Infected cell cultures were fixed and tested with an immunoperoxidase focus detection assay, as described previously (29). The ffu were counted by using a Visiolab 1000 station (Biocom, Paris; ref. 25).

Blocking Assays. To evaluate the blocking activity of integrin ligands and antibodies, and of RGD and RGE peptides, MA104 or CHO cells were washed twice with MEM and incubated with indicated concentrations of the reagents in MEM, for 60 min (90 min for antibodies) at 37°C. For all incubations with vitronectin, MEM containing 400 μM Mn^{2+} instead of Ca^{2+} was used (30), whereas for all other procedures, including washings, regular MEM with Ca^{2+} was used. After the incubation step, reagents were removed and the cells were infected as described above. To evaluate whether antibodies to β_3 and vitronectin were able to inhibit rotavirus infectivity if added after the virus had been adsorbed, MA104 cells in 96-well plates were washed twice with MEM and chilled on ice for 5 min, and the virus was adsorbed at 4°C for 60 min. The cells were then washed twice with ice-cold MEM, and either vitronectin (1.5 μg/ml) or anti-β_3 antibodies (Santa Cruz Technology, 20 μg/ml) were added, and the mixture was incubated for 1 h at 4°C. The cells were washed once with MEM and maintained for 14 h at 37°C before immunostaining for virus. As control for these experiments, vitronectin or anti-β_3 antibodies were added for 1 h at 4°C before addition of the viruses for 1 h at 4°C, or were added to the cells after the virus had been adsorbed for 1 h at 37°C.

Binding Assay. The binding assay was carried out as described by Zárate *et al.* (31). Briefly, a suspension of 5×10^4 cells, preincubated either with 20 μg/ml of a goat polyclonal antibody to the β_3 integrin subunit (Santa Cruz Technologies) or with 1.5 μg/ml of vitronectin for 1 h at 4°C, were mixed with 300 ng of purified virus in MEM/1% BSA in a final volume of 200 μl and incubated for 1 h at 4°C with gentle mixing. The cell–virus complexes were washed three times with ice-cold PBS containing 0.5% BSA. In the final wash, the cells were transferred to a fresh tube, and then treated with 50 μl of lysis buffer (50 mM Tris, pH 7.5/150 mM NaCl/0.1% Triton X-100). The virus present in the lysates was quantified by an ELISA (31). In all binding assays, a binding control with no cells was performed.

Flow Cytometry. MA104 and CHO cells grown to 80% confluence were washed and brought into a single-cell suspension by incubation with 0.5 mM EDTA in PBS at 37°C and dispersed by gentle pipetting. Cells were collected by low-speed centrifugation ($200 \times g$) and resuspended in ice-cold MEM without serum, and the cell concentration was determined with a hemocytometer. In each experiment, 2.5×10^5 cells were incubated with either mAb LM609 or IgG1 control antibody (5 μg/ml) for 1 h at 4°C, washed twice with 2% (vol/vol) FCS in PBS, and then incubated with fluorescein-conjugated anti-mouse IgG antibodies (12 μg/ml; Zymed) for 1 h at 4°C. Antibody binding was analyzed by using a FACScan flow cytometer and CELLQUEST software (Becton Dickinson) with appropriate gating parameters.

Results

Antibodies to $\alpha_v\beta_3$ Integrin Inhibit Rotavirus Infectivity. Several protein bands with the ability to block rotavirus infection were isolated by preparative gel electrophoresis from MA104 cell extracts obtained with the nonionic detergent octyl β-glucoside (25). Tryptic peptides from one of these bands, with an apparent molecular mass of 110 kDa, were sequenced; one of them was found to be identical to amino acids 266–279 of the human β_3 integrin subunit, whereas two other peptides were derived from filamin and spectrin proteins. Given this finding, antibodies to β_3 were tested for their ability to block the infectivity of the SA-dependent simian rotavirus RRV, its NA-resistant variant nar3, and the natural NA-resistant HRV strain Wa. A monoclonal antibody (mAb 26) to this integrin inhibited the infectivity of all three rotavirus strains by 40–45%, depending on the virus strain (Fig. 1*A*). Because β_3 is known to associate with integrin subunits α_v and α_{IIb} (9), we tested the blocking activity of antibodies to these integrin subunits. A polyclonal antibody to α_v, or a mAb (LM609) that recognizes both α_v and β_3 subunits, inhibited the infectivity of rotaviruses (Fig. 1*B*), whereas a mAb to subunit α_{IIb} had no effect (not shown).

Because $\alpha_2\beta_1$, $\alpha_4\beta_1$, and $\alpha_x\beta_2$ integrins have been suggested to play a role during rotavirus infection (25), the blocking activity of antibodies directed against each subunit of these integrins was compared with the activity of antibodies to α_v and β_3 (Fig. 1*B*). Antibodies to α_2, α_4, and β_2 inhibited the infectivity of all three rotavirus strains by 22–44%, depending on the antibody and the virus strain tested, whereas mAbs to α_x and β_1 had low or no inhibitory capacity, depending on the virus strain. On the other hand, antibodies to either α_v or β_3 inhibited all strains by 44–50%, with the exception of nar3, which was reduced by 27% by the α_v antibody. Antibodies to integrin subunits α_1, α_3, α_5, α_6, β_4, β_5, and α_{IIb} did not block the infectivity of any of the three viruses by more than 9% (not shown).

The Block in Infectivity by mAbs to α_2 and β_3 Integrins Is Additive. When antibodies directed against each subunit of a given integrin heterodimer were mixed, no additive inhibition of infectivity was observed (not shown). However, when combinations of antibodies directed against different integrins were tested, antibodies to $\alpha_2\beta_1$ and $\alpha_v\beta_3$ had a clear additive blocking effect (Student's t test, $P < 0.001$), suggesting that these integrins are involved in different stages of rotavirus infection. None of the other integrin antibody combinations blocked the infectivity of the viruses additively (Fig. 2).

Inhibition of Rotavirus Infectivity by Integrin Ligands. The incubation of cells with various integrin ligands showed that vitronectin, which is known to interact with $\alpha_v\beta_3$, blocked rotavirus infectivity by 60–70% at 0.5 μg/ml (Fig. 3). Fibronectin, which is also an $\alpha_v\beta_3$ ligand, inhibited infectivity by 30–50% when used at 20 times the above concentration, whereas collagen type I, which binds to $\alpha_2\beta_1$, blocked virus infectivity by 20% at 10 μg/ml. Other integrin ligands and glycoproteins, such as laminin, chondroitin sulfate, glycophorin A, and BSA, had no effect on rotavirus infectivity when incubated with cells before virus infection (Fig. 3).

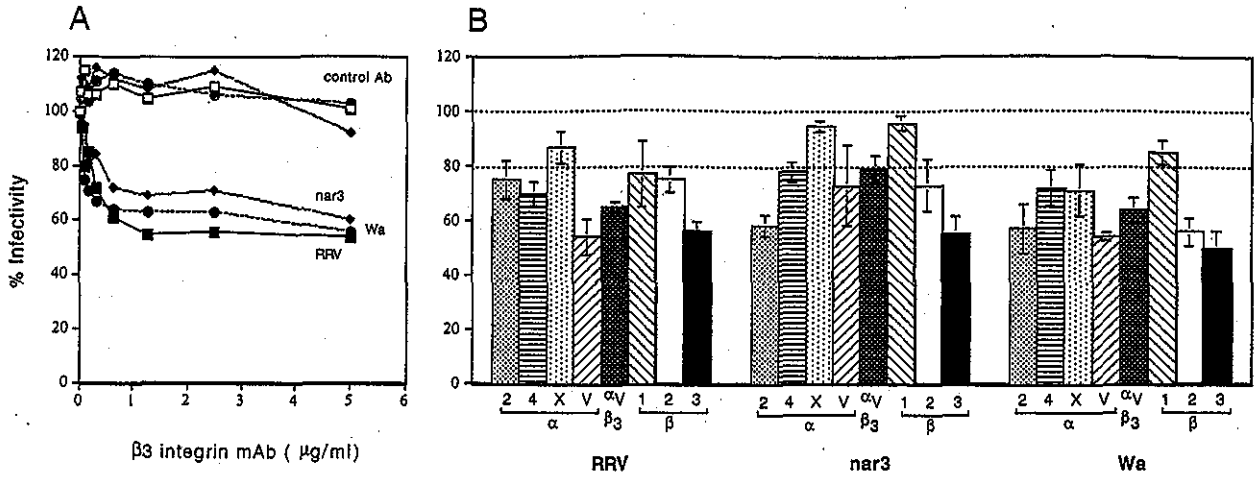

Fig. 1. Rotavirus infectivity is inhibited by antibodies to $\alpha_v\beta_3$ integrin. Antibodies to β_3 integrin (*A*) or to different integrin subunits (*B*) were added to monolayers of MA104 cells for 90 min at 37°C. After incubation with antibody, the cells were washed twice with MEM, and then RRV, nar3, or Wa viruses were adsorbed for 45 min at 4°C, the viral inoculum was removed, and the cultures were maintained for 14 h at 37°C. Cells were then fixed and immunostained. Data are expressed as percentage of the virus infectivity obtained when the cells were preincubated with MEM as control. The average numbers of foci representing 100% infectivity were 134, 122, and 139 in *A*, and 83, 98, and 86 in *B*, for RRV, nar3, and Wa, respectively. The bars represent the standard error of at least three independent experiments performed in duplicate. In *B*, the dotted lines at 80% and 100% infectivity are shown for reference. The antibody used in *A* was mAb 26. The antibodies used in *B* were as follows: polyclonal goat antibodies to α_4, α_v, α_v, and β_3 (20 μg/ml); mAb 4B7R to β_1 (20 μg/ml); mAb LM609 to $\alpha_v\beta_3$ (10 μg/ml); mAb P1E6 to α_2 (3.2 μg/ml); and MHM23 to β_2 (41 μg/ml).

Rotaviruses Interact with a Region of $\alpha_v\beta_3$ Different from its RGD-Binding Site. Typically, $\alpha_v\beta_3$ integrin recognizes its ligands through the tripeptide RGD (9); however, neither VP4 nor VP7 proteins of any of the tested rotavirus strains have this consensus sequence. To evaluate whether rotaviruses were interacting with this integrin by an RGD sequence formed in the three-dimensional structure of the viral proteins, or by an RGD-independent binding site, an RGD peptide was used to block viral infectivity. Incubation of the cells with this peptide inhibited infectivity of all three rotavirus strains by 20%, as compared with 70% inhibition caused by vitronectin (Fig. 4). Incubation of the cells with RGD before the addition of vitronectin relieved the blocking capacity of this protein, indicating that RGD efficiently blocked the attachment of vitronectin to $\alpha_v\beta_3$. A control peptide, RGE, neither blocked rotavirus infectivity nor relieved the blocking effect of vitronectin. These results indicate that rotaviruses bind to $\alpha_v\beta_3$ through an alternative region, different from the RGD-binding site, and suggest that vitronectin might be blocking rotavirus infectivity through steric hindrance. The fact that the RGD peptide was able to block rotavirus infectivity at a low level suggests that the virus binds to $\alpha_v\beta_3$ through a site proximal to the RGD-recognition domain.

Rotaviruses Interact with $\alpha_v\beta_3$ at a Postattachment Step. To determine whether the interaction of rotaviruses with $\alpha_v\beta_3$ occurred during attachment or at a postattachment step, rotavirus binding inhibition experiments with vitronectin and antibodies to β_3 were carried out. Neither vitronectin nor antibodies to the β_3 subunit inhibited the binding of any of the rotavirus strains tested (Fig. 5*A*). These results suggest that $\alpha_v\beta_3$ is not used by rotaviruses for

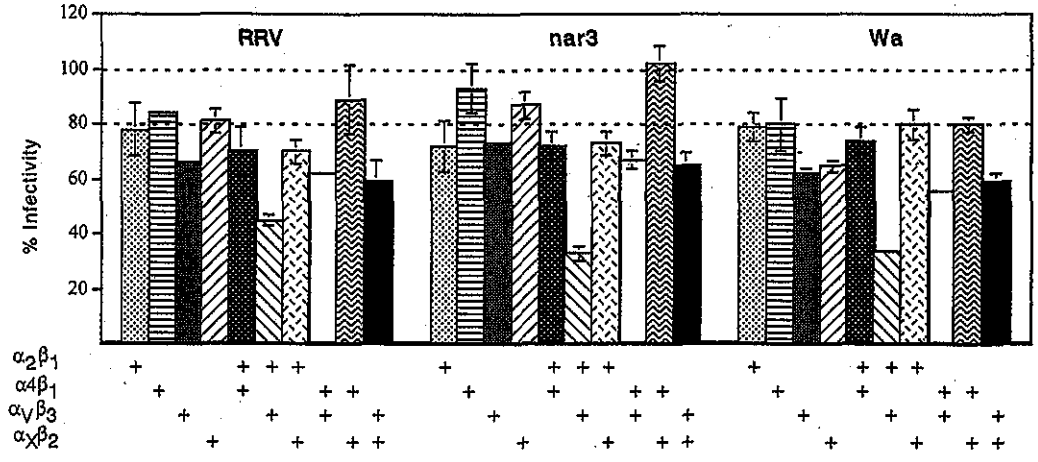

Fig. 2. Antibodies to $\alpha_v\beta_3$ and $\alpha_2\beta_1$ integrins inhibit rotavirus infectivity additively. Combinations of antibodies directed against different integrins were tested for their ability to block rotavirus infectivity in MA104 cells, as described in the legend for Fig. 1. Data are expressed as percentage of the virus infectivity obtained when the cells were preincubated with MEM as control. The average numbers of foci representing 100% infectivity were 128, 123, and 141 for RRV, nar3, and Wa, respectively. The bars represent the standard error of at least three independent experiments performed in duplicate. The dotted lines at 80% and 100% infectivity are shown for reference. The antibodies used were the same as described in the legend for Fig. 1, except for mAb LM609, which was not used.

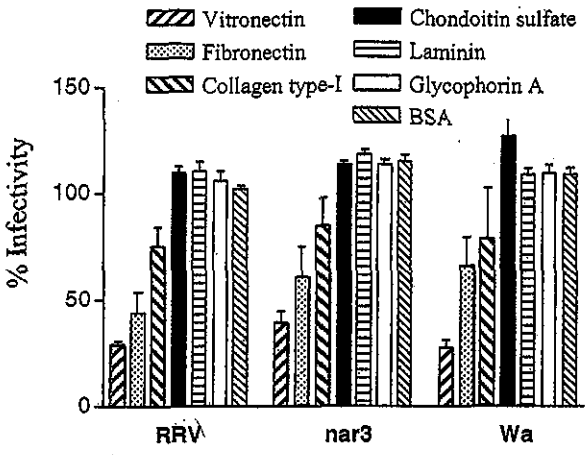

Fig. 3. Inhibition of rotavirus infectivity by integrin ligands. MA104 cells were incubated with either 0.5 μg/ml of vitronectin or 10 μg/ml of fibronectin, collagen type I, chondroitin sulfate, laminin, glycophorin A, or BSA for 60 min at 37°C, washed, and infected with rotaviruses as described in the legend for Fig. 1. Data are expressed as percentage of the virus infectivity obtained when the cells were preincubated with MEM as control. The average numbers of foci representing 100% infectivity were 128, 123, and 141 for RRV, nar3, and Wa, respectively. The bars represent the standard error of at least three independent experiments performed in duplicate.

their initial attachment to the cell surface. In addition, if vitronectin or the anti-β_3 antibody was added to the cells after the viruses had been adsorbed at 4°C, infectivity inhibition still occurred (Fig. 5*B*). Of interest, the inhibitory effect of the antibody was greater when added after adsorption of the virus than when added before the virus. On the other hand, if the virus was adsorbed for 60 min at 37°C (a temperature that allows the internalization of the virus into the cell) before vitronectin or the anti-β_3 antibody was added, no inhibitory effect was observed (not shown).

Recombinant β_3 Integrin Promotes Rotavirus Infection of CHO Cells. CHO cells, which are about 1000-fold less susceptible to viral infection than MA104 cells, and the stable transfected CHO

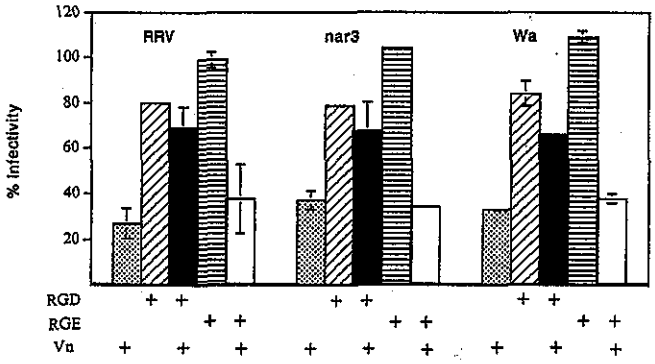

Fig. 4. Rotaviruses attach to a site on $\alpha_v\beta_3$ different from the integrin RGD-recognition domain. MA104 cells were incubated with MEM or peptides GRGDSP (RGD) or GRGESP (RGE) (400 μg/ml) for 60 min at 37°C. The cells were washed and vitronectin (Vn, 1.5 μg/ml) was subsequently added to control (MEM) or peptide-incubated cells for 60 min at 37°C. The cells were then washed and infected with rotaviruses as described in the legend for Fig. 1. Data are expressed as percentage of the virus infectivity obtained when the cells were preincubated with MEM as control. The average numbers of foci representing 100% infectivity were 109, 161, and 114 for RRV, nar3, and Wa, respectively. The bars represent the standard error of at least two independent experiments performed in duplicate.

variant cell lines VNRC and A5, which express the $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$ integrins, respectively (26), were used to determine whether β_3 integrin expression facilitates rotavirus infectivity. Both VNRC and A5 cells were 3 to 4 times more susceptible to rotavirus infection than parental CHO cells. This increase in infectivity was shown to be blocked by incubation with either an antibody to β_3 or vitronectin (shown in Fig. 6*A* for VNRC cells), indicating that the augmented infectivity observed in these cells is due to the expression of β_3 integrin. The level of $\alpha_v\beta_3$ cell surface expression in VNRC cells as compared with parental CHO and MA104 cells is shown in Fig. 6*B*.

Discussion

Comparative characterization of three rotavirus strains, the SA-dependent simian rotavirus RRV, its NA-resistant variant nar3, and the HRV strain Wa, which is naturally resistant to NA treatment of cells, has been used to understand the early events of rotavirus infection. At least three cell surface sites seem to be involved in the rotavirus–MA104 cell interaction during the early steps of infection with these three viruses (8, 32, 33). In the present study, $\alpha_v\beta_3$ integrin has been identified as a postbinding receptor for rotavirus in these cells.

Rotavirus binding to $\alpha_v\beta_3$ has been shown to be RGD-independent, consistent with the fact that neither of the surface proteins, VP4 or VP7, nor the protein that forms the intermediate layer of the virus, VP6, has an RGD sequence motif. Hantavirus cell entry has also been shown to be mediated by β_3 integrins (22). Like that of rotaviruses, their entry is not blocked by RGD peptides and is still mediated by $\alpha_{IIb}\beta_3$-integrin mutants defective in ligand binding, indicating that their interaction with β_3 is independent of the integrin binding to physiologic ligands. The binding of $\alpha_v\beta_3$ to sequences other than RGD is not without precedent, since such an interaction has been reported for other proteins, including matrix metalloproteinase 2, basic fibroblast growth factor, and ADAM 23/MDC3 human disintegrin (34).

Rotavirus nar3 binds to MA104 cells through the VP5 domain of VP4 (31), and more recently we have found that this attachment is mediated by $\alpha_2\beta_1$ integrin (32). We also found that RRV interacts with this integrin after initially binding to a SA-containing compound through the VP8 domain of VP4 (32). The fact that antibodies to $\alpha_2\beta_1$ and $\alpha_v\beta_3$ blocked rotavirus infection in an additive manner suggests that these integrins play a role at different stages of virus entry, an observation consistent with the idea that regardless of the primary cell molecule recognized by these three rotavirus strains, they all engage in a postattachment interaction with integrin $\alpha_v\beta_3$. It is of interest to note that rotaviruses bind to essentially every cell line that has been tested, although they efficiently infect only cells of intestinal or renal origin (3). Thus, the interaction with $\alpha_v\beta_3$ could be responsible, at least in part, for the restricted cell range of these viruses.

By flow cytometry $\alpha_v\beta_3$ integrin has been found in MA104, COS7, and Caco-2 cells (Fig. 6*B* and ref. 35), which are all susceptible to rotavirus infection. On the other hand, this integrin was not detected, or was barely detected, in CHO, BHK, and K562 cells (Fig. 6*B* and ref. 36), which are 100- to 1000-fold less susceptible to infection by these viruses. Thus, there seems to be a correlation between the susceptibility to rotavirus infection and the presence of $\alpha_v\beta_3$ integrin. Analysis of a larger panel of permissive and semipermissive cell lines is, however, needed to confirm this observation.

Rotavirus infection was initially reported to be mediated by integrins by Coulson's group (23, 24). They demonstrated that rotavirus SA11 binding to the human myelogenous leukemic cell line K562 increases with integrins $\alpha_2\beta_1$ and $\alpha_4\beta_1$ expression via transfection (24), and that the binding increase resulted in an augmented infection of the transfected cells. It was also shown that treatment of K562 cells with the phorbol ester phorbol 12-myristate 13-acetate (PMA) significantly increases cell infec-

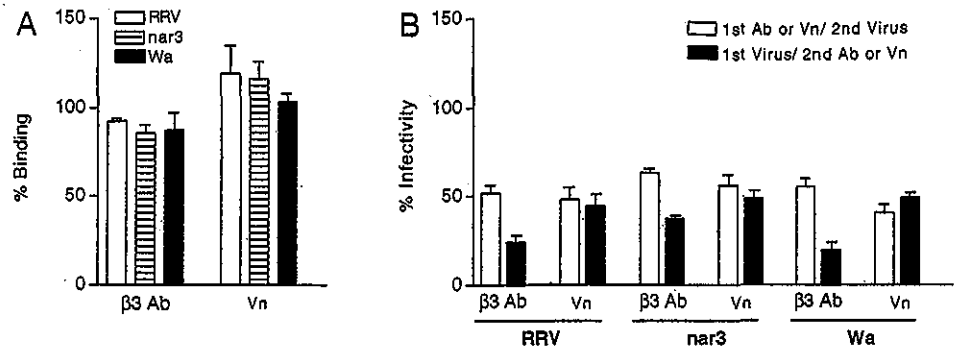

Fig. 5. Rotaviruses interact with $\alpha_v\beta_3$ at a postattachment step. (*A*) MA104 cells in suspension were incubated with a goat polyclonal antibody (20 μg/ml) against β_3 integrin, or with vitronectin (Vn, 1.5 μg/ml) for 1 h at 4°C. The cells were washed once with PBS and subsequently mixed with purified RRV, nar3, or Wa rotaviruses for 1 h at 4°C. After washing, the cells were lysed and the attached viruses were quantified by an ELISA (31). (*B*) MA104 cells were incubated in 96-well plates with rotaviruses for 1 h at 4°C, and then either β_3 antibodies or vitronectin (at the same concentrations used in *A*), were added for 1 h at 4°C (1st virus/2nd Ab or Vn). In the control experiment (1st Ab or Vn/2nd virus), vitronectin and antibodies were added before the viruses, using the same incubation conditions. The cells were then further incubated for 14 h at 37°C and immunostained for the virus. Data are expressed as percentage of the virus binding, or infectivity, when the cells were preincubated with MEM as control. The average numbers of foci representing 100% infectivity were 130, 99, and 114 for RRV, nar3, and Wa, respectively. The bars represent the standard error of at least two independent experiments performed in duplicate.

tion, without increasing virus binding levels, leading to the conclusion that the induction of the endogenous $\alpha_2\beta_1$ gene expression was the most likely basis for the augmented infectivity. It is known, however, that in addition to the induction of $\alpha_2\beta_1$, PMA also induces high expression levels of $\alpha_v\beta_3$ integrin in K562 cells (36). The fact that Hewish *et al.* (24) did not detect an enhancement in SA11 virus binding to PMA-treated cells suggests that the infectivity increase was the result of a post-binding interaction. Thus, it is likely that at least a fraction of the infectivity increase in PMA-treated K562 cells could have been the result of the induced $\alpha_v\beta_3$ expression. Because CHO cells do not express $\alpha_2\beta_1$ on their surface (unpublished observation), the rotavirus infectivity enhancement observed in the VNRC and A5 β_3-expressing cells is likely to be due only to the enhanced expression of this integrin.

Integrin subunit β_3, as well as $\alpha_2\beta_1$ integrin, has been reported to be present in murine and human enterocytes, primarily associated with the basolateral cell surface (37–39). Thus, if these integrins are involved in facilitating rotavirus cell entry in a natural infection, they might be initially available for virus interaction in limited amounts, unless the cell–cell contact regions are disrupted to expose their basolateral surface. In this regard, in a recent study rotaviruses were shown to induce structural and functional alterations in tight junctions of polarized intestinal Caco-2 cell monolayers (40). In addition, in polarized MDCK cells, $\alpha_2\beta_1$ integrin is exposed apically to the tight junctions (41). Finally, it is also relevant that enteroviruses such as coxsackievirus A9 and echovirus 9 can employ $\alpha_v\beta_3$ integrin as cellular receptor (14, 16), and that adenovirus-mediated gene delivery to the intestinal epithelium is dependent on, or is significantly increased by, the presence of $\alpha_v\beta_3$, among other integrins (35, 38).

Rotavirus cell infection involves a delicate cell and tissue tropism which may require the specific presence of several cell molecules organized in a precise manner. This idea is consistent with the fact that although expression of $\alpha_v\beta_3$ integrin in CHO cells (present study) or $\alpha_2\beta_1$ integrin in K562 cells (24) enhances the susceptibility of these cells to rotavirus infection, the level of permissiveness achieved does not attain that of MA104 cells, indicating that other cell surface molecules important for rotavirus entry are absent from the former cell lines. It remains to be established, among other things, whether any of the receptor molecules described so far are irreplaceable, and if, in fact, there exists a unique infectivity pathway for rotaviruses, with distinct entry points for different virus strains.

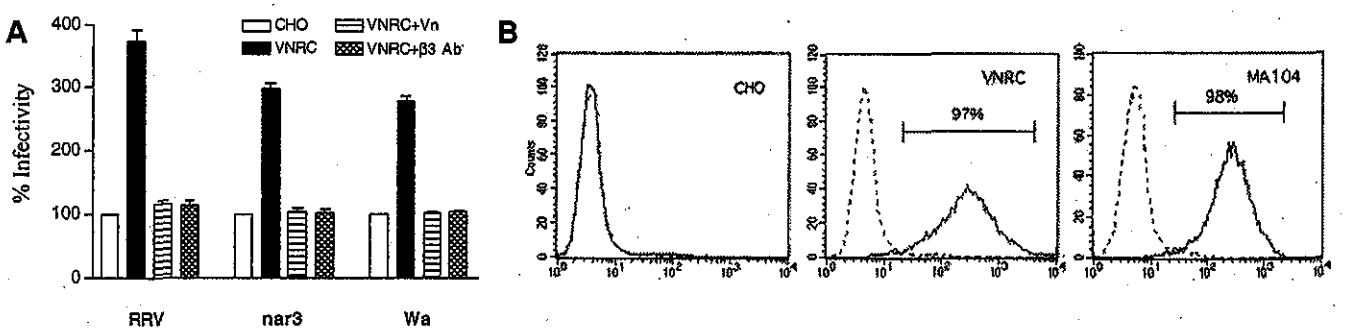

Fig. 6. (*A*) Recombinant β_3 integrin promotes CHO cell infection. Parental CHO cells (CHO) or stably transfected CHO cells expressing the α_v and β_3 integrin subunit genes (VNRC) (26) were infected in 96-well plates with rotaviruses. VNRC cells were also infected after they had been preincubated with either vitronectin (1.5 μg/ml; VNRC+Vn) or β_3 goat polyclonal antibodies (20 μg/ml; VNRC+β_3 Ab). Data are expressed as percentage of the virus infectivity obtained when the cells were preincubated with MEM as control. The average numbers of foci representing 100% infectivity were 72, 157, and 43 for RRV, nar3, and Wa, respectively. CHO cells are about 1000-fold less infectable than MA104 cells by the three viruses tested, and therefore dilutions of virus stocks were adjusted accordingly, to count the above-indicated number of infected cells in control wells. The bars represent the standard error of at least four independent experiments performed in duplicate. (*B*) Flow cytometric analysis of $\alpha_v\beta_3$ integrin surface expression in parental CHO, VNRC, and MA104 cells. mAb LM609 (solid lines), which recognizes the $\alpha_v\beta_3$ heterodimer, and the isotype IgG1 control antibody (dashed lines) were used at 5 μg/ml.

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D. La proteína de choque térmico hsc70 está involucrada en la entrada de rotavirus a la célula huésped

En este trabajo se caracterizó como receptor de los rotavirus a la proteína de choque térmico hsc70. A pesar de que esta proteína se considera de localización citoplásmica y nuclear, existen reportes de que puede encontrarse en la superficie de las células (60); sin embargo, dado que carece de señales de exportación hacia la membrana citoplasmática, el mecanismo por el cuál esta proteína llega a la superficie no ha sido dilucidado. Por todo ello, uno de los objetivos de este trabajo fue mostrar que la proteína hsc70 efectivamente se encuentra en la superfice de las células MA104, mediante citometría de flujo. Además, encontramos que anticuerpos dirigidos contra la proteína hsc70 bloquean la infectividad de los rotavirus; por otro lado, mostramos que estos virus fueron capaces de unirse específicamente a la proteína hsc70 recombinante expresada en bacteria e inmovilizada en placas para ELISA.

Encontramos que los anticuerpos dirigidos contra hsc70 no fueron capaces de prevenir la unión de ninguna de las tres cepas de rotavirus utilizadas en este trabajo (RRV, nar3 y Wa), y que anticuerpos dirigidos contra las proteínas virales VP7 y VP4 bloquearon la unión de los rotavirus con hsc70 en ensayos de ELISA. Este trabajo se publicó en el artículo "Heat shock cognate protein hsc70 is involved in rotavirus cell entry" (28).

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Heat Shock Cognate Protein 70 Is Involved in Rotavirus Cell Entry

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In this work, we have identified the heat shock cognate protein (hsc70) as a receptor candidate for rotaviruses. hsc70 was shown to be present on the surface of MA104 cells, and antibodies to this protein blocked rotavirus infectivity, while not affecting the infectivity of reovirus and poliovirus. Preincubation of the hsc70 protein with the viruses also inhibited their infectivity. Triple-layered particles (mature virions), but not double-layered particles, bound hsc70 in a solid-phase assay, and this interaction was blocked by monoclonal antibodies to the virus surface proteins VP4 and VP7. Rotaviruses were shown to interact with hsc70 at a postattachment step, since antibodies to hsc70 and the protein itself did not inhibit the virus attachment to cells. We propose that the functional rotavirus receptor is a complex of several cell surface molecules that include, among others, hsc70.

Rotaviruses are formed by a triple-layered protein capsid (12). In the surface of the virus there are two proteins, VP4 and VP7, which are responsible for the initial interactions of the virus with the host cell. VP4, the viral attachment polypeptide, is cleaved by trypsin into subunits VP5 and VP8, and this cleavage is associated with the penetration of the virion into the cell (12).

Rotavirus strains can be divided, with regard to their requirements to attach to the host cell, into neuraminidase (NA)-sensitive (those requiring sialic acid) and NA-resistant strains (those that either do not require sialic acid or bind to sialic acid molecules resistant to the NA treatment). Many of the strains isolated from animals, including the rhesus rotavirus RRV, are NA sensitive (9, 16, 28, 33), while a number of animal rotaviruses and most, if not all, human rotavirus strains, including the human rotavirus Wa, are resistant to NA (9, 16, 34). Some NA-sensitive rotavirus strains have been suggested to bind ganglioside GM3 containing *N*-glycolyl neuraminic acid as the sialic acid moiety, which is sensitive to NA treatment (11, 47), while some human rotaviruses have been proposed to use GM1, an NA-resistant ganglioside, to attach to cells (21). In addition, it has recently been shown that rotavirus nar3, a variant of RRV that, unlike the parental virus, does not require sialic acid to bind to cells, uses integrin $\alpha 2\beta 1$ as its docking molecule on the cell surface (54).

Regardless of the cell molecule employed to initially attach to the cell surface, it has been shown that both NA-sensitive and NA-resistant rotaviruses interact with integrin $\alpha v\beta 3$ at a postattachment step (19). These findings have led to the hypothesis that rotavirus cell entry is a multistep process (1, 34).

We recently showed that an octyl-β-glucoside extract of MA104 cells, obtained under nonlytic conditions, has the ability to efficiently block rotavirus infectivity when preincubated with the virus before cell infection (20). From this detergent extract, we isolated a protein band of about 73 kDa which showed a high blocking specific activity for rotaviruses (20). Four tryptic peptides derived from this band were sequenced; two of them corresponded to α-actinin, while the other two had a 100% identity with the human heat shock cognate protein hsc70, at amino acid regions 160 to 171 and 221 to 236.

hsc70 is present on the surface of rotavirus-susceptible and nonsusceptible cells. Even though hsc70 does not have an export signal sequence, it has been shown that it can be exposed on the surface of various cell types (see below). In this work, the presence of this protein on the surface of MA104 cells was demonstrated by flow cytometry (Fig. 1A) and by immunofluorescence (Fig. 1B), where nonpermeabilized cells showed the ringlike pattern of staining that has been previously observed for human 5838 Ewing's sarcoma cells expressing hsc70 on their surface (37). In addition, we analyzed by flow cytometry the presence of this protein on the surface of various cell lines which differ in their susceptibility to the virus. We assayed Caco-2 cells, which are infected as efficiently as MA104 cells by rotaviruses RRV, nar3, and Wa; Hep2 cells, which are not infected by human rotavirus Wa and are about 10- and 1,000-fold less susceptible than MA104 for rotaviruses nar3 and RRV, respectively; and BHK cells, which are about 10,000-fold less susceptible to infection by all three viruses (data not shown). Hsc70 was detected on the surface of these three cell lines, being more abundant in Hep2 and BHK cells than in Caco-2 cells (Fig. 1C).

Antibodies to hsc70 block rotavirus infection. Given these findings, monoclonal antibodies (MAbs) to hsc70 were evaluated for their ability to block rotavirus infectivity when preincubated with MA104 cells before virus infection. An anti-α-actinin MAb was found not to affect rotavirus infectivity, while MAbs either specific for hsc70 or which cross-react with both hsc70 and hsp70 blocked the infectivity of all three viruses by about 50 to 60%, depending on the MAb and the virus strain (Fig. 2A). On the other hand, one MAb specific for hsp70

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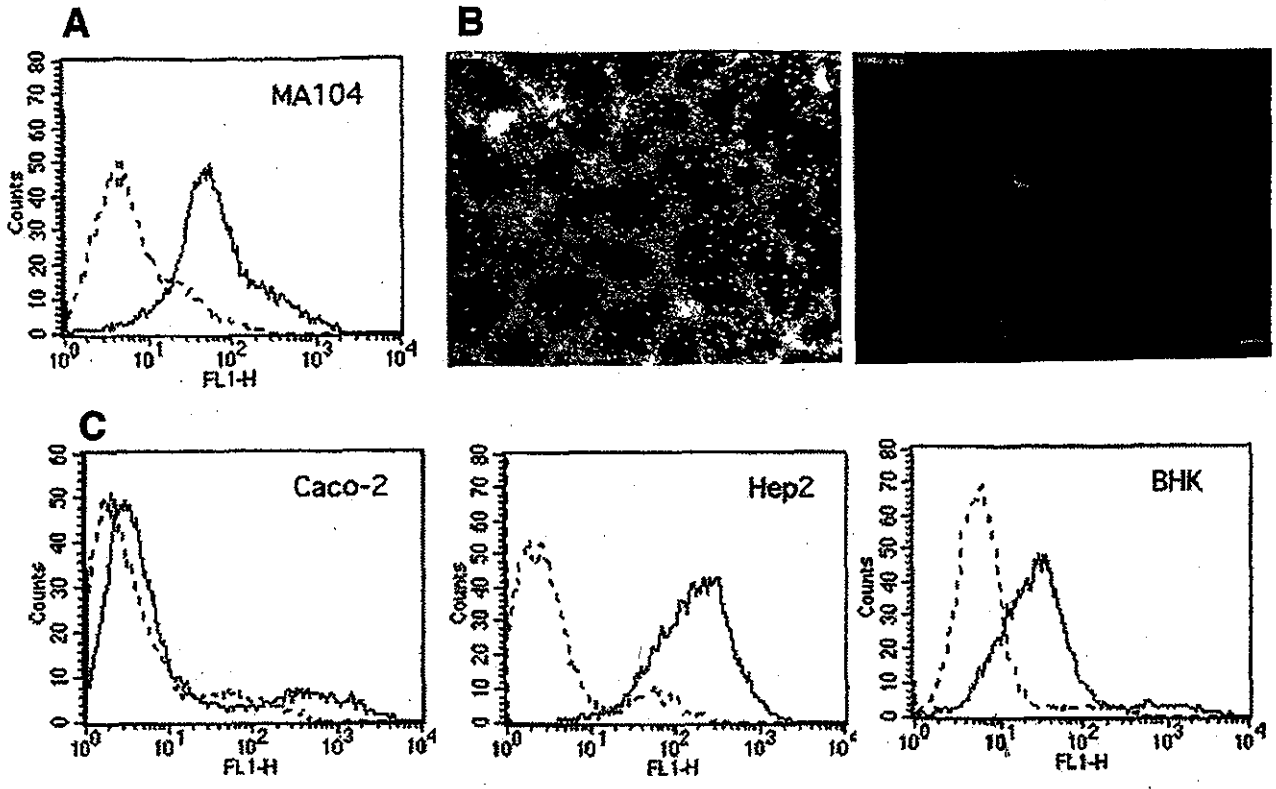

FIG. 1. hsc70 is present on the surface of cells. (A) Flow cytometry analysis of hsc70 surface expression in MA104 cells. MAb MA3-014, specific for hsc70 (solid line), was used at a 1:25 dilution. The control antibody of isotype immunoglobulin M (dashed line) was used at 20 μg/ml. The assay was performed as described previously (29). (B) Immunofluorescence of nonpermeabilized MA104 cells incubated with a MAb to hsc70. MA104 cells were fixed with paraformaldehyde and were incubated with a 1:25 dilution of MAb MA3-014 (left panel) or control immunoglobulin M (10 μg/ml) (right panel) for 90 min at room temperature, followed by staining with a goat anti-mouse immunoglobulin M coupled to fluorescein isothiocyanate, as described earlier (29). (C) Flow cytometry analysis of the presence of hsc70 on the surface of Caco-2, Hep2, and BHK cells, using the same hsc70 and control antibody described above for MA104 cells.

(MAb MA3-009) did not show rotavirus-blocking activity. In addition, a mixture of MAbs to hsc70 inhibited the infectivity of all three rotavirus strains by about 80%, while not affecting the infectivity of poliovirus and reovirus, two other nonenveloped viruses (Fig. 2B). The MAbs and a hyperimmune serum to hsc70, prepared by immunization of rabbits with a recombinant purified human hsc70 protein (see below), did not inhibit rotavirus infectivity if incubated with the cells after the viruses had been adsorbed at 37°C for 1 h (not shown). The rabbit polyclonal antibodies to hsc70 were used to test if this protein was involved in the infection by rotaviruses of cells other than MA104. These antibodies inhibited the infectivity of rotaviruses Wa, nar3, and RRV in Caco-2 cells as efficiently as in MA104 cells (Fig. 2C) and also blocked the infectivity of nar3 in Hep2 cells (Fig. 2C). The inhibition of the infectivity of rotaviruses RRV and Wa in Hep2 cells and of all three strains in BHK cells could not be evaluated reliably since only a few cells were infected. These findings suggest that hsc70 is involved in rotavirus infection of cells other than MA104 but also indicate that hsc70 by itself is not the protein that determines the tropism of rotaviruses.

hsc70 binds to rotaviruses and inhibits their infectivity. A recombinant human hsc70 protein containing a COOH-terminal histidine tail was produced in bacteria and purified by nickel column affinity. This protein, as well as a natural bovine hsc70 protein (StressGen Biotechnologies), was able to block rotavirus infectivity in a dose-dependent manner when preincubated with the viruses before infection. At a concentration of 50 ng/ml, they inhibited the infectivity of the viruses by about 40 to 50% (Fig. 3A and B). In contrast, α-actinin (Fig. 3A), spectrin (Fig. 3B), and bovine serum albumin (not shown) had no effect on the infectivity of the viruses.

To confirm that rotaviruses bind directly and specifically to hsc70, purified triple-layered particles (TLPs) of RRV or virus particles lacking the surface proteins (double-layered particles, DLPs) were tested for their ability to bind the recombinant human hsc70 protein in an enzyme-linked immunosorbent assay. The TLPs bound to hsc70 in a dose-dependent manner, while the DLPs failed to bind this protein at the concentrations assayed (Fig. 4A). The binding of TLPs to hsc70 was shown to be specific, since it was efficiently competed by MAbs 159 to VP7 ($P = 0.02$) and 2G4 to VP5 ($P = 0.009$) but not by MAbs 7A12 to VP8 ($P = 0.14$) and 255/60 to VP6 (Fig. 4B).

Neither antibodies to hsc70 nor the hsc70 protein blocks the binding of rotaviruses to cells. The hsc70 polyclonal serum blocked the infectivity of the viruses in MA104 cells to an extent similar to that achieved with the MAbs to this protein; however, it did not significantly block the binding of rotaviruses to cells ($P = 0.29$, 0.50, and 0.47 for rotaviruses Wa, nar3, and RRV, respectively; Fig. 5A). Similarly, despite its ability to

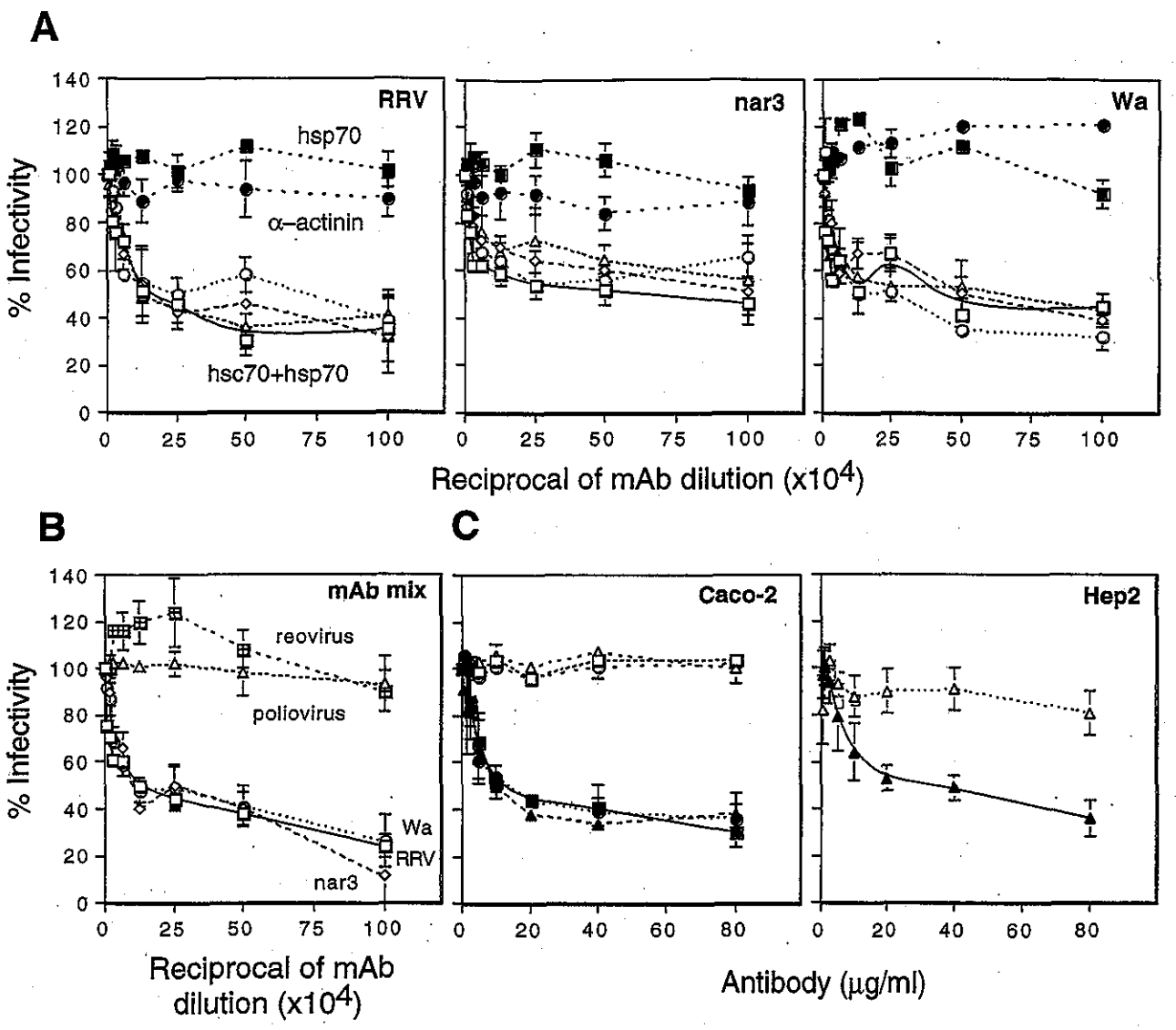

FIG. 2. Rotavirus infectivity is inhibited by antibodies to hsc70. MAbs to hsc70 (A) or a mixture of MAbs to hsc70 (B) was added to monolayers of MA104 cells for 90 min at 37°C. After incubation with antibody, the cells were washed, and then RRV, nar3, or Wa viruses (or reovirus and poliovirus in panel B) were adsorbed for 45 min at 4°C, the viral inoculum was removed, and the cultures were maintained for 14 h at 37°C. Cells were then fixed and immunostained as described earlier (20). Data are expressed as percentage of the virus infectivity obtained when the cells were preincubated with minimal essential medium as control. The average number of foci counted, representing 100% infectivity, was 107, 173, and 100 in panel A and 189, 129, and 112 in panel B for RRV, nar3, and Wa, respectively. The bars represent the standard error of at least three independent experiments performed in duplicate. The antibodies used in panel A were MA3-001 (open triangles), MA3-006 (open squares), MA3-007 (open circles), MA3-008 (open diamonds) (hsc70 + hsp70), MA3-009 (closed squares) (hsp70), and a MAb to α-actinin (closed circles). The mixture of antibodies used in panel B contained MAbs MA3-001, MA3-006, MA3-007, MA3-008, and MA3-014. All these antibodies were from Affinity Bioreagents Inc. (C) Rotavirus infectivity in Caco-2 and Hep2 cells is inhibited by antibodies to hsc70. Rabbit preimmune (open symbols) or hyperimmune (closed symbols) purified polyclonal antibodies to hsc70 were added to monolayers of Caco-2 and Hep2 cells, followed by addition of the viruses using the same incubation conditions described above. The average number of foci counted representing 100% infectivity was 118, 113, and 99 for rotaviruses RRV, nar3, and Wa, respectively, in Caco-2 cells and 80 for nar3 in Hep2 cells. The bars represent the standard error of at least two independent experiments performed in duplicate.

block the infectivity of rotaviruses, the hsc70 protein did not significantly affect the binding of the viruses to cells ($P = 0.11$, 0.74, and 0.50 for rotaviruses Wa, nar3, and RRV, respectively; Fig. 5A). These results suggest that rotaviruses interact with hsc70 on the cell surface at a postattachment step. This observation is supported by the fact that if antibodies to hsc70 or the hsc70 protein is added after the viruses have been adsorbed to cells at 4°C, conditions where the virus particles attach to the cell surface but do not enter the cell, they are still able to efficiently inhibit infectivity (Fig. 5B).

hsc70 is a constitutive member of the heat shock-induced hsp70 protein family that functions in normal cellular physiology. The proteins in this family are highly conserved nucleocytoplasmic ATPases which have been associated to a number

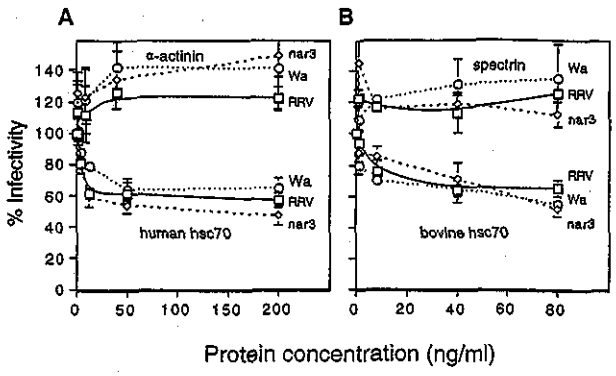

FIG. 3. Rotavirus infectivity is blocked by protein hsc70. The indicated concentrations of either recombinant human hsc70, bovine hsc70, α-actinin, or spectrin were incubated with the viruses for 90 min at 37°C. The virus-protein mixtures were added to MA104 cells for 45 min at 4°C. The cells were washed and the cultures were maintained for 14 h at 37°C. Cells were then fixed and immunostained as described earlier (20). Data are expressed as the percentage of the virus infectivity obtained when the viruses were preincubated with minimal essential medium as control. The average number of foci counted, representing 100% infectivity, was 134, 127, and 116 in panel A and 114, 94, and 118 in panel B for RRV, nar3, and Wa, respectively. The bars represent the standard error of at least three independent experiments performed in duplicate.

of functions, including protein folding, translocation across biological membranes, and assembly and disassembly of oligomeric complexes. In response to different stress conditions, these proteins prevent the formation of protein aggregates by stabilizing unfolded intermediates which are subsequently refolded to the native state or degraded (23, 32, 36). In particular, hsc70 has been shown to favor protein transport across organellar membranes, bind nascent polypeptides, and dissociate clathrin from clathrin coats (40).

It is well documented that infection of animal cells by viruses often results in alterations of the cellular stress response, which is characterized by elevation and relocalization of heat shock proteins (26). In most cases the induced stress proteins have been shown to be members of the hsp60, hsp70, and hsp90 families, depending on the type of virus and host cell (26). In some cases, a direct interaction between the stress proteins and viral polypeptides has been documented, and stress proteins have been described to be present in the mature virions of rabies (48) and human immunodeficiency (52) viruses. In particular, hsp70 and hsc70 have been reported to interact with capsid proteins from poliovirus (30), vesicular stomatitis virus (22), Sindbis virus (39), vaccinia virus (27), simian virus 40 (50), adenovirus (51), hepatitis B virus (44), reovirus (56), and polyomavirus (10). These interactions have been shown to facilitate different stages of the viruses' life cycle, including the transport into the nucleus of adenoviral DNA (51) and polyomavirus proteins (10); the activation of the polymerase of hepatitis B virus (42); the enhancement of virus gene expression in measles and canine distemper viruses (41, 43); and, in general, the packaging of the virus particles. In the case of adenovirus, it has been shown that the induction of hsp70 and hsp40 is essential for the replication of the virus (18).

Despite their typical nucleocytoplasmic residence and the fact they do not contain obvious endoplasmic reticulum-Golgi targeting signal sequences, hsc70 and other heat shock proteins have been reported to be present on the surface of tumor cell lines (5, 15, 37, 38) and in cells infected with viruses (7, 8), as well as in mammalian spermatogenic cells (6, 35), epidermal cells (46), and monocytes and B cells (31). The type of association that the heat shock proteins establish with the cell surface is not known; however, it has been recently shown that hsc70, hsp70, and other stress proteins interact with specific receptors in antigen-presenting cells and are internalized by receptor-mediated endocytosis (3, 4). hsc70 is also known to interact with lipids, and it has been shown that this protein is able to form cation channels in acidic phospholipid membranes (2).

With regard to the interaction of stress proteins with viral polypeptides at the cell surface level, an hsp60-like protein located on the surface of freshly isolated human monocytes, as well as in established monocytic and T-cell lines, has been shown to interact with the human immunodeficiency virus gp41 protein, and it has been suggested that this interaction might facilitate the virus infection (52). Also, hsc70 has been described as an enhancement factor on the surface of murine fibroblasts and in a human T-cell line for the syncytium formation induced by human T-cell lymphotropic virus type 1 (13, 49). In this work, we have shown that hsc70 seems to be involved in the cell entry of rotaviruses. To our knowledge, this is the first report to provide evidence to support the role of a stress protein during the early steps of a viral replication cycle.

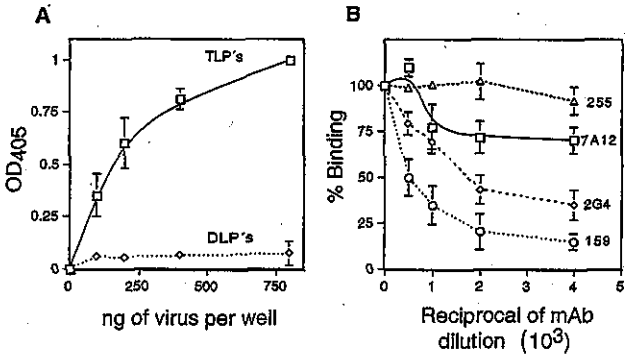

FIG. 4. Binding of rotavirus RRV to hsc70. (A) The indicated amounts of purified RRV TLPs and DLPs were added to microtiter plates to which 500 ng of nickel column affinity-purified recombinant human hsc70/well had been previously adsorbed. The bound virus was detected by incubation with a rabbit hyperimmune serum to rotavirus, followed by an alkaline phosphatase-conjugated secondary antibody, as described earlier (55). The phosphatase activity was detected with the Sigma 104 substrate, reading the optical density at 405 nm (OD_{405}). (B) Binding of RRV to hsc70 in the presence of antirotavirus MAbs. Purified RRV TLPs (300 ng) were preincubated for 1 h at room temperature with the indicated dilutions of MAbs (ascites fluid). After incubation with the MAbs, the virus-antibody mixture was added to the hsc70-coated wells and the bound virus was detected as described above. The bars represent the standard deviation of the mean of two independent experiments performed in duplicate. The MAbs used were 255 to VP6, 7A12 to VP8, 2G4 to VP5, and 159 to VP7. By Student's *t* test, the *P* values for antibodies 2G4 and 159 showed that the blocking of binding for the two highest concentrations of these antibodies was significantly different from the control MAb 255 to VP6 ($P = 0.02$ and 0.05 for 2G4; $P = 0.009$ and 0.04 for 159). In the case of antibody 7A12, the *P* values were 0.14 and 0.07.

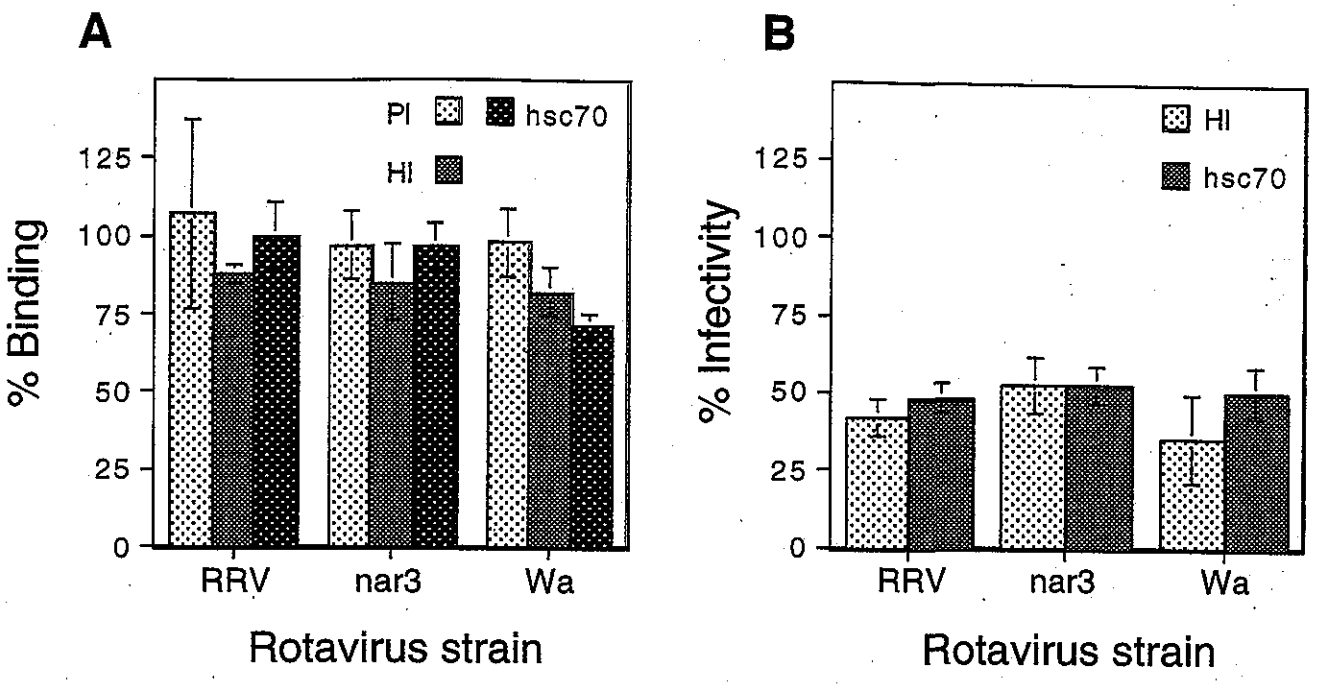

FIG. 5. Rotaviruses interact with hsc70 at a postattachment step. (A) MA104 cells in suspension were preincubated with 80 μg of preimmune (PI) or hyperimmune (HI) sera/ml to human hsc70 (partially purified by ammonium sulfate precipitation) during 1 h at 4°C. The excess, unbound antibody was removed, and then 300 ng of either RRV-, nar3-, or Wa-purified TLPs was added and the mixture was further incubated for 1 h at 4°C. To assay the binding blocking activity of the hsc70 protein (hsc70), 300 ng of the corresponding virus was incubated with the recombinant human hsc70 (10 μg/ml) for 1 h at room temperature, and then the virus-protein mixture was adsorbed to cells as described above. The amount of virus bound to cells was determined by an enzyme-linked immunosorbent assay as described earlier (55). By Student's *t* test, the *P* values for the hyperimmune serum and the hsc70 protein showed that their blocking activity for the binding of all three viruses was not significantly different from that of the control preimmune serum. The *P* values ranged from 0.11 for hsc70 incubated with rotavirus Wa to 0.74 for hsc70 incubated with virus nar3. (B) MA104 cells were incubated in 96-well plates with rotaviruses for 1 h at 4°C, and then either a hyperimmune (HI) serum to hsc70 (80 μg/ml) or the protein itself (hsc70) was added for 1 h at 4°C h. The cells were then further incubated for 14 h at 37°C and were immunostained for the virus as described earlier (20). Data are expressed as the percentage of the virus binding or infectivity when the cells were preincubated with minimal essential medium as control. The average numbers of foci representing 100% infectivity were 109, 119, and 145 for RRV, nar3, and Wa, respectively. The bars represent the standard deviation of the mean of at least two independent experiments performed in duplicate.

Similar to what was found with integrin αvβ3, rotaviruses seem to interact with hsc70 at a postattachment step. This observation is supported by the fact that (i) antibodies to hsc70 which block the infectivity of the viruses when preincubated with the cell before infection do not block their cell attachment; (ii) preincubation of the viruses with a recombinant human hsc70 protein inhibits the virus infectivity without notoriously affecting the binding of the viruses to cells; and (iii) when antibodies to hsc70, or the protein itself, are added to cells after the virus has been allowed to adsorb to the cell surface at 4°C, they still efficiently inhibit virus infectivity. These findings indicate that the interaction of rotaviruses with hsc70 may represent a late and common interaction of the viruses with the cell, after their initial attachment to other cell surface molecules. Given the major conformational changes that viral surface proteins have been shown to undergo during the cell entry of several enveloped and nonenveloped viruses (14, 17, 24, 45), it is reasonable to hypothesize that the rotavirus outer layer proteins, VP4 and VP7, also undergo conformational rearrangements during one or more of the multiple contacts that are proposed to take place between the virus particles and cellular molecules on the cell surface or during the cell entry and/or uncoating of the viruses. In this scenario, it is tempting to speculate that a protein with chaperone activity, like hsc70, could have a pivotal role to help in these processes (19, 25).

It has been shown that rotavirus-nonsusceptible cells stably transfected with the genes for α2, α4, or αv and β3 integrin subunits are only a few times more susceptible than the parental cell lines.

This limited increase in susceptibility indicates that integrins alone, as seems to be the case for hsc70, are not the only molecules needed to transform a poorly permissive cell line into one fully susceptible (like MA104) to the virus. Altogether, these data suggest that hsc70 is involved in the entry of rotaviruses, by working in combination with other proteins, most likely integrins, although other, hitherto unidentified cell receptors cannot be discarded.

It has recently been shown that sphingolipids and cholesterol are important for rotavirus infection and has been proposed that a complex of proteins immersed in the lipid microdomains known as rafts might serve as the functional receptor for rotaviruses (1, 20). Integrins and hsc70 might be components of this complex. In this regard, there has been an interesting, recent observation that bacterial lipopolysaccharide (LPS) from gram-negative bacteria and lipoteichoic acid (LTA) from

the outer cell wall of gram-positive bacteria bind to CD14, a glycosylphosphatidylinositol-linked protein which functions as their cell surface receptor on human monocytic and endothelial cell lines. After their initial binding to CD14, LPS and LTA are rapidly transferred to the heat shock proteins hsp70 and hsp90 on the cell membrane. Antibodies to these heat shock proteins were found to block the transfer of LPS and to inhibit interleukin 6 production upon LPS stimulation. These data indicate that LPS transfers from CD14 to hsp70 and hsp90, which may be part of an LPS-LTA multimeric receptor-transducing complex that might be present in lipid microdomains (53).

It remains to be determined if, during the infection of cells by rotaviruses, hsc70 serves only as an anchor on the membrane for the viruses during their transit to the cell's interior or if the chaperone activity of the protein is important for this event.

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DISCUSION

Recientemente se ha hecho evidente que la entrada de los virus a su célula huésped es un proceso que involucra varios pasos y en el que diversas proteínas, tanto celulares como virales, están involucradas. La necesidad de establecer varias interacciones con receptores específicos en la superficie celular se ha observado en virus pertenecientes a diferentes familias. Así, se sabe que el virus de la inmunodeficiencia humana, el virus del Ébola, y el virus de Herpes, por mencionar sólo algunos, utilizan más de un receptor para entrar a su célula huésped (70). Además, se ha observado que las primeras interacciones virus-célula generalmente inducen cambios conformacionales en la partícula viral que le permiten exponer nuevos sitios en las proteínas virales que participan en el proceso de penetración del virus hacia el citoplasma celular, donde se inicia el ciclo de replicación viral.

En el caso de los rotavirus, se ha encontrado que la entrada de estos virus es también un proceso de varios pasos, en el que existen cuando menos cuatro moléculas celulares identificadas como receptores. El estudio de las interacciones iniciales del virus con la célula, realizado a lo largo de este proyecto, permitió determinar que la proteína VP4 es la responsable de la unión inicial del virus a la superficie de la célula. Previamente se había mostrado que el rotavirus RRV se unía, a través del dominio VP8 de la proteína VP4, a un receptor en la superficie de la célula que contiene AS (22, 36, 46); más recientemente se ha propuesto que esta molécula receptora pudiera ser el ganglíosido GM3 (31, 66). En este trabajo encontramos que la variante nar3 se une a un receptor resistente al tratamiento de las células con NA, esta interacción es mediada por el dominio VP5 de la proteína VP4 (80). Previamente, Méndez y col habían mostrado que a pesar de su insensibilidad al tratamiento con NA, la variante nar3 aún tiene la capacidad de unirse a AS, puesto que es capaz de aglutinar eritrocitos a través de los AS presentes en la superficie de éstos (55). Sin embargo, encontramos que la variante nar3 no utiliza su dominio de unión a AS, aún en células no tratadas con NA, y que se une inicialmente a la célula a través de la proteína VP5, puesto que la proteína recombinante GST-VP5 bloquea la unión de nar3, mientras que la proteína GST-

VP8 no tiene ningún efecto. Además, los AcM dirigidos contra VP8, que son capaces de bloquear tanto la infectividad como la unión de la cepa RRV, no afectan a la variante nar3, a pesar de que son capaces de reconocer al virus nar3 en ensayos de ELISA (55).

El análisis de la secuencia del gene de VP4 de la variante nar3 mostró tres cambios en la secuencia de aminoácidos con respecto a la cepa parental RRV, concretamente en las posiciones 37 (Leu-Pro), 187 (Lys-Arg) y 267 (Tyr-Cys) (56). Los primeros dos cambios se encuentran dentro del dominio VP8, en particular el residuo 187 está situado en el dominio de hemaglutinación (interacción con AS); la caracterización de virus revertantes mostró que este cambio es el más relevante para obtener el fenotipo de resistencia al tratamiento con NA (56). El tercer cambio en la proteína VP4 de la variante nar3 cae dentro del dominio VP5; Cuadras y col, en nuestro laboratorio, encontraron que la Cys introducida en la posición 267 está involucrada en la formación de un puente disulfuro alterno con la Cys 318 de la proteína VP4 (10), y plantearon la posibilidad de que este puente disulfuro alterno pudiese cambiar la conformación de VP4 en la variante nar3, este cambio le permitiría interaccionar con la célula directamente a través de VP5, de modo diferente a la interacción que se observa para el virus parental.

Por otra parte, a pesar de que el anticuerpo 2G4 (dirigido contra VP5) no bloquea la unión a la superficie de la célula del rotavirus RRV, sí es capaz de neutralizar la infectividad de este virus. Esto indica que después de que el virus RRV se une a un receptor sensible a NA, a través de VP8, ocurre una segunda interacción (facilitada por el primer contacto) mediada por VP5; esta segunda interacción es bloqueada por el AcM 2G4. En conjunto, estos resultados sugieren que la proteína VP4 de la variante nar3 sea conformacionalmente distinta a la VP4 de la cepa parental RRV; ésto explicaría por qué el virus nar3 es capaz de interaccionar con la célula directamente a través de VP5. No obstante, hay que señalar que las posibles diferencias entre la estructura de ambas proteínas parecen ser sutiles, puesto que, como mencionamos anteriormente, el virus nar3 conserva la capacidad de aglutinar eritrocitos

y los anticuerpos dirigidos contra VP8 reconocen a esta variante tan bien como lo hacen con RRV (56).

El mapeo del sitio de unión a la superficie de la célula presente en VP5 se centró, en primer lugar, en las regiones que habían sido descritas como potencialemente funcionales dentro de esta proteína. Como se describe en la introducción, estas regiones son el tripéptido DGE (localizado en los aa 308-310), que es capaz de mediar la unión a integrinas del tipo $\alpha2\beta1$ (8) y la región hidrofóbica (en los aa 384-401), que posee actividad de fusión de liposomas *in vitro* (13, 16). Utilizamos proteínas recombinantes mutagenizadas en estas regiones y péptidos sintéticos que contienen estas secuencias en ensayos de unión a células MA104; estos experimentos nos permitieron establecer que la región que contiene el tripéptido DGE es responsable de la unión de nar3, a través de la proteína VP5, a la superficie de la célula. Además, identificamos directamente a la integrina $\alpha2\beta1$ como el receptor inicial de nar3, puesto que anticuerpos dirigidos contra esta integrina son capaces de bloquear la unión inicial de la variante nar3. Encontramos que la integrina $\alpha2\beta1$ también está involucrada en la infectividad de la cepa RRV, puesto que AcM contra esta integrina, así como el péptido DGE, bloquean la infectividad de RRV, pero no su unión a la superficie celular. Estos datos refuerzan nuestro modelo de interacciones sucesivas, según el cual hemos propuesto que el virus RRV se une en primer lugar a un receptor sensible al tratamiento con NA, y posteriormente interacciona con la integrina $\alpha2\beta1$, a través de VP5; por otro lado, la variante nar3 se une inicialmente con la integrina $\alpha2\beta1$ evitando la interacción inicial con el receptor sensible a NA (79).

Como ya mencionamos en los resultados, en el laboratorio se aisló y caracterizó un AcM dirigido contra la superficie de las células MA104, el cual bloquea la infectividad de los rotavirus; este AcM, llamado 2D9, es una inmunoglobulina del tipo M. Aunque el comportamiento de este AcM en los experimentos de infectividad y de unión de rotavirus es muy similar al que se observó con el AcM dirigido contra la integrina $\alpha2\beta1$ (dado que bloquea la unión de nar3, pero no la de RRV, y que es capaz de bloquear la infectividad de

ambos virus), el AcM 2D9 no parece reconocer a esta integrina, ya que por ensayos de inmunotinción de vellosidades intestinales el AcM 2D9 y el AcM anti-integrina $\alpha 2\beta 1$ presentan patrones distintos de tinción, indicando que reconocen diferentes tipos celulares. Sin embargo, en células MA104 la estructura celular que reconoce el AcM 2D9 parece estar físicamente cercana a la integrina $\alpha 2\beta 1$, puesto que desplaza la unión de anticuerpos contra esta integrina en ensayos de citometría de flujo (Isa y col, datos no publicados). Es posible que el virus nar3 pueda utilizar al antígeno que reconoce el AcM 2D9 como un receptor alternativo, ya que células que carecen de la integrina $\alpha 2\beta 1$, pero que son reconocidas por el AcM 2D9 pueden ser infectadas con este virus, aunque con muy baja eficiencia (49).

Además de haber descrito a la integrina $\alpha 2\beta 1$ como el receptor de la variante nar3, en este trabajo encontramos dos resultados interesantes. Por un lado, el péptido que representa la región hidrofóbica (RH) de VP5 bloquea la infectividad de los virus RRV y nar3, pero no afecta la unión de estos virus, ni la de la proteína recombinante GST-VP5. El paso, durante la ruta de entrada, que es bloqueado por el péptido RH, parece ocurrir después de la unión inicial del virus a la célula. Recientemente se ha descrito que la proteína VP5 expresada en bacterias, tiene la capacidad de fusionar liposomas, y que muy probablemente esta actividad se encuentra en la región hidrofóbica, puesto que al mutagenizar residuos dentro de esta región se pierde la actividad de fusión (13, 16). Es posible que la capacidad de VP5 para interaccionar com membranas lipídicas esté involucrada en el mecanismo de entrada de los rotavirus, facilitando este proceso.

Por otro lado, encontramos que al mutagenizar el tripéptido DGE en la proteína recombinante GST-VP5 (responsable de la unión a la integrina $\alpha 2\beta 1$) no se elimina la capacidad de esta proteína de unirse a la superficie de la célula, lo que nos llevó a estudiar la posible existencia de otra región de unión en la proteína VP5.

El uso de péptidos sintéticos que representan distintas regiones de VP5 nos permitió identificar una región, entre los aa 642-659 (péptido 5), como la responsable de esta segunda interacción de VP5 con la superficie de la célula; además, la construcción de una proteína

VP5 truncada, que sólo contiene los últimos 300 aa de la proteína (VP5-COOH) nos permitió establecer que este segundo sitio de unión es independiente de la región de unión a la integrina $\alpha 2\beta 1$. El hecho que el péptido 5 bloquea la infectividad de los rotavirus RRV y nar3, pero no su unión, apunta hacia la posibilidad de que se trate de una interacción post-unión para ambos virus. Recientemente Jolly y col. reportaron una serie de regiones de VP4 capaces de unirse a la superficie de las células MA104; estas regiones fueron identificadas utilizando la técnica de despliegue en fagos. Dentro de los fagos seleccionados en ese trabajo se encontraron tres que representan una región entre los aa 650-657 (38); esta región cae dentro de la secuencia representada en el péptido 5 (aa 642-659). La identificación de este dominio de unión de VP5 a la superficie celular, mediante dos estrategias distintas, confirma este hallazgo.

De manera paralela a este trabajo, en nuestro laboratorio encontramos que la proteína de choque térmico hsc70 está involucrada en la infectividad de los rotavirus, en una interacción post-unión (28). Quedaba pues por demostrar si la interacción mediada por el extremo carboxilo de VP5 es responsable de la interacción de los rotavirus con esta proteína. En este trabajo encontramos que, efectivamente, la región de VP5 localizada entre los aa 642-659, es responsable de esta interacción, que ocurre como un paso posterior a la unión del virus con la integrina $\alpha 2\beta 1$, que es mediada por la secuencia DGE de VP5.

El papel de la interacción virus-hsc70 durante el proceso infeccioso no es claro aún; una posibilidad es que la proteína hsc70 sirva como un punto de anclaje que favorezca la interacción de los rotavirus con otras moléculas celulares que pudiesen ser las responsables de la entrada de estos virus a la célula. Sin embargo, el hecho que la proteína hsc70 tenga actividad de chaperona sugiere que podría jugar un papel más activo en la entrada de los rotavirus; por ejemplo, favoreciendo cambios conformacionales en la partícula viral que le permitieran a los rotavirus penetrar hacia el citoplasma de la célula, o favoreciendo la perdida de la capa externa de la partícula viral, lo cual es un paso necesario para que estos virus inicien el proceso de transcripción.

Recientemente se publicó una predicción de la estructura secundaria de la proteína VP4 basada en datos de criomicroscopía electrónica de los rotavirus realizada en presencia de los AcM 7A12 y 2G4 (75); estos AcM están dirigidos contra las proteínas VP8 y VP5, respectivamente. Este trabajo dio información respecto a la distribución de la proteína VP4 en la espícula. Como se muestra en la figura 14, el polipéptido VP8 forma la cabeza de la espícula, y justo debajo de ésta se encuentra la región hidrofóbica reconocida por el AcM 2G4 (alrededor del aa 393). Esto significaría que la región de interacción con la integrina $\alpha 2\beta 1$ (DGE), que se encuentra entre los aa 308-310 no está alejada de la punta de la espícula, puesto que se encuentra entre VP8 y la región hidrofóbica. Por otro lado, la región de VP5 representada en el péptido 5 (aa 642-659) debería encontrarse en la parte baja de la espícula, cercana a la región que está en contacto con VP7. Siguiendo el modelo de interacciones sucesivas se podría pensar que, para el caso del virus RRV, después de la interacción inicial con AS y con la integrina $\alpha 2\beta 1$, que se llevan a cabo con la punta de la espícula (es decir con VP8 y con la región DGE de VP5), la partícula viral sufre un cambio conformacional que le permite interaccionar con la proteína hsc70 a través de una región que se encuentra cercana a la base de la espícula. Se sabe que una porción de VP5 en la espícula se encuentra oculta por la proteína VP7 y que tiene contactos con la proteína de capa intermedia VP6; sin embargo, suponemos que la región del péptido 5 debería encontrarse expuesta, puesto que los rotavirus pueden interaccionar con la proteína hsc70 purificada. Esta idea es congruente con la observación de que el AcM 159, dirigido contra VP7, es capaz de bloquear la interacción de los rotavirus con la proteína hsc70. Proponemos entonces que la región de interacción con la proteína hsc70 (que está representada en el péptido 5) se encuentra cerca de la superficie del virión formada por VP7; ésto explica por qué el AcM 159 bloquea casi por completo la unión del rotavirus RRV a la proteína hsc70 purificada, mientras que el anticuerpo 2G4, que se une en la punta de la espícula sólo bloquea esta interacción en un 50%, probablemente debido a impedimentos estéricos y no a un bloqueo directo de la región involucrada en esta interacción.

A

B

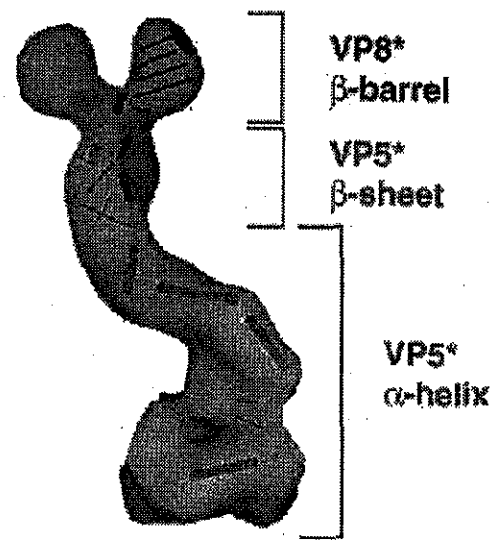

Figura 14. (A) Predicción de la estructura secuendaria de VP4, se muestra el consenso obtenido utilizando seis métodos de predicción distintos. La región de VP8 (en rojo) está basada en la estrucutra cristalográfica reportada (Dormtitzer et al. 2002), el dominio VP5 se muestra en verde. Las flechas corresponden a las regiones estructuradas como hojas β y los cilindros corresponden a hélices α. (B) Predicción de la organización de VP4 en la espícula viral, basada en datos de criomicroscopía electrónica de rotavirus en presencia de AcM, figura tomada de Thiova et al. 2001.

Otro aspecto de la unión de los rotavirus que nos interesaba estudiar era determinar si el corte con tripsina afecta, en alguna medida, este evento. Previamente se había descrito que la unión de los rotavirus a la superficie celular es independiente del corte con tripsina (7, 40). En este trabajo encontramos que efectivamente, la unión de los rotavirus a las células MA104, es independiente de que la partícula viral haya sido cortada o no con tripsina, puesto que las partículas que poseen la proteína VP4 intacta (sin cortar) se unen a la célula de manera específica y con un perfil de saturación muy similar al de las partículas cortadas. Sin embargo, cuando hicimos una caracterización de las proteínas virales responsables de la unión de los rotavirus cortados o no con tripsina, encontramos diferencias. Mientras que el virus RRV se une a la superficie de la célula a través de VP8, sin importar si ha sido tratado o no con tripsina, la variante nar3 se comporta de manera distinta. Cuando las partículas virales de nar3 han sido cortadas con tripsina se unen a la célula a través del dominio DGE de VP5, pero la unión del virus nar3 no cortado es mediada por la glicoproteína viral VP7.

Previamente habíamos establecido que la integrina αvβ3 participa durante la entrada de los rotavirus, en un paso post-unión, y habíamos encontrado que esta interacción es independiente del motivo canónico (RGD) de unión a este tipo de integrinas (29). En este trabajo encontramos que el virus nar3 no cortado se une a la integrina αvβ3 a través de VP7, ya que AcM neutralizantes dirigidos contra VP7 bloquean la unión del virus nar3 no cortado y un anticuerpo dirigido contra la integrina β3 tiene el mismo efecto.

Por otro lado, se ha reportado que los hantavirus también interaccionan con la integrina β3 de manera independiente del tripéptido RGD (26). La comparación entre la secuencia de la proteína VP7 de los rotavirus y la proteína G1G2 de los hantavirus, nos permitió encontrar una región de nueve aminoácidos en la que estas proteínas son idénticas en un 66.7 %. Resalta el hecho que un péptido sintético (llamamdo CNP) que representa esta región, bloquea la infectividad de los rotavirus RRV y nar3, así como la unión del virus nar3 no cortado. Además se obtienen resultados muy similares utilizando el péptido derivado de la

secuencia de la proteína G1G2 de los hantavirus, lo que indica que ambos péptidos son capaces de unirse a la misma entidad molecular, que probablemente es la integrina αvβ3.

También encontramos que el péptido CNP es capaz de unirse a la integrina αvβ3 inmovilizada en placas de ELISA. Además, este péptido puede bloquear la unión del virus nar3 no cortado en este mismo tipo de ensayo. Finalmente, encontramos que el sitio de unión del péptido CNP en la integrina αvβ3 es distinto del sitio utilizado por el tripéptido RGD, ya que los péptidos CNP y RGD no compiten entre sí por la unión a la integrina αvβ3.

La unión de virus nar3 no cortado nos ha servido como modelo para estudiar la interacción directa ente los rotavirus y la integrina αvβ3. Esta interacción es mediada por la proteína VP7, concretamente por la región representada en el péptido CNP (aa 161-169). Pero la interacción entre los rotavirus y la integrina αvβ3 no es evidente cuando el virus está cortado con tripsina (donde ya habíamos encontrado que la interacción inicial es o con AS, mediada por VP8, o con la integrina α2β1, mediada por VP5, dependiendo de la cepa que se trate), por lo que suponemos que es necesario que ocurran cambios en la partícula viral, posiblemente facilitados por los contactos previos del virus con la superficie de la célula, para que el virus cortado se una a esta integrina mediante la región CNP de VP7 y se lleve a cabo una infección productiva.

Recientemente, se publicó un análisis comparativo de criomicroscopía electrónica entre rotavirus que habían sido cortados o no con tripsina. Este estudio mostró que los virus no cortados no tienen las espículas de VP4 bien definidas, sugiriendo que éstas se encuentran menos ordenadas y que el corte con tripsina da lugar a espículas bien ordenadas, que se observan claramente en la microscopía (9) (ver figura 2), lo que podría ser importante para la entrada del virus. Otra observación interesante señalada en este estudio es que aparentemente existen diferencias en la conformación de la proteína VP7 entre los virus cortados y no cortados, a pesar de que el tratamiento con tripsina no produce ningún corte en la proteína VP7; por ello se ha propuesto que estas diferencias podrían ser un reflejo del cambio conformacional que ocurre en VP4 con el tratamiento proteolítico. El hecho que el virus nar3

no cortado pueda unirse a la célula a través de VP7, mientras que el virus cortado lo haga a través de VP5, podría deberse a las diferencias encontradas en las conformaciones de VP4 y VP7 causadas por el corte con tripsina.

Este trabajo nos ha permitido describir por primera vez una función para la proteína viral VP7. Además, hemos encontrado un nuevo dominio peptídico capaz de interaccionar con integrinas del tipo αvβ3, que es utilizada por los rotavirus y que es diferente del motivo de unión canónico RGD. Queda por determinar si esta secuencia también puede ser utilizada por los hantavirus para unirse a la integrina αvβ3. Por otro lado, existe la posibilidad de que este motivo de unión a integrinas de tipo αvβ3 pueda ser utilizado por otras proteína virales o celulares para interaccionar con esta proteína.

En conjunto, los resultados obtenidos durante el desarrollo de este trabajo nos han permitido apuntalar y definir varias de las primeras interacciones de los rotaviruus con su célula huésped, que se resumen en el siguiente modelo de trabajo; éste integra las observaciones que se han hecho a lo largo de varios años en nuestro laboratorio (Figura 15) y en el que proponemos que:

a) El rotavirus de origen animal RRV interacciona en primer lugar con un receptor que contiene AS (sensible al tratamiento con NA); este receptor no ha sido identificado directamente, pero un candidato es el gangliósido GM3 (31, 66). Esta interacción es mediada por el sitio de unión a AS presente en VP8, entre los aminoácidos 93-208.

b) Después de esta interacción, el virus RRV se une con la integrina α2β1 a través del motivo DGE presente en la región amino terminal de VP5 (307-309). Esta interacción representa la unión inicial del virus nar3.

c) El AcM 2D9 bloquea la unión del rotavirus nar3, pero aún no se concoce la molécula contra la que va dirigido. Suponemos que esta entidad molecular se encuentra físicamente cerca de la integrina α2β1, y es posible que funcione como un receptor alternativo de la variante nar3 en células que carecen de esta integrina.

d) Las interacciones iniciales de los rotavirus RRV y nar3, que son mediadas por regiones localizadas en la región distal de la espículas, les permiten interaccionar posteriormente con la proteína hsc70 y con la integrina αvβ3, utilizando para ésto la región carboxilo de VP5 (que suponemos se encuentra cerca de VP7) y la región de VP7 que contiene al péptido CNP, respectivamente.

e) En el caso del rotavirus de origen humano Wa, se ha encontrado que AcM dirigidos contra VP7 son capaces de bloquear su unión a la superficie de la célula (Villatoro y col, datos no publicados) y es posible que su receptor inicial sea un ganglιósido cuyos AS sean resistentes al tratamiento con NA, como por ejemplo el ganglιósido GM1 (11, 31). Sin embargo se sabe que este virus interacciona con la integrina αvβ3 y con la proteína hsc70 en un paso posterior a su unión inicial.

f) Las integrinas α4β1 y αxβ2 también están involucradas en el proceso de entrada de los rotavirus y es posible que puedan funcionar como receptores alternativos en diferentes líneas celulares.

g) La región hidrofóbica de VP5 podría estar involucrada en los primeros eventos de la infección, probablemente durante el proceso de entrada.

Todos estos datos apoyan la idea de que la entrada de los rotavirus es un proceso que involucra varios pasos, así como una serie de receptores que son utilizados de manera sucesiva por el virus. Esta serie de eventos finalmente le permitiría a la partícula viral llegar al citoplasma.

Una integración de los datos que han sido aportados hasta ahora en este campo se puede encontrar en la revisión "Early events of rotavirus infection: the search for the receptor(s)" que se presenta en el anexo (1).

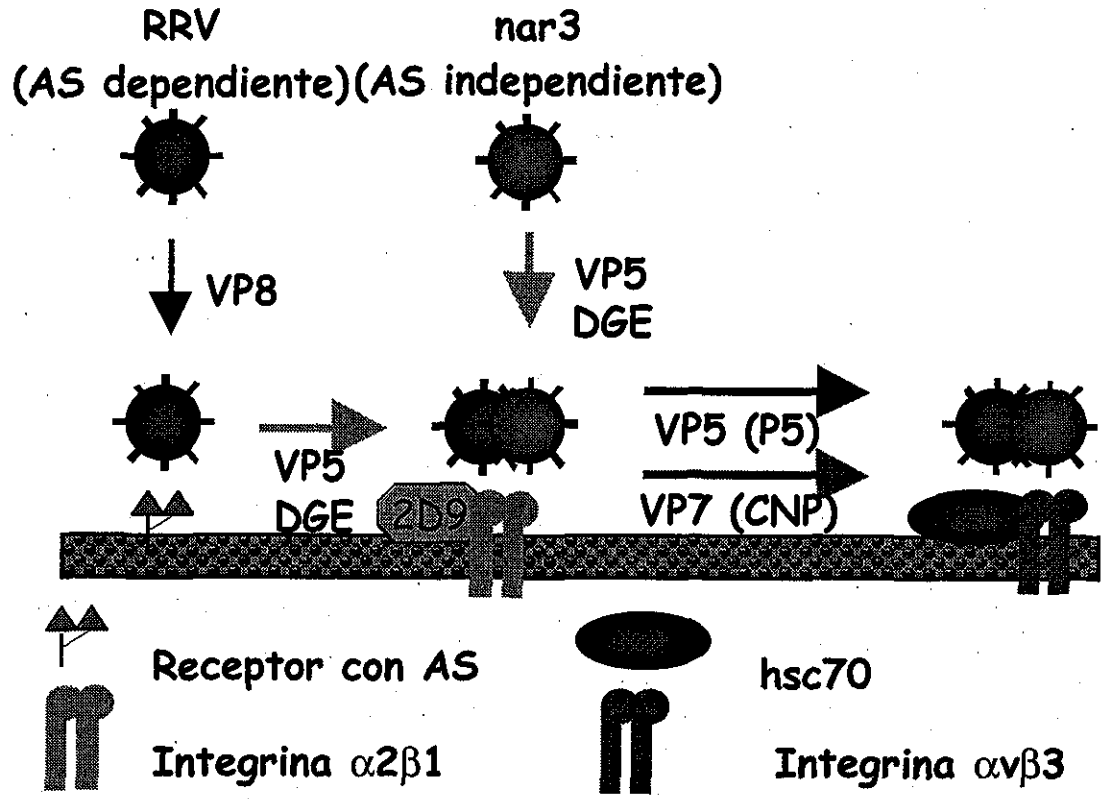

Figura 15. Modelo de las primeras interacciones de los rotavirus. El rotavirus silvestre RRV se une incialmente con un receptor que contiene AS, a través del dominio VP8 de la proteína VP4. Después de esta primera interacción, que puede producir un cambio conformacional en VP4, el virus RRV se une a un receptor resistente a NA, la integrina α2β1. Esta interacción es mediada por el tripéptido DGE presente en VP5 (aa 308-310). Esta segunda interacción podría facilitar las interacciones posteriores con la integrina αvβ3 y con la proteína de choque térmico hsc70; estas interacciones están mediadas por la región CNP de VP7 (aa 161-167) y por la región del péptido 5 de VP5 (aa 642-658), respectivamente. La variante resistente a NA, nar3, se une inicialmente con la integrina α2β1, y posteriormente interacciona con la integrina αvβ3 y con hsc70. El antígeno que reconoce el AcM 2D9 no se ha identificado, pero suponemos que se encuentra físicamente cerca de la integrina α2β1.

PERSPECTIVAS

Los resultados obtenidos hasta el momento nos han permitido establecer que existen varios receptores para rotavirus y que el proceso de entrada implica varios pasos. Sin embargo, para comprender mejor este mecanismo es necesario estudiar los cambios conformacionales que suponemos sufre la partícula viral durante este proceso. Resultados preliminares obtenidos en nuestro laboratorio indican que el anticuerpo no neutralizante HS2 (dirigido contra VP5) es capaz de neutralizar la infección del virus cuando éste ya se encuentra unido a la superficie celular; ésto sugiere que el AcM HS2 es capaz de reconocer el epítope contra el que va dirigido, debido posiblemente a un cambio en la estructura de la partícula viral, mediado por el contacto inicial con la superficie celular. Por otro lado, también es necesario estudiar los efectos que tienen las interacciones iniciales del virus sobre la célula huésped; por ejemplo, es posible que los contactos del virus con las integrinas inicien cascadas de señalización dentro de la célula que afecten de algún modo el curso de la unión y la penetración. También sería interesante estudiar el papel de la proteína hsc70 en el proceso de entrada, puesto que es muy posible que participe activamente en los cambios conformacionales de la partícula viral durante su paso hacia el citoplasma.

Por otro lado, es necesario identificar a los receptores iniciales de los rotavirus naturalmente resistentes a NA, ya sean de origen humano o animal, y a sus contrapartes en las proteínas virales. A pesar de los esfuerzos realizados en nuestro laboratorio para caracterizar la unión de la cepa de origen humano Wa, no ha sido posible identificar a su receptor, pero se ha podido establecer que la proteína VP7 de este virus es probablemente responsable de mediar la interacción inicial del virus Wa con la célula. Queda por determinarse cual es la región de VP7 responsable de dicha interacción y el receptor inicial de esta cepa.

Sabemos que el tropismo de los rotavirus es restringido, sin embargo las moléculas que se han identificado como receptoras tienen una distribución más amplia que el tropismo observado para estos virus. Es lógico suponer que la sola presencia de estas moléculas no es, por lo tanto, responsable de la suceptibilidad a la infección por rotavirus, sino que la presencia

de todas estas moléculas en conjunto sería necesaria. De hecho, datos de nuestro laboratorio muestran que la disociación de microdominios lipídicos, conocidos como rafts, utilizando cilodextrina bloquea la infección por rotavirus, sugiriendo que al menos algunas de las moléculas receptoras podrían estar asociadas con ellos. Por lo anterior, una línea de investigación que actualmente se sigue en nuestro laboratorio es determinar si las moléculas que han sido identificadas como receptores de los rotavirus se encuentran formando un complejo, y el papel de los microdominios lipídicos, en la infección por rotavirus.

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A N E X O

Early events of rotavirus infection: the search for the receptor(s)

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Abstract. The entry of rotaviruses into epithelial cells seems to be a multistep process. Infection competition experiments have suggested that at least three different interactions between the virus and cell surface molecules take place during the early events of infection, and glycolipids as well as glycoproteins have been suggested to be primary attachment receptors for rotaviruses. The infectivity of some rotavirus strains depends on the presence of sialic acid on the cell surface, however, it has been shown that this interaction is not essential, and it has been suggested that there exists a neuraminidase-resistant cell surface molecule with which most rotaviruses interact. The comparative characterization of the sialic acid-dependent rotavirus strain RRV (G3P5[3]), its neuraminidase-resistant variant nar3, and the human rotavirus strain Wa (G1P1A[8]) has allowed us to show that $\alpha 2\beta 1$ integrin is used by nar3 as its primary cell attachment site, and by RRV in a second interaction, subsequent to its initial contact with a sialic acid-containing cell receptor. We have also shown that integrin $\alpha V\beta 3$ is used by all three rotavirus strains as a co-receptor, subsequent to their initial attachment to the cell. We propose that the functional rotavirus receptor is a complex of several cell molecules most likely immersed in glycosphingolipid-enriched plasma membrane microdomains.

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Group A rotaviruses are non-enveloped viruses that possess a genome of 11 segments of double-stranded RNA contained in a triple-layered protein capsid. The outermost layer is composed of two proteins, VP4 and VP7. The smooth external surface of the virus is made up of 780 copies of glycoprotein VP7, while 60 spike-like structures, formed by dimers of VP4, extend from the VP7 surface (Estes 1996).

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VP4 has essential functions in the virus life cycle, including receptor binding and cell penetration (Crawford et al 1994, López et al 1985, Ludert et al 1996). The properties of this protein are therefore important determinants of host range, virulence and induction of protective immunity. The role of VP7 during the early interactions of the virus with the cell is not clear, although it has been shown that it can modulate some of the VP4-mediated virus phenotypes, including receptor binding (Beisner et al 1998, Méndez et al 1996), and it has been suggested that it might interact with cell surface molecules after the initial attachment of the virus through the spike protein (Coulson et al 1997, Estes 1996, Méndez et al 1999). For rotaviruses to enter the cell, VP4 has to be cleaved by trypsin into two subunits, VP5 and VP8 (Arias et al 1996, López et al 1985).

Rotaviruses have a very specific cell tropism, infecting only the enterocytes on the tip of villi of the small intestine, suggesting that specific host receptors must exist. *Invitro*, they also display a strict tropism, binding to a variety of cell lines, but infecting efficiently only those of renal or intestinal epithelium origin.

Different rotavirus strains display different requirements to bind, and thus infect, susceptible cells. The cell attachment of some rotavirus strains isolated from animals (other than humans) is greatly diminished by treatment of cells with neuraminidase (NA), indicating the need for sialic acid (SA) on the cell surface (Ciarlet & Estes 1999, Fukudome et al 1989, Keljo & Smith 1988, Méndez et al 1993). The interaction with a SA-containing receptor, however, does not seem to be essential, since variants which no longer need SA to infect the cells can be isolated from the SA-dependent strains (Ludert et al 1998, Méndez et al 1993). In addition, many animal rotavirus strains are NA-resistant and most, if not all, strains isolated from humans are also NA-resistant (Ciarlet & Estes 1999, Fukudome et al 1989, Méndez et al 1999). Thus, there is a great interest in identifying the NA-resistant cellular receptor(s) for rotavirus, and to determine the role it (they) may have on the narrow tropism observed for this virus. In this context, it is also of importance to define the viral proteins, and their specific domains, involved in contacting the cell receptor(s).

To understand the early events of rotavirus infection we have undertaken the comparative characterization of three rotavirus strains: the SA-dependent simian rotavirus RRV, its NA-resistant variant nar3, and the human rotavirus (HRV) strain Wa, which is naturally resistant to NA. A summary of the advances and approaches we have taken to characterize the early events of infection of these viruses is presented.

The interaction of rotavirus with its host cell is a multistep process

Several lines of evidence suggest that rotaviruses need to interact with more than one cell surface molecule to enter the cell, using during this process different

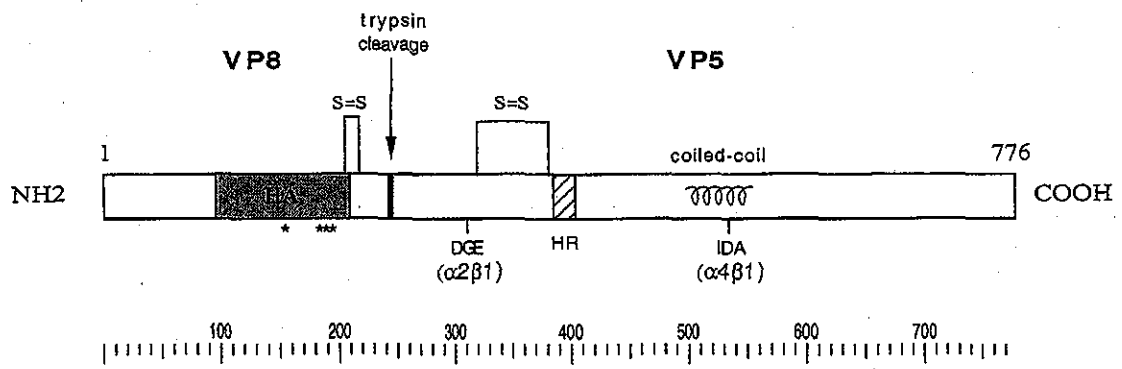


FIG. 1. Distinctive structural features of the outer shell protein VP4. The trypsin cleavage region is indicated by an arrow, which defines the boundary between VP8 and VP5. In VP8, the haemagglutination domain (HA) (aa 93 to 208) is shadowed; the asterisks below this domain indicate aa 155 and 188–190, which are important in the SA binding activity of this protein. The disulfide bridges between Cys203 and Cys216, and between Cys318 and Cys380, are indicated by S=S. In VP5, the position of the DGE and IDA tripeptide sequence binding motifs which might putatively be recognized by integrins $\alpha 2\beta 1$ and $\alpha 4\beta 1$, respectively, are shown. The hydrophobic region (HR), which has been proposed to be a putative fusion domain, and a predicted heptad repeat (coiled-coil) which might form part of a coiled-coil structure are also depicted.

domains of the virus surface protein VP4 (Fig. 1). The following studies, which support these multiple interactions, were carried out in the rhesus monkey kidney epithelial cell line MA104, which is highly susceptible for rotavirus infection.

(a) In an infection assay designed to detect competition for cell surface molecules at both attachment and post-attachment steps (Méndez et al 1999), it was found that HRV Wa efficiently competed with the infectivity of the SA-dependent porcine rotavirus strain YM, and that of the variant nar3 both in untreated, as well as in NA-treated cells. This competition was non-reciprocal since YM and nar3 did not compete with the infectivity of Wa. In contrast, a two-direction competition between the variant nar3 and a SA-dependent strain was found. The fact that the competition between the two NA-resistant strains nar3 and Wa was not reciprocal indicates that they bind to different molecules. In addition, the SA-dependence phenotype clearly differentiates strains, like RRV or YM, from nar3 and Wa. Altogether, these findings suggest the existence of at least three cellular structures involved in rotavirus cell infection, with at least one being shared by human, SA-dependent, and animal, NA-resistant, variant strains.

(b) The comparison of the binding characteristics of wild-type RRV (wtRRV) and nar3 to MA104 cells showed that both the SA-dependent and SA-independent interactions of these viruses with the cell are mediated through two different domains of VP4 (Méndez et al 1993). It was shown that RRV

TABLE 1 Inhibition of binding and infectivity of RRV and nar3 viruses by MAbs to VP4 and by VP8 and VP5 recombinant proteins

		% Binding and infectivity in the presence of the indicated MAbs or recombinant proteins					
	Virus	*no MAb*	*αVP8 (7A12)*	*αVP5 (2G4)*	*GST*	*GST–VP8*	*GST–VP5*
Binding[a]	RRV	100	9	84	102	25	97
	nar3	100	72	9	99	100	24
Infectivity[b]	RRV	100	8	9	87	44	102
	nar3	100	95	16	110	104	50

[a]Expressed as the percentage of virus binding in the absence of antibodies or recombinant proteins.
[b]Expressed as percentage of the virus infectivity obtained in the absence of antibodies or recombinant proteins. The arithmetic means from two independent experiments performed in duplicate are shown.

attaches to the cell through VP8, while nar3 does so through the VP5 domain of VP4 (Zárate et al 2000a). This observation is supported by the fact that neutralizing antibodies to VP8 block the attachment to cells of RRV, but not of its variant nar3, while a monoclonal antibody (MAb) to VP5 (2G4) inhibits the binding of nar3, but not that of RRV. In addition, recombinant VP8 and VP5 proteins produced in bacteria as fusion products with glutathione S-transferase (GST), are capable of inhibiting the binding and infection of wild-type and variant viruses, respectively, when pre-incubated with the cell (Table 1, Zárate et al 2000b). While nar3 only needs to interact (through VP5) with the NA-resistant receptor, wtRRV seems to engage in the two interactions described in a sequential manner, since MAb 2G4, despite selectively blocking the binding of nar3, efficiently neutralizes the infectivity of both viruses (see also below).

(c) The sequential interaction of RRV with two molecules on the surface of MA104 cells is further supported by the observation that MAb 2D9, which is directed to a cell surface antigen, specifically blocks the infectivity of both wtRRV and nar3, but competes only with the attachment of the variant, indicating that wtRRV is blocked at a post-binding step (López et al 2000). Since MAb 2D9 also blocks the infectivity of nar3 in NA-treated cells, and prevents the cell attachment of the recombinant protein GST–VP5, but does not affect the binding of GST–VP8 (Fig. 3), it would seem to be directed to the NA-resistant receptor used by nar3 to attach to the cell, or to a molecule closely associated with it.

Multiplicity of rotavirus receptors

Despite the advances in the molecular and structural biology of these viruses, little is known about the rotavirus cell receptors. A number of glycoconjugates have been shown to bind to, and to block the infectivity of, SA-dependent animal rotavirus strains, and some of them have been suggested to play a role as possible receptors, like GM3 gangliosides in newborn piglet intestine (Rolsma et al 1998), GM1 in LLC-MK2 cells (Superti & Donelli 1991), and 300–330 kDa glycoproteins in murine enterocytes (Bass et al 1991). It has also been suggested that the NA-resistant ganglioside GM1 may act as a receptor for some HRV strains in MA104 cells (Guo et al 1999). Recently, it was reported that VP4 contains the DGE and IDA tripeptide sequence motifs known to interact with integrins $\alpha2\beta1$ and $\alpha4\beta1$, respectively (Fig. 1), while VP7 contains the $\alpha X\beta2$ integrin ligand site GPR, and the $\alpha4\beta1$ binding motif LDV (Coulson et al 1997, Hewish et al 2000). Antibodies to the integrin subunits $\alpha2$, $\beta2$ and $\alpha4$, as well as peptides that mimic the ligand sites were shown to block the infectivity of the SA-dependent rotavirus SA11 and the HRV strain RV5 (Coulson et al 1997). It was also shown that integrins $\alpha2\beta1$ and $\alpha4\beta1$ can mediate the attachment and entry of rotavirus SA11 into the human myelogenous leukemic cell line K562 (Hewish et al 2000).

As part of the biochemical characterization of the rotavirus cell receptors, we have recently shown that the infectivity of rotaviruses RRV, nar3 and Wa is

TABLE 2 Effect of metabolic inhibitors, cell membrane cholesterol depletion, and octyl-β-glucoside on the infectivity and binding of rotaviruses in MA104 cells

	% Infectivity			*% Binding*		
Inhibitor[a]	*RRV*	*nar3*	*Wa*	*RRV*	*nar 3*	*Wa*
No treatment	100	100	100	100	100	100
PDMP (25 μg/ml)	20	40	23	110	46	104
Tunicamycin (2 μg/ml)	56	48	–	111	101	94
BenzylGalNAc (2 mM)	101	150	147	ND	ND	ND
Octyl-β-glucoside (0.2%)	41	41	39	32	40	33
β-cyclodextrin (10 mM)	9	6	5	112	109	116
OG extract[b] (20 μg/ml)	5	3	4	60	59	57

[a]MA104 cell monolayers were incubated with the indicated concentration of inhibitor for 1 h (β-cyclodextrin), 24 h (tunicamycin), or 72 h (PDMP and BenzylGalNAc) at 37 °C, or for 90 min (octyl-β-glucoside) at room temperature, before virus infection.
[b]Rotaviruses were incubated with either 20 or 400 μg/ml of OG-extracted proteins, for the binding and infectivity inhibition assays, respectively. At 20 μg/ml the binding of all three viruses was inhibited by about 40%.
The mean of at least three independent experiments carried out in duplicate is shown.

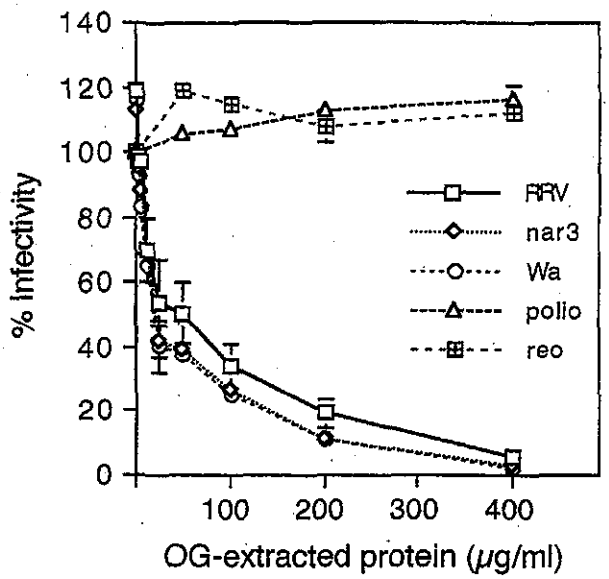


FIG. 2. Inhibition of rotavirus infectivity by the OG extract. The indicated concentrations of OG-extracted protein were incubated with the viruses for 90 min at 37 °C. The virus–protein mixtures were used to infect MA104 cell monolayers, after an adsorption period at 4 °C, the inoculum was removed and the infection was left to proceed for 14 h at 37 °C. At this time the cells were fixed, and the infectious titre was determined by an immunoperoxidase focus assay. Percentage infectivity is referred to the infectivity of the viruses incubated in 0.2% OG. Error bars represent one standard error of the mean of three independent experiments carried out in duplicate.

partially blocked by metabolic inhibitors of *N*-glycosylation (tunicamycin), and glycolipid synthesis (PDMP), while it is not affected by the inhibition of the cellular *O*-glycosylation (Guerrero et al 2000a). In addition, we also showed that depletion of cholesterol from the cell membrane with methyl-β-cyclodextrin reduced the infectivity of the three viruses by more than 90%, while not affecting their binding to the cell (Table 2). The involvement of *N*-glycosylated proteins, glycolipids, and cholesterol in rotavirus infection suggest that the virus receptor(s) might be forming part of the cell membrane glycosphingolipid-enriched lipid microdomains, termed rafts (Simons & Ikonen 1997).

In a different approach we showed that treatment of MA104 cells with the non-ionic detergent octyl-β-glucoside (OG), under non-lytic conditions, renders the cells largely refractory to binding and infection by rotaviruses (Table 2) (Guerrero et al 2000a), most probably due to the extraction of the rotavirus receptor(s). Accordingly, pre-incubation of the viruses with the OG extract inhibited infectivity by more than 95% (Fig. 2). Five protein bands with the ability to block rotavirus infectivity were purified by preparative electrophoresis from these extracts, and amino acid sequence analysis of the band of 110 kDa, revealed the presence, among other proteins, of the β3 integrin subunit.

$\alpha 2\beta 1$ integrin mediates the cell attachment of the NA-resistant RRV variant nar3

The initial interaction of nar3 with the cell surface is likely to be with integrin $\alpha 2\beta 1$, through the DGE integrin binding domain present in VP5, since: (i) antibodies to the $\alpha 2$ subunit reduce by 30% the infectivity of both wtRRV and nar3, but only block the cell attachment of nar3; (ii) MAbs to $\alpha 2$ block the attachment of the GST–VP5 fusion protein but not that of GST–VP8 (Fig. 3); (iii) GST–VP5 specifically displaces up to 75% of the cell binding of nar3, while a GST–VP5 mutant polypeptide in which the $\alpha 2$ integrin binding motif DGE was changed to AGE no longer displaces it (Zárate et al 2000b); and (iv) a synthetic VP4 peptide which comprises the $\alpha 2\beta 1$ integrin binding motif DGE efficiently inhibits the attachment of nar3, but not that of RRV (Fig. 3) (Zárate et al 2000b).

Even though the behaviour of MAb 2D9 is similar to that of $\alpha 2\beta 1$ integrin antibodies (Fig. 3), 2D9 is probably not directed to this integrin, since its pattern of staining of mouse small intestinal cells is quite different from that obtained with $\alpha 2\beta 1$ MAbs (R. Espinoza, C. F. Arias & S. López, unpublished data). Nevertheless, the cell structure recognized by 2D9 must be in close proximity to integrin $\alpha 2\beta 1$ on the surface of MA104 cells, since MAb 2D9 displaces the binding of antibodies to $\alpha 2\beta 1$ by flow cytometry (P. Isa, C. F. Arias & S. López, unpublished results). 2D9 might serve as an alternative cell receptor for the variant nar3, since cells that lack $\alpha 2\beta 1$ but are 2D9-positive, like L or CHO, can be infected by this virus, albeit at much lower efficiency (P. Isa, C. F. Arias & S. López, unpublished data).

Integrin $\beta 3$ functions as a co-receptor for rotaviruses

The relevance of $\beta 3$ integrin for rotavirus infection was established by the fact that antibodies to this integrin subunit reduced by 50% the infectivity of RRV, nar3 and Wa rotaviruses. In accordance to this finding, when vitronectin, a $\beta 3$ integrin ligand, was pre-incubated with cells, it specifically blocked rotavirus infectivity up to 70% (Guerrero et al 2000b).

Since integrins $\alpha 2\beta 1$, $\alpha 4\beta 1$ and $\alpha X\beta 2$ have been suggested to play a role during rotavirus entry (Coulson et al 1997), we performed blocking experiments using mixes of antibodies directed to these integrins and to $\alpha V\beta 3$. A clear additive blocking effect was found when antibodies to integrins $\alpha 2\beta 1$ and $\alpha V\beta 3$ were mixed, suggesting that these two integrins might be involved in different stages of rotavirus infection (Guerrero et al 2000b).

The expression of $\beta 3$ integrin into the poorly permissive CHO cells was shown to facilitate the infectivity of rotaviruses. CHO cells stably transfected with the $\beta 3$ integrin gene (Díaz-González et al 1996), overexpressing either

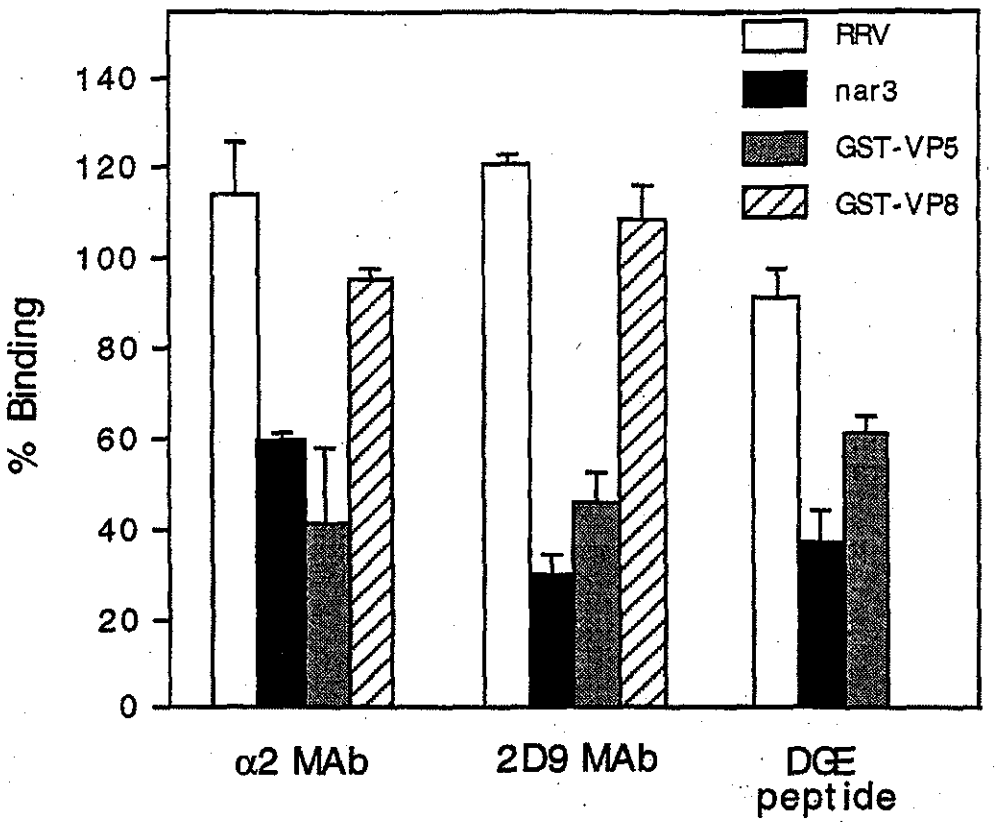


FIG. 3. Effect of antibodies to the cell surface, and of a VP4 peptide, on the binding of RRV and nar3 viruses. MA104 cells were preincubated for 1 h at 37 °C with a MAb to integrin subunit α2, with MAb 2D9 or with peptide DGE. After incubation, these cells were washed, and purified RRV or nar3 viral particles or affinity purified GST–VP8 and GST–VP5 fusion protein were adsorbed for 60 min at 4 °C with gentle shaking. The amount of cell bound virus, or fusion protein, was determined by an ELISA, as described (Zárate et al 2000a). The VP4 synthetic peptide evaluated comprises amino acid residues 300 to 321 of the protein, and contains the DGE sequence binding motif for integrin α2β1. Data are expressed as the percentage of virus or recombinant protein binding, in the absence of antibodies or peptide. The arithmetic means and standard deviations of two independent experiments are shown.

αIIbβ3 or αVβ3 integrins, were three to four times more susceptible to rotavirus infection than the parental CHO cell line. This increase in infectivity was shown to be blocked by incubation of the cells with either MAbs to β3 or vitronectin (Fig. 4) (Guerrero et al 2000b). Furthermore, it was shown that the interaction of rotaviruses with αVβ3 is at a post-attachment step, probably penetration, since vitronectin and antibodies to β3 do not, or only slightly, inhibit rotavirus cell attachment. Also, the interaction of rotaviruses with β3 integrin was found to be RGD-independent, as expected from the fact that neither VP4 nor VP7 have this integrin binding motif (Guerrero et al 2000b).

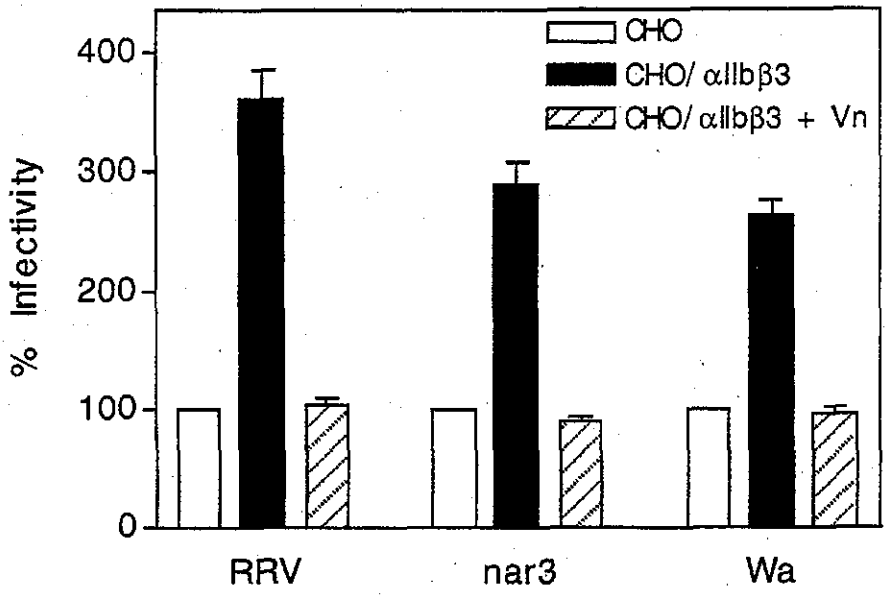


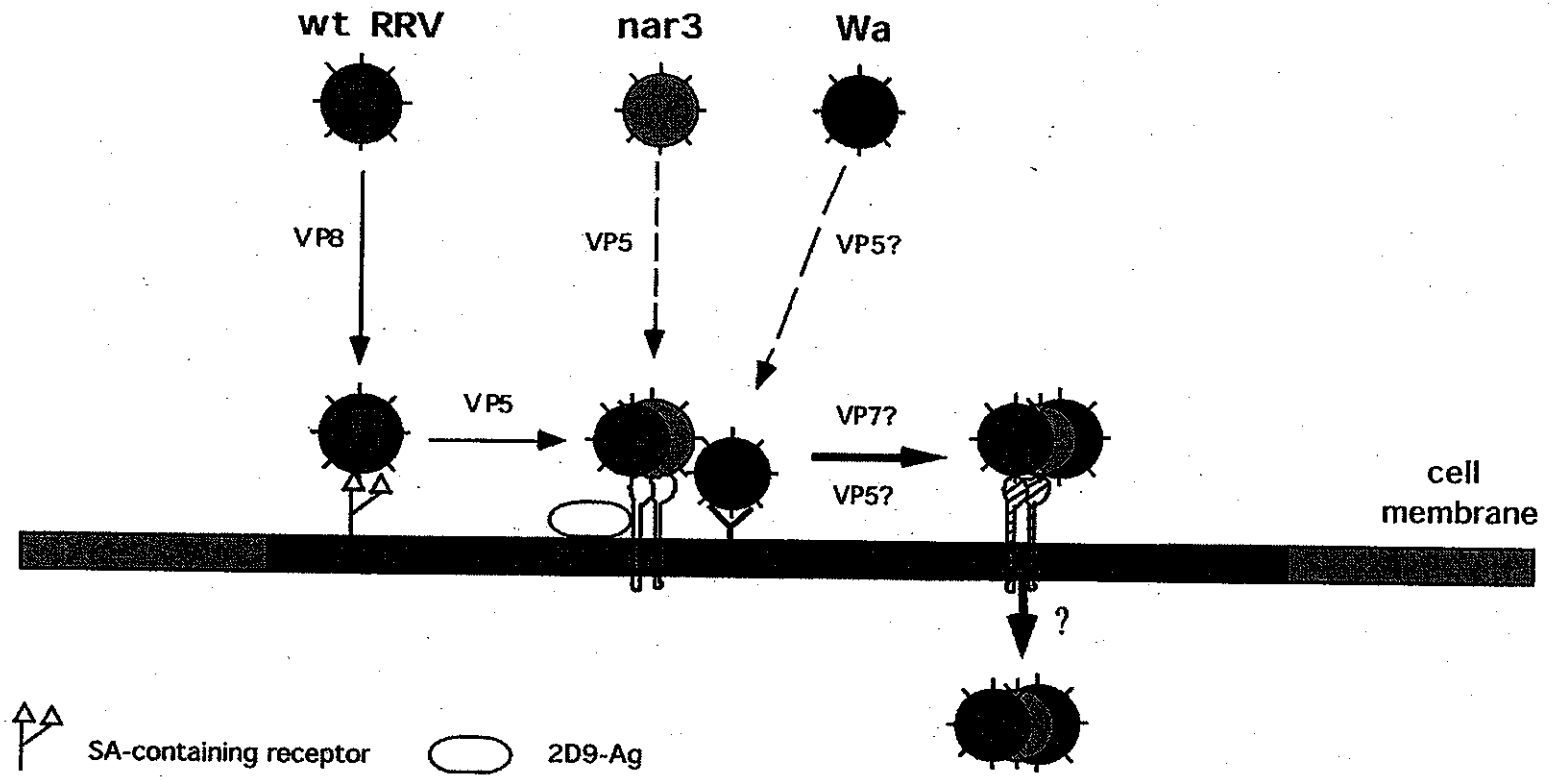
FIG. 4. The expression of $\beta 3$ integrin in CHO cells facilitates rotavirus cell infection. Monolayers of control CHO cells or CHO cells expressing integrin αIIb$\beta 3$ (Díaz-González et al 1996), in 96-well plates, were infected with 2×10^3 ffu's of RRV, nar3 or Wa viruses per well. After 60 min adsorption at 37 °C, the infection was left to proceed for 16 h at 37 °C, at which time the cells were fixed and immunostained. In the condition where the cells were preincubated with vitronectin (CHO/αIIb$\beta 3$ + Vn), the integrin ligand (1.5 μg/ml) was added for 1 h at 37 °C before virus infection. Data are expressed as percentage of the virus infectivity obtained in the CHO cells. The arithmetic mean from two independent experiments performed in duplicate are shown. The standard error is shown.

A model for the early interactions of rotaviruses with MA104 cells

As a summary of the data presented here, we propose the following working model (Fig. 5), which takes into account the currently available information:

(a) Wild type RRV interacts primarily with a SA-containing cell receptor through the VP8 domain of VP4. The identity of the SA-containing molecule has not been determined, although good candidates are ganglioside GM3 (Guo et al 1999, Rolsma et al 1998), or the SA present in the integrin molecules (see below). The SA-binding domain of VP8 is located between amino acids 93 to 208, with residues 155, and 188 to 190, having an important role in this function (Fig. 1) (Fiore et al 1991, Fuentes Panana et al 1995, Isa et al 1997).

(b) Subsequent to the initial interaction with SA, RRV interacts with a second cell receptor, most probably $\alpha 2\beta 1$ integrin, through the DGE integrin-binding motif located in the VP5 subunit of VP4 (Zárate et al 2000b). The ability of



the NA-resistant variant nar3 to interact directly with this integrin is likely to be the result of a slight different conformation of its VP4 protein, compared to that of the wtRRV protein (Méndez et al 1993, 1996). Although the present data clearly indicate the existence of two different interactions between wild-type RRV and the cell surface, it has not yet been established whether two cell molecules, or two sites in the same molecule (e.g. $\alpha 2\beta 1$), interact with VP8 and VP5. The fact that in infection competition assays the wild-type and variant viruses compete with each other reciprocally, suggests that if it is not the same cellular entity, the two cell molecules must be in close proximity.

(c) Integrins $\alpha 4\beta 1$ and $\alpha X\beta 2$ have been implicated in rotavirus cell infection (Coulson et al 1997, Guerrero et al 2000b, Hewish et al 2000). The role of these integrins has not been determined yet, however, given that no additivity was observed when mixes of antibodies to these and other integrins were tested (Guerrero et al 2000b), they may represent alternative interaction sites for rotaviruses.

(d) The results obtained in the infection competition assays described above suggest that HRV Wa initially attaches to a cell surface molecule that is used by RRV and nar3, in a subsequent step after their interaction with $\alpha 2\beta 1$ integrin. It can not be ruled out, however, that the attachment receptor for Wa is not actually used by RRV and nar3, but that HRV Wa interferes with the infectivity of these viruses by binding to a molecule that might be located in close proximity to either $\alpha 2\beta 1$, or to co-receptor $\alpha V\beta 3$. The cellular molecule used by HRV Wa to bind to cells has not yet been characterized, although ganglioside GM1 seems to be a good candidate (Guo et al 1999).

FIG. 5. A model for the early interactions of rotaviruses with MA104 cells. Wild type RRV interacts primarily with a SA-containing cell receptor through the VP8 domain of VP4. Subsequent to this initial interaction, which might induce a conformational change in VP4, the virus interacts with a second, NA-resistant cell receptor, here proposed to be $\alpha 2\beta 1$ integrin. This interaction is through the DGE binding motif of VP5, present at aa 308–310. This second virus–cell interaction might facilitate a third interaction of the virus with $\beta 3$ integrin. The SA-independent variant nar3 is proposed to interact directly, through VP5, with the $\alpha 2\beta 1$ integrin. For the sake of clarity, the SA-containing and NA-resistant cellular receptors are depicted here as two separate entities (the first possibly being ganglioside GM3), however, they could be two domains of the same receptor molecule (see text). The nature of the attachment site for the HRV strain Wa has not been determined, however, we propose that it binds to a molecule that is in close proximity to $\alpha 2\beta 1$, probably GM1 (see text). The antigen recognized by MAb 2D9 (2D9-Ag) has not been identified, but we assume that it should also be close to the $\alpha 2\beta 1$ integrin (see text). After their initial contact with the cell, all three rotavirus strains are proposed to interact with $\beta 3$ integrin, this interaction might mediate the penetration of the viruses into the cell's interior. In this model most, if not all, of the molecules involved in rotavirus binding and entry are proposed to form a complex, probably embedded in glycosphingolipid-enriched lipid microdomains on the cell surface.

Also, the viral protein domain responsible for this interaction has not been determined.

(e) We have found that integrin $\alpha V\beta 3$ plays an important role for infection of all three rotavirus strains at a post-attachment step, most likely penetration; however, the precise function of this protein has yet to be characterized.

(f) The essential components of the glycosphingolipid-enriched membrane domains, termed rafts, are glycoproteins, glycosphingolipids and cholesterol. Since these three components have been found to be important for the initial steps of rotavirus infection (Guerrero et al 2000a), and $\alpha V\beta 3$ integrin has been observed to be present in rafts (C. A. Guerrero, S. López & C. F. Arias, unpublished data, Green et al 1999), we propose that some or all of the various virus-cell interactions described above might take place in these lipid microdomains.

The data presented here are consistent with the existence of several rotavirus receptors which might be tightly organized, maybe forming a complex in glycosphingolipid-enriched rafts. The requirement for several cell molecules to be present and organized in a precise fashion, might explain the exquisite cell and tissue tropism of these viruses. It remains to be established which, if any, of the receptor molecules described so far is indeed non-replaceable, and if in fact there exists a unique pathway of infectivity for rotaviruses, with distinct entry points for different strains.

In conclusion, much remains to be learned about the process of binding and penetration of rotaviruses. The characterization of the nature of the interactions that occur between the cellular and viral partners, and the signal transduction pathways potentially triggered by the early virus–cell contacts, should give insight into the elaborated mechanism used by these viruses to enter cells.

Acknowledgements

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