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Papel del asa 3 del Dominio II de las toxinas Cry1A's de *Bacillus thuringiensis* en el mecanismo de toxicidad: un blanco potencial para modificar el reconocimiento de sus receptores

T E S I S

QUE PARA OBTENER EL GRADO DE

D O C T O R E N C I E N C I A S

P R E S E N T A

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D I R E C T O R D E T E S I S

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TABLA DE CONTENIDO

thuringiensis Cry toxins

ÍNDICE DE FIGURAS

ÍNDICE DE TABLAS

ABREVIATURAS

ALP	Fosfatasa alcalina
APN	Aminopeptidasa-N
BSA	Albúmina sérica bovina
Bt	*Bacillus thuringiensis*
Bt-R1	Receptor 1 de *Bacillus thuringiensis* (Caderina de *Manduca sexta*)
CD	Dicroísmo circular
cDNA	Ácido desoxirribonucleico complementario
CDR	Región determinante de complementariedad
CR	Repetido de Caderina
DO_{600}	Densidad óptica a 600 nanómetros
DNA	Ácido desoxirribonucleico
DTT	Ditiotritiol
EDTA	Etilen-diamino-tetra-acetato
EGTA	Etilen-glicol-tetra-acetato
ELISA	Ensayo inmunoabsorbente ligado a enzimas
FPLC	Cromatografía de flujo en fase líquida
GalNac	N-acetil-galactosamina
GPI	Glucosil-fosfatidil-inositol
IgG	Inmunoglobulina gama
IPTG	Isopropil-tio-galactopiranósido
K*d*	Constante de afinidad
LB	Medio de cultivo Luria Bertani
LC	Concentración letal
p/p	Proporción peso a peso
p/v	Proporción peso a volumen
PBS	Solución amortiguadora de fosfatos
PCR	Reacción en cadena de la polimerasa
pfu	Unidad formadora de colonias
PFT	Toxina formadora de poros
pH	Potencial de hidrógeno
PMSF	Fenil-metano-sulfonil-fluoruro
PVDF	Polivilideno bifluoruro
RNA	Ácido ribonucleico
RNAi	Ácido ribonucleico interferente
rpm	Revoluciones por minuto
RT-PCR	Transcripción reversa en combinación con PCR
scFv	Fragmento variable de cadena sencilla
SDS	Dodecil-sulfato de sodio
SDS-PAGE	Electroforesis en gel de poliacrilamida en presencia de SDS
SP	Medio de esporulación
TBR	Región de unión a la toxina
VMMA	Vesículas de la microvellosidad media apical

v/v	Proporción volumen a volumen

ABSTRACT

Bacillus thuringiensis (Bt) produces proteins with entomopathogenic properties during its sporulation phase; these proteins are called Cry toxins. This family of proteins is widely used as a biopesticide for insect control in agriculture and in the control of mosquitoes, which are disease vectors. The mode of action of Cry toxins is by forming holes on membranes of midgut cells in susceptible insect larvae. One important specificity determinant of these toxins is the recognition of receptors present in the insect. The study of this interaction will provide knowledge in understanding resistance by the insects or lead to engineering toxins against non-susceptible insects.

In this work, we characterized mutations in the Cadherin receptor from *Manduca sexta* that affect its recognition by the Cry1A's toxins and identify its binding site in the CR12. Such mutations could generate resistant insect populations, since a single mutation significantly affects binding. In previous work, it was established that the presence of recombinant fragments of a toxin-binding Cadherin increases the activity of Cry toxins. Using truncated versions of toxin-binding sites Cadherin (CR7, CR11, CR12 and CR7-CR12) and mutant fragments (L1418R and I1422R), we established that this increased toxicity correlates with oligomerization of the Cry1A's toxins. Further, we observed a correlation between forming oligomers using Cadherin fragments and synergism.

Finally, we characterized the effect of mutations at loop 3 of Cry1Ab toxin. These mutations affected the recognition of Cadherin and Aminopeptidase-N receptors differentially depending on their monomeric or oligomeric states. Mutations at the loop 3 of Domain II of Cry1Ab toxin as a monomer affected the binding to Aminopeptidase-N, but not to the Cadherin receptor. However, when the binding was analyzed on oligomeric conformation, an opposite phenomenon occurs because these mutations affected the binding to Cadherin, but not to Aminopeptidase-N. These experiments suggest that Cry1Ab toxin has a binding mechanism Ping-Pong like, in which the toxin binds to the receptor with low affinity due to its abundance (Aminopeptidase-N), this allows its location on the membrane facilitating its binding to the high affinity receptor (Cadherin), triggering the oligomerization. Then, the oligomeric structure increases its affinity to Aminopeptidase-N, thus promoting the insertion of the toxin into the membrane.

RESUMEN

Bacillus thuringiensis (Bt) produce proteínas con propiedades entomopatógenas durante el proceso de esporulación denominadas toxinas Cry, esta familia de proteínas es ampliamente usada como biopesticidas para el control de poblaciones de insectos plaga en la agricultura y vectores de enfermedades. El mecanismo de acción de las toxinas Cry por el cual matan a los insectos es mediante la formación de poros en las membranas de las células epiteliales del intestino medio de las larvas de insectos susceptibles. Uno de los determinantes para la especificidad de estas toxinas es el reconocimiento de receptores presentes en el insecto, por lo que su estudio permitirá aprovechar este conocimiento para sobrellevar la resistencia generada por las plagas o dirigir especificidad hacia insectos no susceptibles.

En este trabajo caracterizamos mutaciones en el receptor Caderina de *Manduca sexta* que afectan su reconocimiento por las toxinas Cry1A's y se identificó su sitio de unión en el CR12, este tipo de mutaciones potencialmente podrían generar poblaciones de insectos resistente, ya que una mutación sencilla afecta de manera importante su unión. En trabajos previos se estableció que la presencia de fragmentos recombinantes de este receptor incrementan la actividad de las toxinas Cry en los insectos. Haciendo uso de versiones truncadas del receptor Caderina (CR7, CR11, CR12 y CR7-CR12) y los fragmentos mutantes (L1418R e I1422R), establecimos que este aumento de toxicidad se debe a que dichos fragmentos favorecen la oligomerización de las toxinas Cry1A's, y observamos una correlación en el rendimiento para formar oligómeros utilizando las versiones truncadas del receptor y el sinergismo entre la toxina y los fragmentos de Caderina.

Finalmente caracterizamos el efecto de mutaciones en el asa 3 de la toxina Cry1Ab, estas mutaciones afectan el reconocimiento de los receptores Caderina y Aminopeptidasa-N de manera diferencial dependiendo de su estado monomérico u oligomérico. Mutaciones en el asa 3 del Dominio II de la toxina Cry1Ab en forma de monómero afectan la unión con la Aminopeptidasa-N pero no con el receptor Caderina, sin embargo cuando se analizó la unión en su conformación oligomérica sucede un fenómeno contrario ya que estas mutaciones afectan la unión con Caderina pero no con la Aminopeptidasa-N, con estos experimentos proponemos que la toxina tiene un comportamiento de unión tipo "Ping-Pong", en el cual los primeros

eventos de unión se llevan a cabo con un receptor de baja afinidad pero presente de manera abundante (Aminopeptidasa-N), que permite su localización sobre la membrana facilitando su encuentro con un receptor de alta afinidad (Caderina), al cual se une y desencadena el proceso de oligomerización. Esta estructura oligomérica incrementa su afinidad por la Aminopeptidasa-N uniéndose a esta proteína, la cual favorece la inserción de la toxina en la membrana.

I. INTRODUCCIÓN

I.1 Generalidades de *Bacillus thuringiensis*

En 1901 se aisló una bacteria que causó la muerte de las larvas del "gusano de seda" (*Bombyx mori*) en Japón, esta bacteria fue nombrada como *Bacillus sotto*, debido a las características de la enfermedad en estos insectos ("sotto" = colapso repentino). Diez años más tarde, en Turingia, Alemania, el científico Ernest Berliner aisló esta misma bacteria de "polillas de la harina" (*Ephestia kuehniella*) y fue renombrada como *Bacillus thuringiensis* (Bt).

Bt es una bacteria Gram-positiva, ubicua y aerobia estricta que pertenece a un grupo de bacterias bacilares conocido como grupo *Bacillus cereus*. Dentro de este grupo se encuentran tres especies bien caracterizadas que son *B. thuringiensis*, *B. anthracis* y *B. cereus*, y tres especies menos estudiadas: *B. wihensthephanensis*, *B. mycoides* y *B. pseudomycoides* (Priest, et al., 2004). Este grupo de bacterias se caracteriza por tener un ciclo de vida bifásico; una fase de crecimiento vegetativo donde la bacteria se reproduce por bipartición cada 30-90 minutos, dependiendo del medio y la fase de esporulación, la cual es un programa de diferenciación de bacteria a espora, este programa se activa cuando la bacteria se encuentra en limitación de nutrientes. La espora es una forma de vida latente que puede permanecer en el ambiente por largos periodos de tiempo en ausencia de humedad y nutrientes, hasta que encuentra un medio rico que le permita germinar para iniciar el crecimiento vegetativo (Schnepf, et al., 1998).

Las técnicas morfológicas, bioquímicas y genéticas para clasificar las especies pertenecientes al grupo de *Bacillus cereus* han revelado que éstas son indistinguibles. Sin embargo, presentan un fenotipo totalmente diferente; *B. anthracis* es el agente causal de la enfermedad conocida como ántrax y *B. cereus* se le considera un patógeno oportunista en el humano, mientras que Bt es considerado un patógeno de insectos. La diferencia entre estos fenotipos se debe principalmente a la presencia de plásmidos de alto peso molecular que contienen los factores de virulencia.

Bt se caracteriza por la presencia de un cuerpo paraesporal en forma de cristal de naturaleza proteica con propiedades entomopatógena, estas inclusiones cristalinas son sintetizadas cuando se activa el proceso de esporulación. Las proteínas que

forman el cristal paraesporal son denominadas toxinas Cry, cuyos genes se encuentran integrados dentro de “megaplásmidos” y sus promotores se activan durante el desarrollo de la esporulación.

I.2 Toxinas Cry

A la fecha se han aislado subespecies de Bt que producen toxinas Cry con actividad hacia diferentes ordenes de insectos como Lepidópteros, Coleópteros, Dípteros, Himenópteros, Hemípteros, Ortópteros y Malófagos, así como otros invertebrados como Ácaros, Nemátodos y Protozoarios (Schnepf, et al., 1998).

La definición de toxina Cry se refiere a aquellas proteínas que forman inclusiones paraesporales (“crystal”) que tienen algún efecto tóxico experimentalmente verificable hacia algún organismo blanco, o bien que sea similar en secuencia a las proteínas Cry conocidas. Se tiene registro de alrededor de 188 genes de proteínas Cry clasificadas en 59 grupos (http://www.lifesci.sussex.ac.uk). La nomenclatura para estas proteínas se basa en la identidad de aminoácidos y son 4 rangos los que se les asignan (Crickmore, et al., 1998). El primer rango es dado por un número arábigo y denota una identidad del 45% (Cry1-Cry59), el segundo rango se indica con una letra mayúscula y corresponde a una identidad del 78% (Cry1A, Cry1B, Cry1C, etc.), el tercero indica una identidad del 95% y se simboliza con una letra minúscula (Cry1Aa, Cry1Ab y Cry1Ac), finalmente el cuarto rango se indica con un número arábigo y es utilizado para establecer “alelos” de genes conocidos que difieren en unos cuantos cambios de aminoácidos (Cry1Aa1, Cry1Aa2, etc.).

I.3 Estructura de las toxinas Cry

Mediante cristalografía de rayos X se ha resuelto la estructura de las toxinas Cry1Aa, Cry2Aa, Cry3Aa, Cry3Ba, Cry4Aa, Cry4Ba y Cry8Ea (Li, et al., 1991; Grochulski, et al*.,* 1995; Galitsky, et al., 2001; Morse, et al., 2001; Boonserm, et al*.,* 2005; Boonserm, et al., 2006; Guo, et al., 2009). Estas proteínas tienen actividad hacia Lepidópteros (Cry1Aa), Dípteros (Cry4Aa y Cry4Ba), Coleópteros (Cry3Aa, Cry3Ba y Cry8Ea) y una de ellas con actividad dual hacia Lepidópteros y Dípteros (Cry2Aa), aunque estas toxinas tienen un porcentaje de identidad menor al 40 % (primer rango de su clasificación: Cry1, Cry2, Cry3, Cry4 y Cry8), presentan un patrón

estructural común, todas ellas son proteínas globulares y están compuestas por tres Dominios (Figura 1).

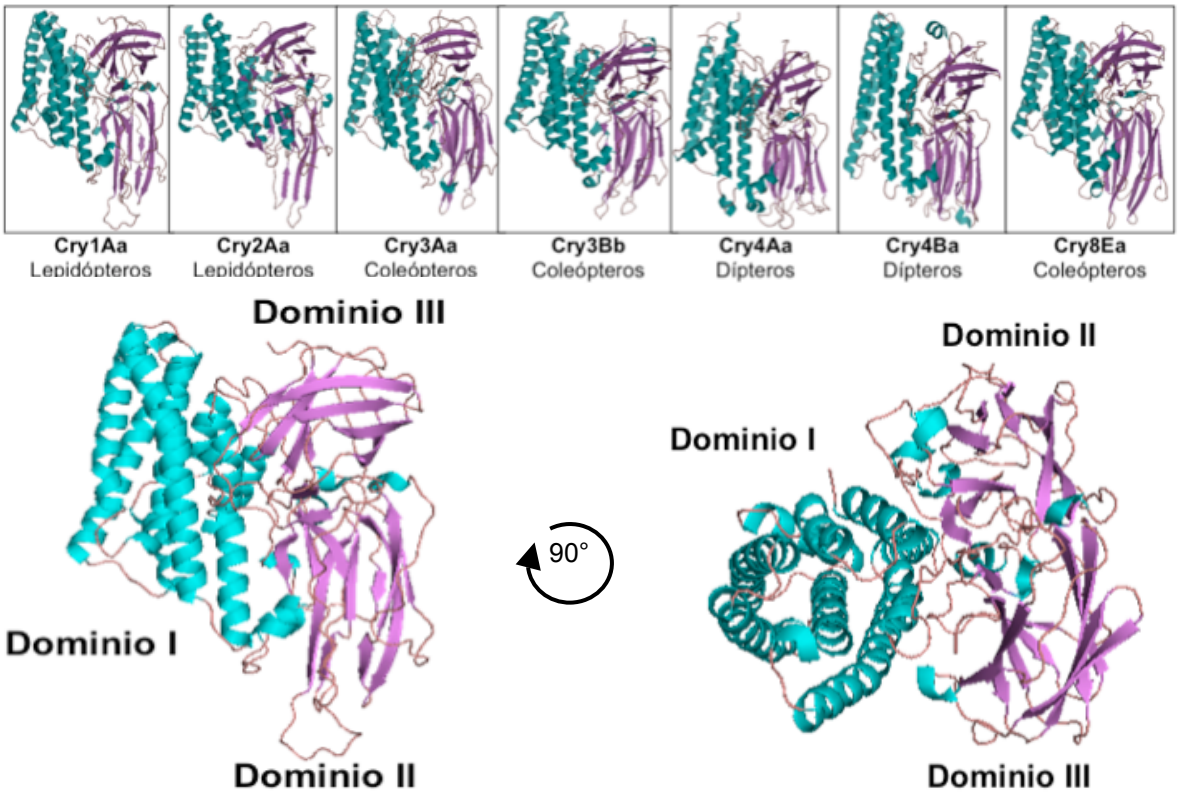

Figura 1. Estructura tridimensional de las toxinas Cry. En la parte superior se muestran las estructuras de 7 toxinas Cry con actividad hacia diferentes ordenes de insectos. En la parte inferior se muestran los tres dominios bien definidos de la toxina Cry1Aa.

Dominio I: Este dominio se encuentra en el extremo N-terminal y está formado por un ramillete de 7 α-hélices antiparalelas, seis de éstas son anfipáticas (α-1, α-2, α-3, α-4, α-6 y α-7) y rodean a una hélice central hidrofóbica (α-5) (Figura 2). El largo de las hélices de este dominio es suficiente para atravesar la bicapa lipídica de la membrana (30 Å). Estructuralmente este dominio tiene similitud con otras proteínas formadoras de poro como la toxina diftérica producida por *Corynebacterium diphtheriae* y con la Colicina-A producida por algunas cepas de *Escherichia coli* (Parker y Pattus, 1993). La Colicina A contiene un dominio de 10 α-hélices, de las cuales 8 rodean un par de hélices hidrofóbicas centrales, este par de hélices forman una horquilla que se inserta a la membrana formando un poro. Dada la similitud con las toxinas Cry se cree que las hélices α-4 y α-5 son las que se insertan en la membrana formando un poro. Además este dominio tiene una función importante en la formación de oligómeros de la toxina, recientemente se observó que la secuencia de la hélice α-3 contiene

motivos para formar estructuras tipo “coil-coiled” y de esta manera asociarse con otras moléculas para formar oligómeros (Jiménez-Juárez, et al., 2007).

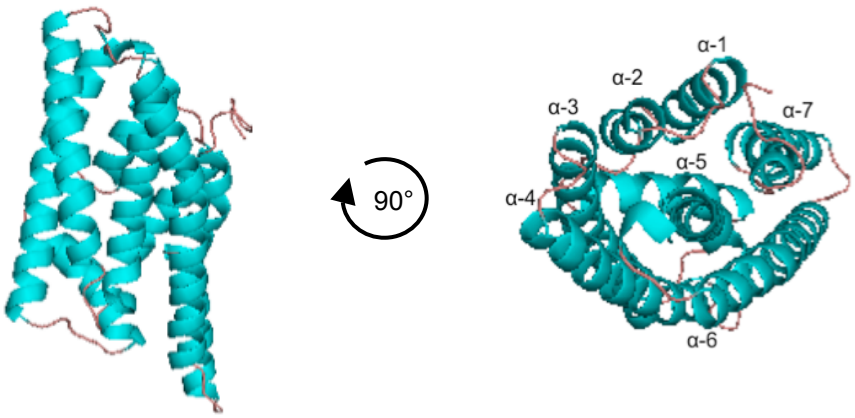

Figura 2. Arreglo estructural del Dominio I. En la figura derecha se observa un ángulo del Dominio I mostrando las 6 α-hélices anfipáticas y la hélice central hidrofóbica α-5.

Dominio II: Este dominio se encuentra en la región central de la estructura primaria de la proteína y esta constituido por tres láminas β formando un arreglo de “prisma”. Cada lámina contiene de 3-4 cadenas β con una topología tipo “llave griega” y estas láminas convergen en el ápice del prisma el cual tiene 3 asas , las asas corresponden a una horquilla que une a dos cadenas β de cada “llave griega” (Figura 3). Particularmente la secuencia y orientación de estas asas son hipervariables entre las toxinas Cry y están involucradas en la unión con su receptor, por lo que tienen una función importante para determinar la especificidad.

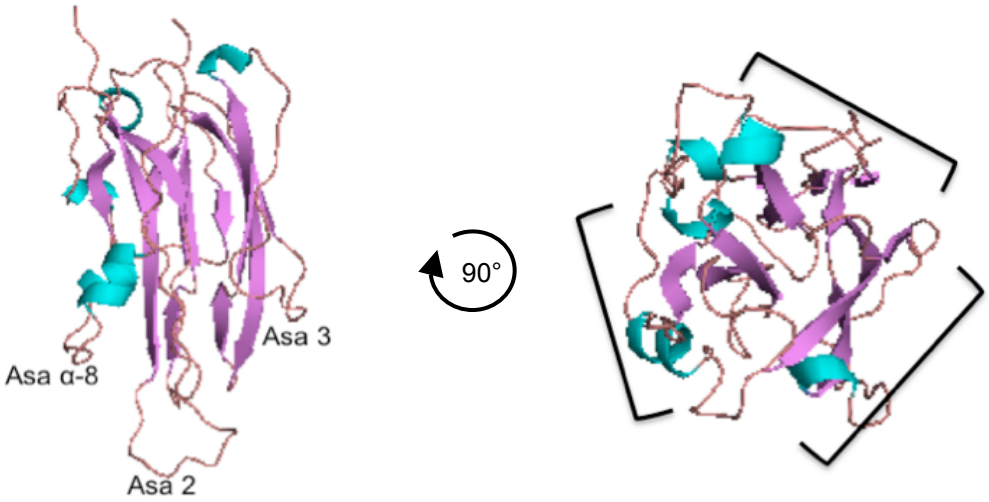

Figura 3. Arreglo estructural del Dominio II. A la derecha un ángulo del Dominio II de la toxina Cry1Aa en el que se observa el ápice del prisma con las 3 asas importantes en unión al receptor. A la izquierda se muestran las 3 láminas β que forman las caras del prisma (corchetes).

Dominio III: Se encuentra en el extremo C-terminal y estructuralmente está formado por 2 láminas β antiparalelas en un arreglo de "emparedado", cada lámina contiene 4-5 cadenas β con topología tipo "jelly-roll" (Figura 4). Este dominio se encuentra en la parte superior del Dominio II formando la base del prisma. Al igual que el Dominio II, también está involucrado en el reconocimiento de los receptores y ambos son estructuralmente parecidos a proteínas de unión a azúcares. Se han hecho estudios sobre la relación filogenética y la función de los tres dominios de las toxinas Cry en los que se ha visto que estos pueden ser compartidos entre varias familias (Bravo, 1997).

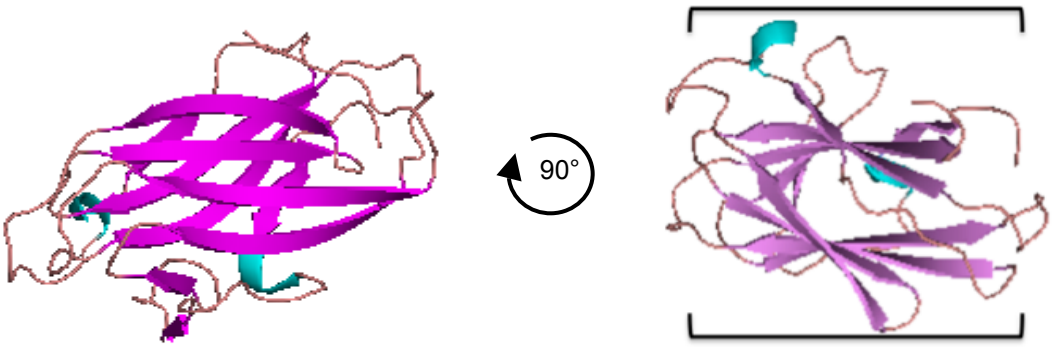

Figura 4. Arreglo estructural del Dominio III. A la derecha se observa el plegamiento tipo "emparedado" con las 2 láminas β (corchetes).

I.4 Mecanismo de acción

Las toxinas Cry pertenecen a un grupo de proteínas formadoras de poros (PFT's) en las membranas de su célula blanco, en este caso las células del epitelio intestinal de los insectos susceptibles. Para que la toxina forme un poro en la membrana, previamente suceden una serie de eventos secuenciales como son: Solubilización, Activación, Unión a receptores, Oligomerización, Inserción y Citólisis (Figura 5).

Solubilización: Para que las toxinas Cry ejerzan su actividad entomopatógena, los cristales deben ser ingeridos por el insecto susceptible, una vez que llegan al intestino los cristales son disueltos bajo las condiciones físico-químicas del lumen intestinal liberando protoxinas (Figura 5, 1). En el caso de Lepidópteros, Dípteros y algunas especies de Coleópteros la solubilización se lleva a cabo en un pH alcalino, también se requiere un ambiente reductor que permite la ruptura de puentes disulfuro que son abundantes en el extremo C-terminal de las proteínas Cry con un peso molecular de 130 kDa. En la mayoría de los cristales con actividad hacia Coleópteros la solubilización se lleva a cabo en un pH neutro o ligeramente ácido (Du, et al., 1994).

Activación: La mayor parte de las toxinas Cry se producen como protoxinas y deben ser activadas para liberar un fragmento tóxico con un peso molecular de 55-65 kDa, esta activación consiste en cortes proteolíticos en los extremos N- y C-terminal, y en algunos casos suceden cortes intramoleculares (Figura 5, 2). Algunos grupos de toxinas Cry tienen un peso molecular de 130-140 kDa como es el caso de las toxinas Cry1; durante su procesamiento, el extremo C-terminal de aproximadamente 500 aminoácidos es eliminado. Por otra parte también se lleva a cabo la proteólisis de un péptido en el extremo N-terminal de aproximadamente 20-40 aminoácidos, este procesamiento es común para la mayoría de los grupos de toxinas Cry. Hay casos especiales en el que las proteínas sufren cortes intramoleculares, ejemplo de ello son las toxinas Cry11A y Cry4B. La toxina Cry11A al ser activada genera dos fragmentos con un peso molecular de 36 y 32 kDa, mientras que la toxina Cry4B es procesada en dos fragmentos de 15 y 45 kDa, en ambos casos éstos permanecen unidos y son necesarios para ejercer su actividad insecticida (Bravo, et al., 2007).

Reconocimiento de receptores y Oligomerización: Una vez activadas, las toxinas reconocen moléculas localizadas en la microvellosidad del epitelio intestinal de los insectos susceptibles llamadas “receptores”, aunque los eventos de solubilización y activación tienen un papel importante en la especificidad de las toxinas Cry, la unión a estas moléculas tiene el mayor peso para determinar su especificidad. La interacción toxina-receptor más conocida es la de las toxinas Cry1A y sus receptores en el Lepidóptero *Manduca sexta*, se han identificado tres moléculas, una proteína transmembranal de tipo Caderina (Vadlamudi, et al., 1995), una Fosfatasa alcalina (ALP) (Jurat-Fuentes, et al., 2004) y una Aminopeptidasa-N (APN) (Knight, et al., 1994); estos receptores tienen una función diferencial y secuencial en el mecanismo de acción. Se ha establecido que el primer reconocimiento de la toxina es con el receptor Caderina, esta interacción favorece la formación de una estructura oligomérica (Figura 5, 3 y 4). Una vez formado el oligómero de la toxina, éste se une a la APN o ALP (Figura 5, 5), este segundo receptor se encuentra anclado a la membrana en regiones llamadas “balsas lipídicas” a través de una unión covalente con el fosfolípido Glucosil-fosfatidil-inositol (GPI); se ha observado que esta interacción permite que la estructura del oligómero de la toxina Cry1Ac se relaje y facilite su inserción a la membrana (Bravo, et al., 2004; Pardo-López, et al., 2006) .

Inserción a membranas: La inserción del oligómero se lleva a cabo en microdominios de membrana ricos en colesterol (balsas lipídicas) en donde forman poros (Figura 5, 6). Se han propuesto varias hipótesis sobre los mecanismos de inserción de las toxina Cry en membranas, sin embargo el más aceptado ha sido el "modelo de sombrilla". Este modelo consiste en que las hélices α-4 y α-5 del Dominio I se insertan en la membrana y forman un poro, cuyo lumen está revestido por la hélice α-4, mientras que el resto de las hélices permanecen acomodadas sobre la superficie junto con el Dominio II y III (Li, et al., 1991).

Citólisis: Una vez que la toxina se inserta y forma un poro, aumenta la permeabilidad de la membrana y permite el paso de iones, agua y moléculas pequeñas (Figura 5, 7). En consecuencia, la membrana celular se despolariza causando un desequilibrio en el transporte de nutrientes en las células columnares del epitelio intestinal, sin embargo el efecto más devastador es la alcalinización del citoplasma, ya que se interrumpen las funciones celulares. Posteriormente la célula aumenta de volumen por la entrada de agua y provoca su lisis (Figura 5, 8), en consecuencia hay una parálisis y destrucción del intestino, inhibición del apetito y finalmente la larva muere. Una vez que el organismo muere, el cadáver proporciona los nutrientes necesarios para que las esporas de Bt pueden germinar y proliferar (Louloudes y Heimpel, 1969).

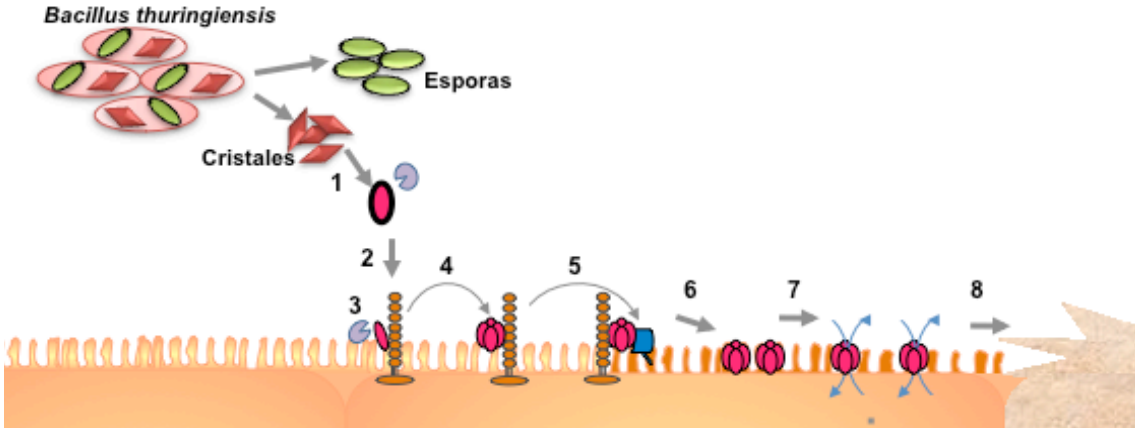

Figura 5. Mecanismo de acción de las toxinas Cry1A's en *M. sexta*. Una vez ingeridos los cristales son solubilizados (1), las protoxinas liberadas se activan proteolíticamente (2) generando una toxina que se une a la Caderina (3), posteriormente oligomeriza (4) y se une a la APN (5). Finalmente el oligómero se inserta en las balsas lipídicas de la membrana (6), formando poros que permiten el paso de iones y moléculas (7) desestabilizando el equilibrio osmótico y la célula muere (8).

I.5 Biotecnología de *B. thuringiensis*: Aplicaciones y riesgos

Bt se ha convertido en uno de los biopesticidas más importantes en el mundo debido a su actividad entomopatógena natural. Las toxinas Cry han sustituido, en gran parte, los agentes químicos utilizados para el control de plagas de insectos, debido a las ventajas que estas proteínas presentan. Por ejemplo, mientras que los compuestos químicos tienen un amplio rango de blancos, las toxinas Cry son especificas, disminuyendo los efectos colaterales en insectos benéficos y otros organismos como mamíferos, aves, anfibios y reptiles, contribuyendo así en la conservación de un ecosistema saludable. Por otra parte se estima que el costo en el desarrollo de una formulación a base de Bt es mucho más económico comparado con el de un pesticida químico sintético, haciéndolo más atractivo para su producción en las compañías industriales (Schnepf, et al., 1998).

Silvicultura: Actualmente hay un gran énfasis en la distribución de pesticidas cuyos efectos colaterales sean mínimos y con ello mantener un ambiente saludable. En este sentido Bt ha sido el mejor pesticida utilizado para el control de Lepidópteros en bosques de coníferas en Estados Unidos y Canadá. Los productos utilizados en la industria forestal han sido basados principalmente en la cepa de Bt HD1 subsp. *kurstaki*, la cual produce las toxinas Cry1Aa, Cry1Ab, Cry1Ac y Cry2Aa, estas toxinas tienen actividad contra varias especies de polillas que son una de las principales plagas de coníferas.

Salud pública: Bt se ha utilizado por más de dos décadas para el control de algunas especies de insectos como *Aedes spp.*, *Culex spp.* y *Anopheles spp.*, los cuales son transmisores de protozoarios y virus, que son el agente causal de enfermedades de importancia en salud pública como malaria, dengue, fiebre amarilla y filariasis. La cepa de elección para el control de las poblaciones de Dípteros es Bt subsp. *israelensis*, esta cepa produce varias toxinas como Cry11A, Cry4Aa, Cry4Ba, Cry10Aa y Cyt1Aa. El éxito que ha tenido esta subespecie de Bt como biopesticida ha sido el efecto sinérgico que existe entre las toxinas Cry y Cyt que contiene el cristal de la bacteria (Wirth, et al., 1997). El nicho ecológico de los estadios larvarios de estos Dípteros son zonas acuáticas y para aumentar la persistencia de las toxinas Cry se han trasferido los genes a otros microorganismos como sistemas alternativos que co-habiten con las larvas y sean una fuente de alimento, como *B. esphericus*,

Caulobacter crescentus, y algunas cianobacterias como *Agmenellum quadruplicatum* y *Synechococcus spp.* (Schnepf, et al., 1998).

Agricultura: La mayor parte de las especies de insectos que provocan daños en cultivos pertenecen al orden de los Lepidópteros y Coleópteros, en las dos últimas décadas la producción de formulaciones para el control de estas plagas ha sido una de las áreas que ha tenido un mayor desarrollo. La aplicación de formulaciones en aerosoles sobre cultivos tiene buenos resultados sobre algunos insectos, sin embargo muchos de ellos invaden órganos internos de la planta como tallos, frutos o raíces, estas zonas no pueden ser alcanzadas por los atomizadores que esparcen las toxinas de Bt. Para sobrellevar este problema se han introducido los genes *cry* en algunas bacterias endofíticas o epifíticas como *Pseudomonas fluorescens*, *P. cepacia*, *Clavibacter xyli*, *Azozpirillum spp.*, *Rhizobium leguminosarum*, *B. cereus*, *B. megaterium, B. subtilis* y *E. coli*. Alternativamente se han introducido los genes *cry* a plantas como tabaco, papa, algodón, maíz, soya, sorgo, arroz, tomate, etc, con la finalidad de que la misma planta produzca la toxina. La generación de estas plantas transgénicas con genes de Bt ha sido uno de los principales intereses en la industria agrícola, el avance biotecnológico en esta área es considerable; actualmente se ha mejorado el nivel de expresión de la proteína con respecto a las primeras generaciones de “plantas Bt”, así como su expresión de manera local en los órganos invadidos por los plagas.

Resistencia de insectos a toxinas Cry: El uso excesivo de toxinas Cry en el campo trae como consecuencia que los insectos evolucionen para sobrevivir y adaptarse a ellas, este proceso de adaptación genera la resistencia a las toxinas de Bt. Este fenómeno es evaluado en el laboratorio estimando la dosis letal que mata el 50% de la población (LC_{50}) de insectos resistentes sobre la LC_{50} de la colonia de insectos sensibles. Una de las estrategias para evitar la generación de poblaciones de insectos resistentes en cultivos de plantas transgénicas es la creación de “refugios”, ésta consiste en cultivar una zona con plantas Bt y en otra zona cercana crear “refugios” con plantas silvestres para mantener poblaciones de insectos susceptibles. En teoría los insectos homocigotos resistentes (RR) generados en las plantas Bt se cruzan con las poblaciones homocigotos sensibles (SS) en refugios y las generaciones heterocigotos (RS) serán sensibles. Por otra parte la aplicación de fórmulas que contengan más de una toxina Cry también ha dado buenos resultados, en teoría

ambas toxinas reconocen diferentes receptores o sitios de unión y para el insecto tendría un elevado costo adaptativo modificar dos proteínas o mutar dos sitios de unión (Bravo y Soberón, 2008). Uno de los principales mecanismos de resistencia de los insectos hacia las toxinas Cry es la modificación de sus receptores, aunque también se han reportado casos debido a cambios en el perfil las proteasas responsables del proceso de activación (Oppert, et al., 1997). Sin embargo, pocos son los casos de resistencia, posiblemente debido a que la pérdida de estas proteínas representa un costo adaptativo muy alto que pone en desventaja a los insectos en su desarrollo (Tabashnik, et al., 2008). Potencialmente existe la posibilidad de que los insectos blanco generen mutaciones en las regiones de unión a las toxinas, de tal manera que dichas mutaciones no comprometan la función de la proteína y sí afecten la unión de las toxinas Cry. El fenómeno de resistencia ha sido estudiado por varios grupos de investigación generando poblaciones de insectos resistentes mediante una continua y creciente exposición a las toxinas Cry a través de varias generaciones. Por otra parte, el monitoreo de insectos en cultivos transgénicos ha permitido recolectar insectos con resistencia generada naturalmente. El estudio de estas poblaciones de insectos resistentes obtenidas en el laboratorio y en el campo, ha permitido conocer sobre los mecanismos de adaptación de los insectos a las toxinas Cry y contribuye en detallar el mecanismo de toxicidad.

Tabla 1. Descripción de algunas especies de organismos con resistencia a toxinas Cry.

Nombre científico	Resistencia a toxinas	Tipo de resistencia	Referencias
Caenorhabditis elegans	Cry5B	Defectos en síntesis de glicolípidos	Griffitts, et al., 2001.
Culex quinquefaciatus	Cry4A, Cry4B, Cry11Aa	Desconocido	Georghiou y Wirth, 1997.
Diatraea saccharalis	Cry1Ab	Desconocido	Forcada, et al., 1999
Ephestia kuehniella	Cry1A, Cry2A	Sobreproducción y tolerancia a lipoforina	Rahman, et al., 2004.
Helicoverpa armiguera	Cry1Ac	Defectos en Caderina y APN1. Sobreproducción de esterasas y hexamerina	Xu, et al., 2005: Zhang, et al., 2009; Gunning, et al., 2005.
Helicoverpa zae	Cry1Ac	Desconocido	Anilkumar, et al., 2008.
Heliothis virescens	Cry1Ac, Cry2Aa	Defectos en Caderina, ALP y proteasas	Gahan, et al., 2001; Jurat-Fuentes, et al., 2004; Forcada, et al., 1999.
Pectinophora gossypiella	Cry1Ac, Cry1Ab	Defectos en Caderina	Morin, et al., 2003.
Plodia interpunctella	*Bt* subsp. entomocidus	Defectos en proteasas	Oppert, et al., 1997.
Plutella xylostella	Cry1Ac, Cry1Ab	Desconocido	Baxter, et al., 2005
Spodoptera exigua	Cry1C	Defectos en APN1	Herrero, et al., 2005.
Trichoplusia ni	Cry1Ac	Desconocido	Janmaat y Myers, 2003.

II. ANTECEDENTES

II.1 Interacción toxina-receptor

Previamente se han establecido las bases moleculares de la especificidad de las toxinas Cry1A's con actividad insecticida hacia Lepidópteros. Como se mencionó antes, se han reportado la presencia de al menos tres proteínas receptoras involucradas en el modo de acción de las toxinas Cry1A's: Caderina, Aminopeptidasa-N y Fosfatasa alcalina.

Varios son los trabajos en los que se describe a proteínas de tipo Caderinas como receptores en los insectos *M. sexta*, *H. virescens*, *Pectinophora gossypiella*, *Ostrinia nubilalis*, *B. mori*, *Helicoverpa armiguera*, *Aedes aegypti, Anopheles gambiae* y *Tenebrio molitor* (Vadlamudi, et al., 1995; Gahan, et al., 2001; Morin, et al., 2003; Flannagan, et al., 2005; Nagamatsu, et al., 1998; Chen, et al., 2009; Hua, et al 2008; Fabrick, et al., 2009). Las Caderinas son proteínas transmembranales con un peso molecular de 200-250 kDa, contienen una región intracelular en el extremo C-terminal, un segmento transmembranal, y una región extracelular con 10-12 dominios repetidos (CR's) en el extremo N-terminal. La afinidad (K*d*) de interacción de las toxina Cry con las Caderinas oscilan en rangos de 1-10 nM. Su papel como receptor se fundamenta en reportes en los que mutaciones en el gen de *caderina* de *H. virescens*, *H. armiguera* y *P. gossypiella* están asociados con la resistencia a toxinas Cry (Gahan, et al., 2001; Xu, et al., 2005; Morin, et al., 2003), además el silenciamiento de esta proteína en *M. sexta* mediante RNA interferente (RNAi) confiere resistencia a las toxinas Cry1A's (Soberón, et al., 2007).

En trabajos previos ya se han identificado los epítopes responsables en la interacción con las toxinas Cry1A's, en *M. sexta* se identificaron dos sitios de unión localizados en los CR7 y CR11, los cuales unen a las asas 2 y α-8 del Dominio II de las toxinas Cry1A's, respectivamente (Gómez, et al., 2002; Gómez, et al., 2003), por otra parte en *H. virescens* se mapeó un sitio en el CR12 que une directamente al asa 3 (Xie, et al., 2005). Utilizando la tecnología de despliegue en fagos se seleccionó un anticuerpo scFv73 (fragmento variable de cadena sencilla) que reconoce el asa 2 del Dominio II de las toxinas Cry1A's (Gómez, et al., 2001). El anticuerpo scFv73 mimetiza al receptor Caderina, además compite la unión de la toxina al receptor y disminuye la toxicidad en *M. sexta* cuando son alimentados con toxina en presencia

del anticuerpo. La interacción del scFv73 con las toxinas Cry1A's se da a través del CDR3 de la cadena pesada y es capaz de oligomerizar a la toxina. Las bases moleculares de la interacción proteína-proteína son poco conocidas, sin embargo en los últimos años se ha acumulado evidencia que las proteínas pueden interactuar a través de secuencias de aminoácidos que tienen un perfil hidropático inverso, a lo que se le conoce como "complementariedad hidropática" (Blalock, 1999). En trabajos anteriores se ha demostrado que las toxinas Cry1A's interaccionan con la Caderina mediante este tipo de reconocimiento en *M. sexta* y *H. virescens* (Gómez, et al., 2002, Gómez, et al., 2003; Xie, et al., 2005).

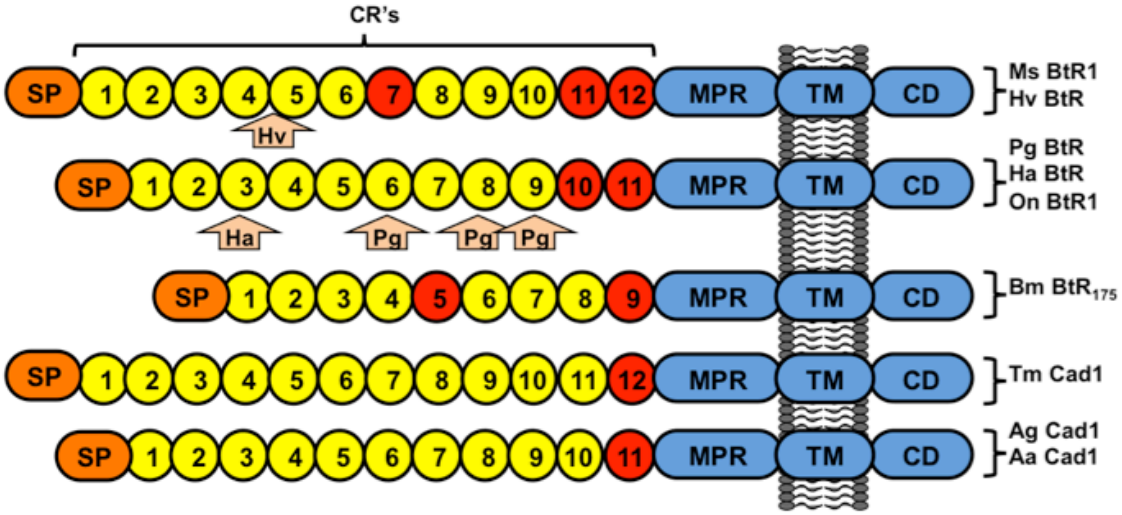

Figura 6. Sitios de unión y alelos mutantes ligados a resistencia en Caderinas de insectos. La localización de las regiones de unión a las toxinas Cry reportadas se muestran en los CR's rojos. Las mutaciones que confieren resistencia a toxinas Cry en *H. virescens, H. armiguera y P. gossypiella* están señaladas con flechas. SP: Péptido señal, CR's: Repetidos de caderina, MPR: Región proximal a la membrana, TM: Región transmembranal, CD: Dominio citoplasmático.

La unión de la toxina a la Caderina desencadena un proceso de oligomerización, el cual involucra un corte proteolítico de la hélice α-1 del Dominio I, se cree que este corte expone regiones hidrofóbicas que permiten la asociación de monómeros (Gómez, et al., 2002). Recientemente se observó que la asociación de estos monómeros de la toxina se debe a la presencia de motivos tipo "coil-coiled" en la hélice α-3, estos motivos consisten en una heptada de residuos que se repiten en una hélice α, esta repetición permite que una de las caras de la hélice sea hidrofóbica y es capaz de unirse a otra hélice con el mismo motivo, además ambos "parches hidrofóbicos" están limitados de cada lado por una serie de residuos con carga que permite establecer puentes salinos formando estructuras muy estables (Jiménez-

Juárez, et al., 2007). En toxinas Cry1A's modificadas mediante ingeniería genética removiendo la hélice α-1 del Dominio I (Cry1A'sMod), son capaces de oligomerizar en ausencia del receptor Caderina y matar insectos con resistencia asociada a mutaciones en este receptor, estos datos confirman que la remoción de la hélice α-1 es necesaria para la formación de oligómeros (Soberón, et al., 2007).

La APN y ALP son metaloproteínas glicosiladas ancladas a "balsas lipídicas" en la membrana por el fosfolípido GPI, tienen un peso molecular aproximado de 120 y 65 kDa, respectivamente, este tamaño es variable dependiendo del insecto. Los modelos de estructura terciaria de la APN de *M. sexta* revelaron contener cuatro dominios (Figura 7), siendo el Domino II el responsable de su actividad de proteasa y el Dominio IV contiene un motivo para unir al fosfolípido GPI en una modificación post-traduccional (Singh y Sivaprasad, et al., 2009). Las afinidades son más bajas comparadas con el receptor Caderina ya que se encuentra en el rango de 80-120 nM.

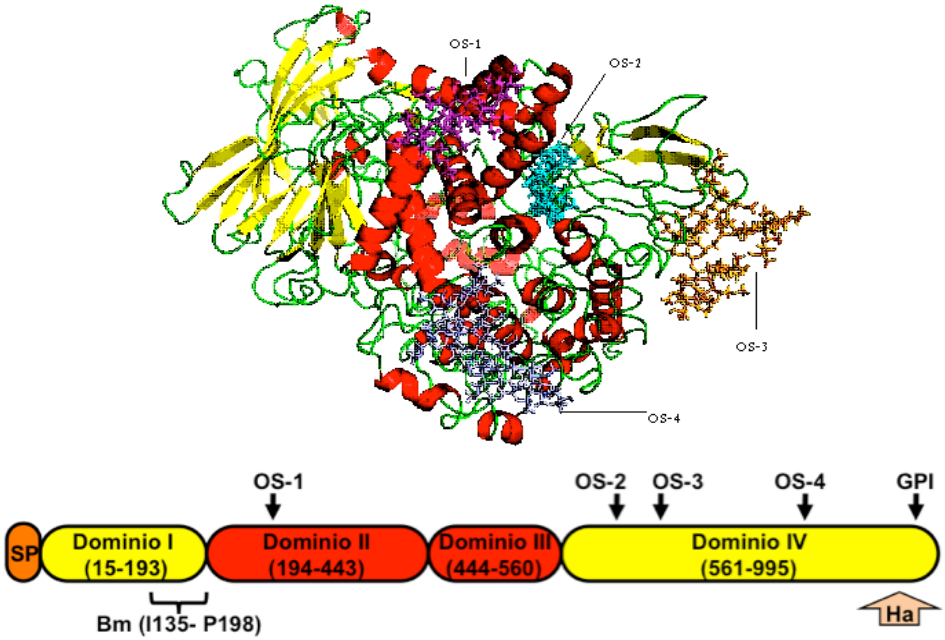

Figura 7. Modelo de la estructura de la Aminopeptidasa-N1 de *M. sexta*. Esquema de los 4 dominios de la proteína indicando con flechas los sitios de glicosilación (OS1-4) y el anclaje a la membrana por el GPI. También se muestra el sitio de unión para la toxina Cry1Aa en *B. mori* y la deleción en el extremo C-terminal que confiere resistencia en *H. armiguera*.

El papel de la ALP como receptor ha sido poco estudiado, se ha visto que una disminución de su presencia en el insecto *H. virescens* confiere resistencia a la toxina Cry1Ac (Jurat-Fuentes, et al., 2004) y recientemente se reportó que la ALP es un receptor para toxinas con actividad hacia Dípteros como la toxina Cry11A y Cry4B

(Chen, et al., 2009). En contraste, se conoce mejor la función de la APN como receptor en Lepidópteros. Existen varias isoformas de esta proteína en los insectos, la carencia de expresión de la isoforma 1 (APN1) en insectos resistentes de *Spodoptera exigua* confiere resistencia a la toxina Cry1Ca (Herrero, et al., 2005) y mutaciones en la APN1 de *H. armiguera* están asociadas con resistencia a la toxina Cry1Ac (Zhang, et al., 2009). Por otra parte, al parecer en *S. litura* la isoforma APN4 es el receptor funcional, pues su silenciamiento con RNAi reduce la susceptibilidad a la toxina Cry1Ca (Rajagopal, et al., 2002).

Los epítopes de unión en el receptor APN aun no han sido bien definidos, en la APN de *B. mori* se identificó una región de 63 aminoácidos (I135 a P198) como sitio de unión a la toxina Cry1Aa (Yaoi, et al., 1999). Por otra parte una deleción de 25 aminoácidos en el extremo C-terminal de la APN1 confiere resistencia a la toxina Cry1Ac en *H. armiguera* y disminuye la unión a la misma toxina, sugiriendo que en esta región se encuentra el sitio de unión (Zhang, et al., 2009). Desde que se describió a la APN como receptor para las toxinas Cry1A's en *M. sexta* (Knight, et al., 1994), se sabía que la unión de la toxina Cry1Ac se veía disminuida en presencia del azúcar N-acetil-galactosamina (GalNac), posteriormente se describió que en el Dominio III de esta toxina existe un motivo de unión a GalNac que une de manera específica a este azúcar presente en el extremo C-terminal de la APN de *M. sexta* y *Lymantria dispar* (Jenkins, et al., 2000; Jenkins, et al., 1999). Por otra parte recientemente se identificaron las regiones β-16 (^{508}STLRVN^{513}) y β-22 (^{582}VFTLSAHV^{589}) del Dominio III de la toxina Cry1Aa involucradas en la unión con la APN1 de *B. mori* (Atsumi, et al., 2005) y una región de la cadena β-16 en la toxina Cry1Ab que une a la APN de *M. sexta* (Gómez, et al., 2006). En previos reportes se han hecho mutaciones en las asas α-8, 2 y 3 del Dominio II de la toxina Cry1Ac que afectan su unión a la APN de *L. dispar*, siendo más dramáticas aquellas mutaciones localizadas en el asa 3 (Jenkins, et al., 2000). Estos datos muestran claramente que los Dominios II y III de las toxinas Cry1A's están involucrados en la unión con la APN. Una vez que la toxina oligomeriza suceden cambios estructurales en las regiones involucradas con la unión a los receptores, esto fue observado utilizando anticuerpos monoclonales que reconocen las asa 2 y 3 del Dominio II de las toxinas Cry1A's, estos anticuerpos reconocen preferencialmente al monómero, mientras que un anticuerpo monoclonal que reconoce la región β-16 del Dominio III reconoce de igual

manera ambas estructuras, indicando que el Dominio III no sufre cambios en su estructura (Gómez, et al., 2006).

II.2 Sinergismo entre toxinas Cry y fragmentos del receptor Caderina

En el mecanismo de acción de las toxinas Cry1A's la primera interacción es con la Caderina, a este receptor en *M. sexta* se le llamó Bt-R_1 (Bravo, et al., 2004, Vadlamudi, et al., 1995). La construcción de versiones truncadas de Bt-R_1 que contienen los sitios de unión para las toxinas Cry1A's (TBR's) ha contribuido en detallar la interacción toxina-receptor. Con el propósito de correlacionar la unión de los TBR's con la toxicidad se realizaron experimentos *in vivo,* en los que estos fragmentos recombinantes inhiben la toxicidad (Gómez, et al., 2003), este fenómeno sucede debido a que la toxina se une a los TBR's formando un complejo Cry1A's-TBR's que impide la interacción con el Bt-R_1 presente en las células del intestino de los insectos. En un intento para validar el CR12 de *M. sexta* como sitio de unión para las toxinas Cry1A's, se construyó una región de Bt-R_1 que contiene el CR12 y la región proximal a la membrana (CR12-MPED), al hacer ensayos de inhibición de toxicidad inesperadamente la presencia de este péptido incrementó la actividad de las toxinas Cry1A's en *M. sexta*, *H. virescens* y *H. zea* (Chen, et al., 2007). Dos hipótesis se propusieron para explicar este "sinergismo", una de ellas propone que la presencia del CR12-MPED modifica la interacción toxina-receptor debido al estado no-plegado del péptido, otra propuesta es que los péptidos se localizan en la microvellosidad del intestino proporcionando una mayor disponibilidad de sitios de unión debido a la elevada afinidad del CR12-MPED por la toxina (9 nM). Más tarde se clonó una Caderina de *A. gambiae* y se analizó su posible papel como receptor para la toxina Cry4Aa, en este trabajo se mapeó el sitio de unión en el CR11 y se clonó obteniendo una fragmento recombinante denominado CR11-MPED, interesantemente la presencia de este péptido en bioensayos incrementó la susceptibilidad de las larvas de *A. gambiae* a la toxina Cry4Ba (Hua, et al., 2008), posteriormente el CR11-MPED y otro fragmento con los CR9-CR11 fueron ensayados en *A. aegypti* obteniendo el mismo efecto (Park, et al., 2009). Este sinergismo también se observó en Coleópteros, en este caso un fragmento recombinante con los CR8-CR10 de la Caderina de *Diabrotica virgifera virgifera* incrementó la actividad de las toxinas Cry3Aa y Cry3Ba en los Coleópteros *D. virgifera virgifera*, *Leptinotarsa decemlineata* y *D. undecimpunctata howardi* (Park, et al., 2009). Sin embargo cuando se analizó el

posible sinergismo en *H. armiguera* de las toxinas Cry1Ac y Cry2Ab con un fragmento de Caderina de este Lepidóptero que contiene el CR11-CR10 y la región proximal a la membrana, se observó una reducción de la actividad en la toxina Cry1Ac y no hubo un efecto para la toxina Cry2Aa (Liu, et al., 2009). Se argumentó que esta reducción de la actividad de la toxina Cry1Ac puede deberse a que este fragmento tiene un plegamiento en el que el sitio de unión se encuentra oculto.

II.3 Tecnología de despliegue en fagos

El despliegue de péptidos sobre la superficie del bacteriófago filamentoso M13 de *E. coli* fue descrito en 1985 por George Smith (Smith y Petrenco, 1997), más tarde se observó que también era posible desplegar exitosamente proteínas como anticuerpos. Este sistema esta basado sobre la ingeniería genética de las proteínas de la cápside viral a las que se le fusiona el gen de la proteína o péptido que se desea desplegar (Figura 8). Durante la fase de propagación del fago en la célula huésped, la proteína de fusión es incorporada a las partículas virales generando fagos con la proteína de interés expuesta sobre su superficie. Tales fusiones pueden ser obtenidas mediante la inserción del gen de interés en el genoma del fago, o bien utilizando un fagémido que exprese la proteína de la cápside del fago en combinación con un "fago ayudador". El sistema de despliegue en fagos es una estrategia que permite tener una conexión directa entre la proteína desplegada (fenotipo) y su gen (genotipo), debido a esta característica es posible obtener y modificar polipéptidos con propiedades de unión a algún ligando mediante un proceso de selección.

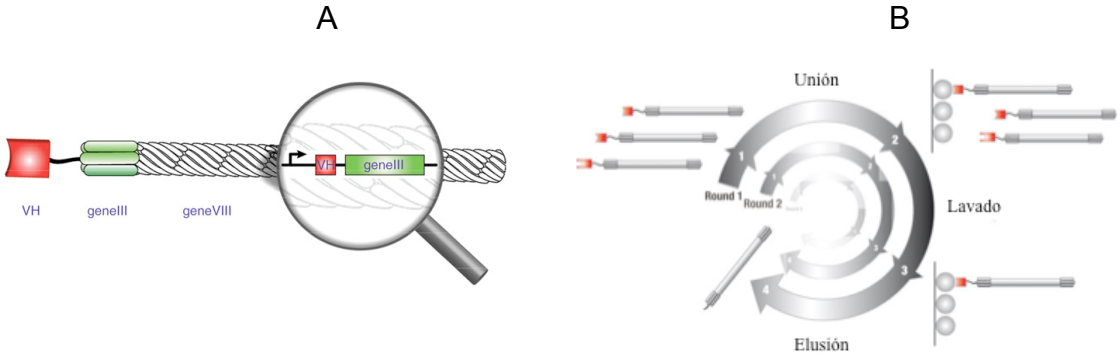

Figura 8. Sistema de despliegue en fagos filamentosos M13. (A) Representación del despliegue de la fracción variable de la cadena pesada de un anticuerpo (VH). (B) Esquema de selección en el que una colección de anticuerpos desplegados son tamizados contra un antígeno fijo sobre una superficie, los fagos que se unen son recuperados y amplificados para realizar subsecuentes rondas de selección.

La utilización de bibliotecas de toxinas Cry desplegadas en fagos es un sistema atractivo para seleccionar toxinas que unan a receptores afectados en unión de insectos resistentes. Inicialmente el fago M13 se utilizó para desplegar las toxinas Cry. El despliegue de versiones truncadas de la toxina Cry1Aa fueron hechos sobre el fago M13 y aunque se observó una eficiencia mínima, la capacidad de unir a los receptores nunca se analizó (Marzari, et al., 1997), además desplegar versiones truncadas de la toxina podría influir en la adaptación de una conformación diferente a la nativa que comprometa su función. Más tarde el mismo sistema de despliegue se utilizó para desplegar la toxina Cry1Ac y se observó una baja eficiencia en el despliegue, sin embargo un dato interesante de este trabajo fue que la toxina desplegada en el fago conservó su actividad contra *M. sexta*, lo cual significa que es capaz de reconocer a sus receptores en el insecto, aunque al analizar su unión en un sistema *in vitro* resultó inespecífica (Kasman, et al., 1998). El despliegue de Cry1Ba sobre el fago M13 también resultó tóxico para *Plutella xylostella* (Nathan, et al*.,* 2006). Todos estos trabajos coinciden en el hecho de un deficiente despliegue de la toxina que no permite una buena selección. Argumentando que esta deficiencia en el despliegue de toxinas Cry se debe a un defecto propio de la célula para exportarlas hacia periplasma, Vilchez y col. (2004) utilizaron el fago λ, cuya morfogénesis se lleva a cabo exclusivamente en el citoplasma, sin embargo en este trabajo, la toxina Cry1Ac se expresó en un vector y posteriormente es incorporada a la cápside viral, esto repercutió directamente en el mismo problema de un despliegue ineficiente (Vílchez, et al., 2004).

Este proyecto consiste en caracterizar la interacción entre las toxinas Cry1A's y el receptor Caderina de *M. sexta*, particularmente nos enfocamos en estudiar el papel del asa 3 del Dominio II y su epítope localizado en el CR12 del receptor. En este sentido, trataremos de establecer una hipótesis sobre los fundamentos moleculares que suceden en el sinergismo entre las toxinas Cry1A's y fragmentos del receptor Caderina. Además, analizaremos cuales son las consecuencias de los cambios estructurales en el asa 3 durante el proceso de oligomerización en el reconocimiento secuencial de los receptores.

III. HIPÓTESIS

Las asas del Dominio II de las toxinas Cry1A's producidas por *Bacillus thuringiensis,* sufren cambios estructurales durante la oligomerización que le permiten tener una unión diferencial entre los receptores Aminopeptidasa-N y Caderina en el insecto *Manduca sexta*, por lo que tienen un papel crucial en el proceso de intoxicación; dada su importancia, estas regiones pueden ser mutagenizadas para modificar su especificidad.

IV. OBJETIVOS

IV.1 Objetivo general

Caracterizar la unión del asa 3 del Dominio II de las toxinas Cry1A's de *Bacillus thuringiensis* con su epítope localizado en el CR12 del receptor Caderina de *Manduca sexta*.

IV.2 Objetivos particulares

Analizar el efecto de mutaciones en el CR12 del receptor Caderina en la unión con las toxinas Cry1A's.

Analizar el efecto de mutaciones en el asa 3 del Dominio II de la toxina Cry1Ab en su modo de acción en el Lepidóptero *Manduca sexta*.

V. MATERIALES Y MÉTODOS

V.1 Cepas, plásmidos y oligonucleótidos

Tabla 2. Descripción de cepas, plásmidos y oligonucleótidos utilizados en este trabajo.

		Descripción
Cepas	Bt 407	*B. thuringiensis serotipo 1 acristalifera(407 Cry⁻)*
	E. coli ER2566	*F- λ- fhuA2 [lon] ompT lacZ::T7 gene 1 gal sulA11 Δ(mcrC-mrr)114::IS10 R(mcr-73::miniTn10-TetS)2 R(zgb-210::Tn10)(TetS) endA1 [dcm]*
	E. coli TG1	*supE thi-1 Δ(lac-proAB) Δ(mcrB-hsdSM)5 (rK- mK-) [F' traD36 proAB lacIqZΔM15]*
Plás	pHT315	*ampR, ErmR, oriC, oriB, pBt1, pBt2(σ^{35}, σ^{28})*
	pET22b	*ampR, oriC, orif1,pT7, lacI, ssPelB, His·Tag*
	pSyn1	*ampR, oriC, oriM13, pLac, ssPelB, His·Tag, myc·Tag*
Oligonucleótid	L3vs23m	5'GGCTTTAGTAATAGTAGTGTAGTTATAATAAGAGCTCCTATGTTCTC3'
	L3vs19m	5'CAATGTTTCGTTCAGGCTTTCGTGTTAGTAGTGTAAGTATAATAAGAGC3'
	L3vs19-23m	5'GTTTCGTTCAGGCTTTCGTGTTAGTATGGTAGTTATAATAAGAGCTCCTATG3'
	L1418RCadMs	5'CTGAACGCTCAAACCGGAGTGCGGACGCTTAATATCCAGCCCACGG3'
	I1422RCadMs	5'CCGGAGTGCTGACGCTTAATCGCCAGCCCACGGCCACGATGCATGG3'

V.2 Preparación de células competentes

Bt 407 competentes: Se realizó un cultivo de Bt 407 en medio líquido BHIG (por litro: Infusión cerebro-corazón 37 gr y Glicerol 0.1%) hasta alcanzar una DO_{600} de 0.5 y después se recuperaron las células centrifugando a 5,000 rpm por 5 min a 4°C. Posteriormente se resuspendió suavemente en una Solución EB (Sacarosa 0.625 M y $MgCl_2$ 1 mM) y se centrifugó igual que en el paso anterior. Las células se resuspendieron en 1 ml de Solución EB. **TRANSFORMACIÓN**: Se mezcló una alícuota de las células competentes (200 µl) con el DNA deseado en celdas de electroporación de 0.4 mm (BioRad) y se dejó reposar durante 5 min a 4°C, posteriormente se dio un pulso eléctrico de 2.5 Volts con una resistencia de 1000 Ω y se dejó reposar por 5 min más. Se agregó medio de cultivo BHIG para obtener un volumen final de 1 ml y se incubó a 30°C durante 1 hr con agitación constante. Finalmente las células se platearon en medio de cultivo sólido LB (por litro: Peptona 10 gr, Extracto de levadura 5 gr, NaCl 10 gr y Agar 15 gr) suplementado con Eritromicina (10 µg/ml).

***E. coli* electrocompetentes:** La cepa de *E. coli* deseada se creció en medio de cultivo líquido YENB (por litro: Extracto de levadura 7.5 gr, Caldo nutritivo 8 gr) hasta

alcanzar una DO_{600} de 0.5-0.9 y se incubaron a 4°C por 5 min. Posteriormente se centrifugó a 5,000 rpm durante 10 min a 4°C y se desechó el sobrenadante. Después la pastilla se resuspendió suavemente en agua fría estéril (4°C) y se repitió la centrifugación. Se desechó el sobrenadante y la pastilla se resuspendió en Glicerol 10% siguiendo la cadena fría. Se centrifugó igual que en los pasos anteriores y finalmente la pastilla se resuspendió en 1 ml de Glicerol 10%. Las células electrocompetentes se almacenaron a -70°C hasta su uso. **TRANSFORMACIÓN:** Se mezcló una alícuota de las células (100 µl) con el DNA deseado en celdas de 0.2 mm (BioRad) y se dio un pulso eléctrico de 2.5 Volts con una resistencia de 200 Ω. Posteriormente se adicionó medio de cultivo SOC (por litro: Triptona 20 gr, Extracto de levadura 5 gr, NaCl 0.58 gr, KCl 0.186 gr, $MgCl_2$ 2.03 gr $MgSO_4$ 24.6 gr y Glucosa 3.6 gr) a un volumen final de 1 ml y se incubó a 37°C durante 1 hr con agitación. Después se platearon las células sobre medio de cultivo LB sólido suplementado con el antibiótico Ampicilina (100 µg/ml).

***E. coli* quimiocompetente:** Se creció la cepa de *E. coli* en medio líquido LB hasta alcanzar una DO_{600} de 0.5, después las células se incubaron a 4°C por 5 min. Posteriormente se centrifugó a 5,000 rpm por 5 min a 4°C y se desechó el sobrenadante. Las células se resuspendieron en una Solución de $CaCl_2$ 60 mM y Glicerol 10%, se incubó por 30 min manteniendo la cadena fría. Finalmente se centrifugó a las mismas condiciones y la pastilla se resuspendió en 1 ml de la solución anterior. Las células se almacenaron a -70°C hasta su uso. **TRANSFORMACIÓN:** Se mezcló una alícuota de 50 µl de células con el DNA deseado y se incubó a 4°C por 30 min. Posteriormente se dio un choque térmico a 42°C por 1 min y se adicionó medio de cultivo SOC a un volumen final de 500 µl y se incubó a 37 °C por 1 hr con agitación. Después se platearon las células sobre medio de cultivo LB sólido suplementado con el antibiótico Ampicilina (100 µg/ml).

V.3 Crecimiento de Bt y purificación de las toxinas Cry

Bt 407 se transformó con el plásmido pHT315-1Ab para la producción de la toxina Cry1Ab y con el plásmido pHT3101 para la toxina Cry1Ac. Las cepas se cultivaron en medio líquido SP pH 7 (por litro: Caldo nutritivo 8 gr, $MgSO_4 \cdot 7H_2O$ 0.25 gr, KCl 1 gr, $MnCl_2 \cdot 4H_2O$ 0.198 mg, $Fe_2SO_4 \cdot H_2O$ 73.2 mg, $CaCl_2$ 147 mg) o en LB suplementados con el antibiótico Eritromicina (10 µg/mL) durante 72 hrs a 30°C con

agitación de 250 rpm. Una vez observada la esporulación y formación de cristales mediante microscopia, la biomasa se recuperó centrifugando el cultivo a 10,000 rpm por 10 min. Posteriormente se realizaron 3 lavados de la pastilla con una Solución NE (NaCl 300 mM y EDTA pH 8.0 10 mM) y después se hicieron 3 lavados con PMSF 1 mM. Finalmente la pastilla se resuspendió en Solución TTN pH 7.2 (Tris-HCl 20 mM, NaCl 300 mM, Triton X-100 1 %).

La purificación de los cristales se realizó por ultracentrifugación a través de un gradiente de Sacarosa. Se prepararon 4 soluciones de Sacarosa al 84, 79, 72 y 67 %, cada una complementada con Tritón X-100 (0.1 %), NaCl (300 mM) y Tris-HCl (20 mM). Con estas soluciones se realizó un gradiente discontinuo quedando en la parte inferior la solución de Sacarosa al 84 %, seguida de las soluciones al 79, 72 y 67 %. En la parte superior del gradiente se agregó la mezcla de esporas-cristales contenidas en la Solución TTN previamente sonicada para liberar a los cristales del los esporangios. Después se centrifugó a 23,000 rpm durante 20 min, con aceleración/desaceleración mínimas. Para eliminar la Sacarosa las fracciones superiores, que generalmente contienen los cristales, se separaron y se diluyeron en Tritón 0.1 %, se centrifugó por 10 min a 10,000 rpm, se recuperó la pastilla y se repitió el procedimiento por 3 veces, finalmente los cristales fueron resuspendidos en Tris-HCl 50 mM y PMSF 1 mM y se almacenaron a 4°C.

V.4 Expresión y purificación de los fragmentos de Caderina

Previamente se clonaron regiones del receptor Caderina de *M. sexta* en el vector de expresión pET22b (Novagen), estas construcciones contienen los CR's con los sitios de unión las toxinas Cry1A's (CR7, CR11, CR12 y CR7-CR12). Estas construcciones se introdujeron a la cepa *E. coli* ER2566 quimiocompetentes con un choque térmico. Posteriormente se realizó un cultivo en medio liquido 2xTY (por litro: Triptona 16 gr, Extracto de levadura 10 gr y NaCl 5 gr) suplementado con el antibiótico Ampicilina 100 µg/ml y se incubó a 37°C con agitación de 250 rpm, cuando se alcanzó una DO_{600} de 0.4-0.6 se adicionó IPTG (1 mM) y se incubó por 4 hrs más a 30°C con la misma agitación. La biomasa se recuperó por centrifugación a 10,000 rpm durante 5 min y la pastilla se resuspendió en una solución de Urea pH 8 (Urea 8 M, $NaPO_4$ 20 mM y NaCl 500 mM). Las bacterias se sonicaron a máxima potencia y

posteriormente se centrifugó a 60,000 rpm durante 15 min. El sobrenadante se recuperó y se almacenó a 4°C hasta la purificación de la proteína.

Para purificar las proteínas se empaquetó una columna con 2 ml de resina Níquel-Agarosa (Quiagen) y se equilibró con Imidazol 20 mM, posteriormente se adicionaron 5 ml del lisado celular previamente preparado y se permitió su paso a través de la resina por gravedad. Se adicionaron 10 ml de Imidazol 30 mM para lavar la resina y finalmente la proteína se eluyó en dos fracciones con 2 ml de Imidazol 250 mM y 2 ml de Imidazol 500 mM. La concentración de proteína se estimó por el método de Bradford utilizando una curva estándar de BSA como referencia. Las fracciones obtenidas se analizaron en SDS-PAGE y se realizó una tinción con azul de Coomassie (Invitrogen) para verificar su pureza.

V.5 Mutagénesis sitio dirigida de toxinas Cry y de fragmentos del receptor Caderina

La mutagénesis se realizó con el "kit" comercial de mutagénesis sitio dirigida QuikChange® Multi (Stratagene) utilizando oligonucleótidos mutagénicos y siguiendo las especificaciones del fabricante. Para obtener las mutantes en el asa 3 de la toxina Cry1Ab se utilizó como templado el plásmido pHT315-1Ab y para la mutagénesis del CR7-CR12 y CR12 se utilizaron los plásmidos pET22b-CR7-CR12 y pET22b-CR12, respectivamente. Los oligonucleótidos utilizados para obtener las mutantes en el asa 3 de la toxina Cry1Ab son: L3vs23m para la mutante S446V (designada como MS), L3vs19m para la mutante S441R, N442V (designada como MD) y L3vs19-23 para la mutante S441R, N442V, S446V (designada como MT). Para realizar la mutagénesis en el CR7-CR12 o en el CR12 se utilizaron los oligonucleótidos: L1418RCadMs para la mutante L1418R, I1422RCadMs para la mutante I1422R y una combinación de ambos para obtener la doble mutante L1418R, I1422R (Tabla 2). Las proteínas mutantes se expresaron y purificaron como se describió arriba. El criterio de mutagénesis fue con base a los perfiles de hidropatía entre los epítope/paratope del CR12 (^{1415}TGVLTLNIQ^{1423}) y el asa 3 de la toxina Cry1Ab (^{439}GFSNSSVS^{I447}) obtenidos con el programa computacional Hypscan.

V.6 Dicroísmo circular de los CR7-CR12 y CR12

Los espectros de Dicroísmo Circular (CD) se obtuvieron en un espectropolarimetro JASCO modelo J-715 equipado con un controlador de temperatura Peltier. Los espectros se colectaron de 200 a 240 nm en celdas de 0.01 cm y se realizaron 8 replicas de cada muestra a 25 °C para mejorar la señal-ruido. Las proteínas recombinantes CR7-CR12 y CR12, mutantes y silvestres, se dializaron en una solución de PBS pH 7.6 (por litro: $NaH_2PO_4 \cdot H_2O$ 0.36 gr, $Na_2HPO_4 \cdot 7H_2O$ 1.02 gr y NaCl 1.62 gr), y la concentración final a la que se tomaron los espectros fue de 0.3 µg/µl. La predicción de estructura secundaria se realizó utilizando el algoritmo CDSSTR, el cual requiere datos colectados en longitudes de onda de 200 a 240 nm.

V.7 Purificación de VMMA de *M. sexta*

Las vesículas de la microvellosidad media apical (VMMA) se obtuvieron por precipitación diferencial con $MgCl_2$. Se disectó el intestino medio de larvas de tercer instar de desarrollo de *M. sexta* y se colocaron en Solución I pH 7.4 a 4°C (Manitol 300 nM, Tris-HCl 17 mM, EGTA 5 mM, DTT 2 mM y PMSF 0.5 mM). Posteriormente se diluyó el tejido en Solución I 1:10 (p/v) y se disgregó con un homogenizador de vidrio manteniéndolo en hielo. Una vez homogenizado se adicionó 1 volumen de $MgCl_2$ 24 mM, quedando a una concentración final de 12 mM, se agitó suavemente y se mantuvo en hielo por 15 min. Después se centrifugó a 4,500 rpm durante 15 min a 4°C en un rotor Beckman JA-20, el sobrenadante se recuperó y se centrifugó a 16,000 rpm durante 30 min a 4°C. La pastilla obtenida se resuspendió en Solución I: $MgCl_2$ 24 mM (1/1, v/v) y se repitieron los dos pasos anteriores de centrifugación. Finalmente la pastilla se resuspendió en Solución I:H_2O (1:1, v/v) y se homogenizó dando 3 golpes a 250 rpm. La concentración de proteína en las VMMA se cuantificó por el método de Lowry (BioRad) tomando como referencia una curva estándar de BSA. Las VMMA se almacenaron a -70°C hasta su uso.

V.8 Purificación de la Aminopeptidasa-N de *M. sexta*

La APN se purificó a partir de VMMA de *M. sexta*. Las VMMA se incubaron en una Solución de solubilización (CHAPS 1%, EDTA 5 nM, NaCl 100 mM, PMSF 1 mM, Tris-HCl 20 mM pH 8.5). Posteriormente la muestra se centrifugó a 10, 000 rpm durante 10 min y el sobrenadante se recuperó y se dializó en una Solución A ($MgCl_2$ 2 mM, KCl 2 mM y Tris-HCl 20 mM pH 8.5). La muestra se cargó dentro de una columna de intercambio iónico (Mono-Q) conectada a un sistema de cromatografía

líquida (FPLC). La proteína unida se eluyó con un gradiente de NaCl de 0-1 M. Las fracciones obtenidas se visualizaron en SDS-PAGE y se realizó una tinción con Azul de Coomassie.

V.9 Expresión y purificación de anticuerpos scFv

Los anticuerpos scFv73 y scFv3L3 se expresaron y purificaron como se reportó antes (Gómez, et al., 2006). El vector pSyn que contiene los genes de los anticuerpos se utilizaron para transformar la cepa *E. coli* TG1 quimiocompetente. Posteriormente se prepararon cultivos en medio líquido 2xTY suplementado con Ampicilina 100 µg/ml y se incubó a 37°C con agitación de 250 rpm hasta alcanzar una DO_{600} de 0.6 y entonces se indujo la expresión de los anticuerpos adicionando IPTG (1 mM), el cultivo se incubó a 30°C durante 4 horas más con agitación de 250 rpm. Una vez terminado el tiempo de inducción se recuperó la biomasa por centrifugación a 10,000 rpm por 10 min. Los anticuerpos se obtuvieron del periplasma celular, esta fracción se obtuvo por un choque osmótico incubando las bacterias a 4°C en una Solución de lisis conteniendo Sacarosa 200 mg/ml, EDTA 1 mM y Tris-HCl 300 mM pH 8. Posteriormente se centrifugó como se indica arriba y el sobrenadante se recuperó. Finalmente los anticuerpos se purificaron por afinidad a la resina Níquel-Agarosa siguiendo el protocolo anterior.

V.10 Experimentos de Western-blot

Las muestras proteicas se mezclaron con una Solución de Laemmli 4X (Tris-HCl 0.125 M, SDS 4%, Glicerol 20%, β-mercaptoetanol 10% y Azul de bromofenol 0.01%) y se incubaron por 3 min a 95°C. Posteriormente las muestras se separaron mediante SDS-PAGE y se electrotransfirieron a una membrana de PVDF durante 45 min a 350 mA. Una vez transferidas las proteínas, la membrana es bloqueada con PBSM (PBS y leche descremada 5 %) durante 1 hr con agitación. Después la membrana se incubó con un anticuerpo primario diluido (1:5000) en PBST (PBS y Tween 20 0.1%) por 1 hr con agitación, terminado el tiempo de incubación se lavó 3 veces con PBST y entonces se incubó con un anticuerpo secundario acoplado a peroxidasa por 1 hr. Finalmente la membrana se lavó 3 veces con PBST y 3 veces con PBS. La unión se reveló utilizando un reactivo luminiscente siguiendo la especificaciones del fabricante (Amersham, Bioscience).

V.11 Activación y oligomerización de las toxinas Cry1A's

Para obtener la toxina en forma monoméricas los cristales purificados se incubaron en una Solución de carbonatos pH 10.2 ($NaCO_4$ 100 mM y β-mercaptoetanol 0.02%) durante 2 hrs a 37°C con agitación constante. Posteriormente las muestras se centrifugaron 5 min a 10,000 rpm y el sobrenadante se recuperó. Se ajustó el pH de la muestra a 8.0 adicionando una solución de Tris-HCl pH 8 y se cuantificó la concentración de proteína. Se utilizó tripsina para activar la protoxina en una proporción 1:30 (p/p) y se incubó por 1 hr a 37°C, una vez terminado el tiempo de activación se inactivó la proteasa adicionando PMSF (1 mM). Las cuantificaciones de proteína se realizaron con el método de Bradford.

Para obtener las estructuras oligoméricas se mezclaron los cristales con la Solución de carbonatos pH 10.2, como fuente de proteasas se adicionó 2.5% (v/v) de jugo digestivo de larvas de *M. sexta* y como receptor se utilizó el anticuerpo scFv73 o los fragmentos recombinantes de Caderina (CR's) en una proporción 1:1 (p/p) de cristales-receptor. La mezcla se incubó a 37°C con agitación suave durante 1 hr. Después se adicionó PMSF (1 mM) y se centrifugó por 10 min a 12,000 rpm. El sobrenadante se recuperó y se utilizó para realizar un Western-blot. Posteriormente los oligómeros se purificaron en una columna de exclusión de peso molecular Superdex-200 HR 10/30 (Amersham Pharmacia Biotech) conectada a un sistema de FPLC, las fracciones obtenidas se analizaron por Western-blot para verificar su pureza. La concentración del oligómero se cuantificó por fluorescencia como se describió antes (Gómez, et al., 2002).

V.12 Ensayos de unión por ELISA y estimación de afinidades relativas (K*d*)

Se fijaron 1 µg de proteína en PBS por cada pozo en placas de ELISA durante 12 hrs a 4°C. Posteriormente se lavaron los pozos 3 veces con PBS y se bloqueó adicionando 200 µl de PBS-BSA 2% por 2 hrs a 37°C. Después se repitió el lavado y se adicionaron 100 µl de PBST con la toxina monomérica u oligomérica y se dejó en incubación por 1 hr a 37°C y entonces se lavó 3 veces con PBST y 2 veces con PBS. Para detectar las toxinas Cry1A's se utilizó un anticuerpo policlonal anti-Cry1A's obtenido previamente por inmunización de un conejo. Después de la incubación con el anticuerpo primario (1:5000), se lavó 3 veces con PBST y 2 veces con PBS y entonces se adicionó el anticuerpo secundario acoplado a peroxidada (1:10,000), anti-

IgG de conejo (Invitrogene). Se incubó por 1 hr a 37°C y después se lavó como se hizo antes. Finalmente se adicionaron 100 µl de sustrato (*orto*-fenilendiamina 2 mM y H_2O_2 0.05% disuelto en una Solución de NaH_2PO_4 100 mM pH 5), una vez que se observó la formación de color se detuvo la reacción adicionando 50 µl de H_2SO_4 1M y se midió la absorbancia a 490 nm en un lector de placas de ELISA.

Para calcular las afinidades relativas se realizaron curvas de unión a saturación fijando 1 µg del receptor sobre la placa de ELISA (APN, CR7-CR12 y CR12). Se realizaron diluciones dobles seriadas de la toxina y el procedimiento de revelado fue igual como se describió arriba. Los datos se analizaron con el programa computacional GraphPad Prism (versión 5.0b) para obtener las curvas de regresión no-lineal, posteriormente los datos se transformaron con la ecuación de Scatchard para ajustar los puntos a una pendiente (Anexo 1).

V.13 Ensayos de unión en solución de las toxinas Cry1Ab a VMMA

Se incubaron 10 µg de proteína de VMMA de *M. sexta* en Solución de unión (PBST y BSA 0.1%) con 5 nM de la toxina Cry1Ab silvestre o mutante en un volumen final de 100 µl y se dejó reposar durante 1 hr a temperatura ambiente. Posteriormente la toxina no unida se eliminó por centrifugación (10 min a 12,000 rpm) y la pastilla se lavó 3 veces con Solución de unión, repitiendo la centrifugación en cada paso de lavado. Finalmente la pastilla se resuspendió en 10 µl de PBS y se realizó un Western-blot para detectar la toxina unida a las vesículas.

Para realizar los ensayos de competencia de unión a VMMA se marcó la toxina Cry1Ab silvestre con biotina utilizando Biotinil-N-hidroxisuccinamida de acuerdo a las especificaciones del fabricante (Amersham, Bioscience). El exceso de biotina no-unida se eliminó separando la toxina Cry1Ab a través de una columna con Sephadex G25. Una vez obtenida la toxina Cry1Ab marcada con biotina se incubaron 10 µg de VMMA con 5 nM de la toxina en ausencia o presencia de varios excesos molares de toxina no marcada silvestre o mutante durante 1 hr. Posteriormente se realizaron 3 lavados como se indica en el procedimiento anterior y para detectar la unión de la toxina Cry1Ab marcada se realizó un Western-blot como se mencionó antes, excepto que en lugar de utilizar anti-Cry1Ab como anticuerpo primario, se utilizó Estreptavidina acoplada a peroxidasa (Amersham, Bioscience). El porcentaje de unión se estimó por

densitometría de las bandas en el Western-blot utilizando el programa computacional ImageJ 1.42q.

V.14 Ensayo de unión por Far-western

Las proteínas CR7-CR12 y CR12 (5 µg) se separaron mediante SDS-PAGE y posteriormente se electrotransfirió a una membrana nitrocelulosa ECL (Amersham, Bioscience). Las membranas se bloquearon con PBS-BSA 1% por 1 hr. Después se adicionó la toxina Cry1Ab a una concentración de 5 nM en PBST-BSA 0.1% y se incubó por 2 hrs. Se lavó la membrana 3 veces con PBST y se adicionó el anticuerpo anti-Cry1Ab en PBST a una dilución de 1:5000 y se incubó por 1 hr, después se lavó la membrana y se adicionó el anticuerpo secundario acoplado a peroxidasa anti-IgG de conejo (1:10,000). Finalmente la unión se reveló con Luminol.

V.15 Bioensayos

Los bioensayos se realizaron con larvas neonatas de *M. sexta* por el método de contaminación de superficie. En placas de 24 pozos se adicionó dieta artificial en cada uno de ellos y se adicionó la toxina sobre su superficie, se dejó secar a temperatura ambiente y después se puso una larva por cada pozo. Una vez transcurridos 7 días se contó el número de larvas muertas para obtener el porcentaje de mortalidad y se comparó con un control negativo en el que en lugar de toxina se adicionó agua sobre la superficie de la dieta. Las dosis LC_{50} y LC_{10} se estimaron con un análisis PROBIT utilizando el programa computacional POLO-PC LeOra.

VI. RESULTADOS

VI.1 Identificación del sitio de unión a las toxinas Cry1A's en el CR12 de *M. sexta*

La Caderina de *M. sexta* es una proteína transmembranal con un peso molecular de 210 kDa que consiste de una región intracelular en el extremo C-terminal, un segmento transmembranal, y una región extracelular que contiene 12 dominios repetidos en el extremo N-terminal (Vadlamudi, et al., 1995). Previamente se identificaron dos regiones discretas localizadas en los CR7 y CR11 en el receptor Caderina de *M. sexta* como sitios de unión a las toxinas Cry1A's, estos sitios son reconocidos por las asas 2 y α-8 del Dominio II, respectivamente (Gómez, et al., 2002, Gómez, et al., 2003). Posteriormente en *H. virescens* se reportó que en el CR12 se encuentra un epítope que es reconocido por el asa 3 de las toxinas Cry1A's (Xie, et al., 2005). En el receptor Caderina de *M. sexta* ya se había observado que las toxinas Cry1A's unen al CR12 (Dorsch, et al., 2002). Al hacer un análisis de las secuencias entre las Caderinas de *M. sexta* y *H. virescens* encontramos que el sitio de unión identificado en *H. virescens* es conservado en ambos insectos, lo cual indica que potencialmente éste podría ser el sitio de reconocimiento del asa 3 en el receptor Caderina de *M. sexta*.

En trabajos anteriores se clonó un fragmento del gen de la Caderina de *M. sexta* que comprende del CR7 al CR12 en el vector pET22b (Soberón, et al., 2007). Esta construcción se utilizó para generar una serie de mutantes sencillas, dobles y triple localizadas en el CR11 y CR12. Una vez obtenidas las mutantes se expresaron de manera heteróloga en *E. coli* y la unión de las toxinas Cry1A's se evaluó mediante Far-western. Extractos proteicos de cultivos de *E. coli* se separaron mediante SDS-PAGE y después se electrotransfirieron a una membrana de nitrocelulosa, posteriormente la membrana se incubó con las toxinas Cry1Ab o Cry1Ac y la unión se reveló utilizando un anticuerpo anti-Cry. Los resultados obtenidos mostraron que las mutaciones sencillas localizadas en el CR12 afectan la unión de las toxinas Cry1A's, y no así las mutaciones en el CR11. Por otra parte las mutantes dobles y triple también están afectadas en unión, sin embargo esto se debe a que todas ellas contienen por lo menos, una mutación sencilla en el CR12 (Figura 9). Estos resultados muestran que, al igual que en *H. virescens*, el sitio de unión para el asa 3 de las toxinas Cry1A's

en el CR12 del receptor Caderina de *M. sexta* es la región ^{1415}TGVLTLNIQ^{1423}, y además que una sola mutación en esta región compromete la unión de las toxinas Cry1A's de manera importante en comparación con las mutaciones generadas en el CR11.

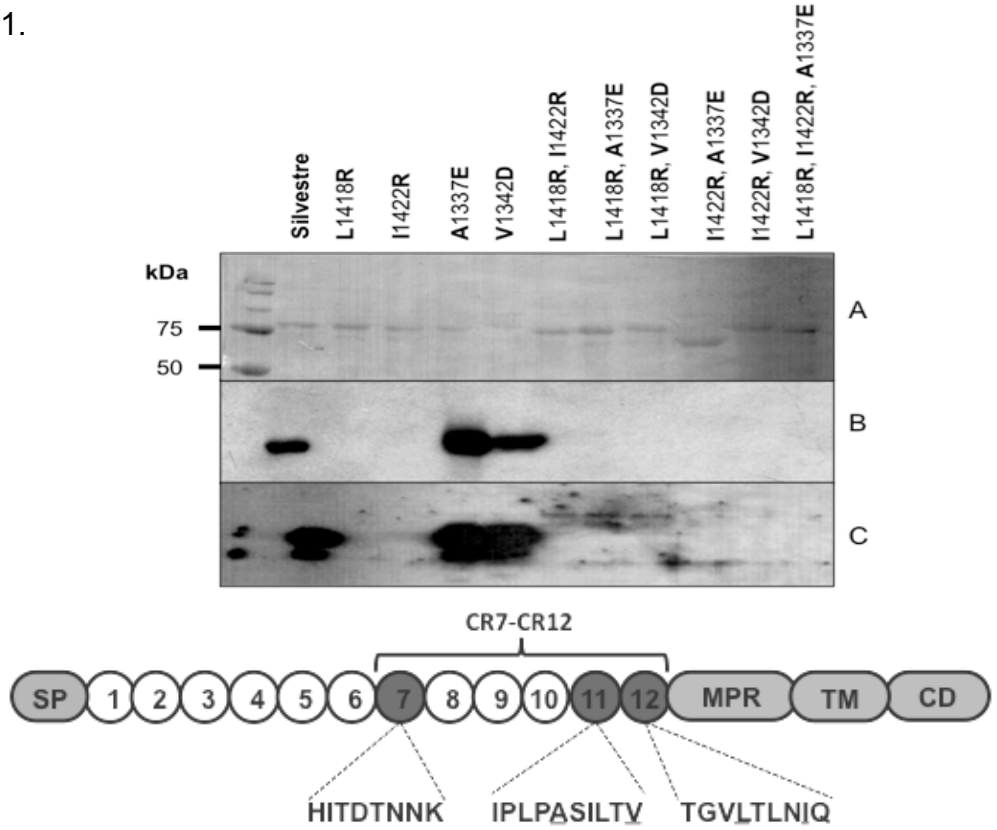

Figura 9. Análisis de unión de las toxinas Cry1A's a mutantes del fragmento CR7-CR12 de *M. sexta*. En la parte superior se muestra una tinción con Azul de Coomassie de las proteínas mutantes (A) y la unión de la toxina Cry1Ab (B) y Cry1Ac (C) analizada por Far-western. En la parte inferior se representa la estructura de la Caderina de *M. sexta* con la secuencia de los tres sitios de unión localizados en el CR7, CR11 y CR12.

En el experimento anterior la unión de las toxinas Cry1A's se analizó mediante una metodología en la que las proteínas tienen que ser desnaturalizadas por SDS-PAGE, sin embargo en condiciones nativas cabe la posibilidad de que las mutaciones generadas en el CR12 afecten su estructura y en consecuencia la unión de las toxinas Cry. Para descartar esta posibilidad se realizó un análisis estructural mediante Dicroísmo Circular. Las mutantes sencillas y doble L1418R e I1422R, se generaron en dos versiones truncadas del receptor Caderina de *M. sexta*, una de ellas contiene los CR7 al CR12 con un peso molecular de aproximadamente 75 kDa (fragmentos analizados anteriormente) y la otra sólo contiene el CR12 de 15 kDa. Estos fragmentos, silvestres y mutantes, se expresaron utilizando el vector pET22b que

permite la fusión de una etiqueta de 6-His en el extremo C-terminal y posteriormente se purificaron por afinidad a Ni-Agarosa. Una vez obtenidas las proteínas se tomaron lecturas a longitudes de onda de 200-260 nm mediante Dicroísmo Circular. Los espectros obtenidos con los fragmentos CR7-CR12 mostraron una señal muy parecida entre la proteína silvestre y las mutantes generadas, indicando que dichas mutaciones no comprometen el plegamiento de la proteína afectando su estructura secundaria (Figura 10). Sin embargo, cuando se compararon los espectros de CD de las mutantes en el contexto del CR12, sí se observaron diferencias significativas entre ellos, aunque los espectros de un plegamiento característico de láminas β permanece (Figura 10). Estas diferencias las atribuimos a que posiblemente estos péptidos contienen un mayor contenido de aminoácidos "no-plegados" comparado con los fragmentos de mayor tamaño (CR7-CR12) que posiblemente contribuyen en la estabilización del plegamiento, aunque en la predicción del porcentaje de la estructura secundaria con el algoritmo utilizado no muestra diferencias significativas (Tabla 3). Por otra parte se ha observado que la separación de dominios de alguna proteína, involucra que estos adquieran una conformación diferente a la nativa cuando se producen de manera independiente.

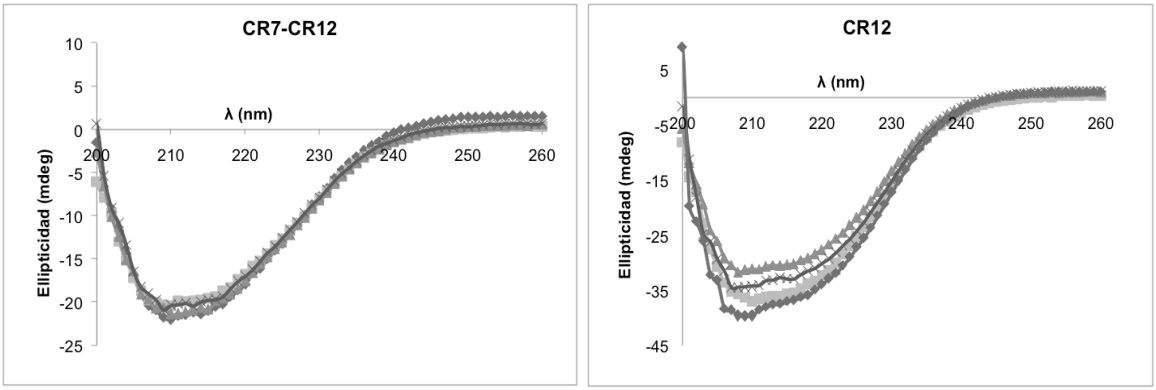

Figura 10. Espectros de Dicroísmo Circular de los fragmentos recombinantes CR7-CR12 y CR12 del receptor Caderina de *M. sexta*. Espectros de CD de las proteínas CR7-CR12 y CR12 silvestres y mutantes. Proteínas silvestres (■), L1418R (▲), I1422R (◆) y L1418R,I422R (✖).

Utilizando las lecturas obtenidos de CD se predijo el porcentaje de estructura secundaria utilizando un algoritmo CDSSTR en la base de datos, mediante este análisis se obtuvo que estas proteínas adoptan una conformación preferencial de estructura secundaria de láminas β (Tabla 3), lo cual correlaciona con las estructuras de la Caderina depositadas en el PDB (Boggon, et al., 2002) y algunas otras previamente analizadas por CD (Pokutta, et al., 1994). El porcentaje de láminas β

observado en la estructura de 5 de los dominios ectoplasmáticos de la Caderina de *Xenopus laevis* fue del 36%, un valor muy parecido al obtenido en este trabajo con los espectros de CD del fragmento CR7-CR12, sin embargo al comparar las hélices α obtuvimos un valor superior al esperado, ya que en la estructura cristalográfica resultó del 1% mientras que en los nuestros fue del 14% (Baggon, et al., 2002). Por otra parte, la estructura de un solo dominio de la Caderina de *Mus musculus* reveló contener 38% de láminas β y 3% de hélices α (Shapiro, et al., 1995), mientras que los datos obtenidos en este trabajo por CD del CR12 fueron del 31% y 18%, respectivamente. Es interesante notar que ambas metodologías arrojan resultados muy similares en el porcentaje de estructura de láminas β, aunque el porcentaje de hélices α es diferente, quizás esta desigualdad se deba a que los fragmentos utilizados en este trabajo se purificaron bajo condiciones desnaturalizantes; y entonces, posiblemente en el proceso de re-naturalización permita que la proteína adopte plegamientos helicoidales, además la estructura resuelta por cristalografía se hizo en presencia de iones como el Calcio que estabilizan el plegamiento de las Caderinas, lo que pudiera contribuir en estas diferencias (Pokutta, et al., 1994).

Tabla 3. Porcentaje de estructura secundaria de los fragmentos recombinantes de la Caderina a partir de los datas de CD.

Estructura secundaria	CR7-CR12				CR12			
	Silvestre	L1418R	I1422R	L1418R I422R	Silvestre	L1418R	I1422R	L1418R I422R
Hélices-α	14 %	12%	13 %	12 %	18 %	17 %	13 %	12 %
Cadenas-β	36 %	36 %	34 %	42 %	31 %	29 %	34 %	35 %
Turns (giros)	23 %	25 %	26 %	19 %	26 %	25 %	22 %	26 %
No estructurado	27 %	27 %	27 %	27 %	21 %	21 %	31 %	25 %

Una vez obtenidos los fragmentos purificados silvestres y mutantes en las versiones truncadas del receptor (CR7-CR12 y CR12) se analizó su capacidad de unión a las toxinas Cry1A's mediante ELISA y Far-western. En la Figura 11 se observa que ambas técnicas mostraron que la toxina Cry1Ac reconoce a los fragmentos silvestres, en contraste con las mutaciones sencillas y doble generadas en los CR7-CR12 y CR12 que afectan considerablemente su reconocimiento por la toxina. Aunque se observó en los espectros de CD que las mutantes en el fragmento CR12 tienen diferencias en el plegamiento del péptido, esto es irrelevante en la unión de las toxinas Cry1A's, ya que el epítope no depende de la conformación adoptada

por la proteína, debido a que la toxina presenta el mismo perfil de reconocimiento en ensayos de unión en condiciones nativas (ELISA) y desnaturalizantes (Far-western).

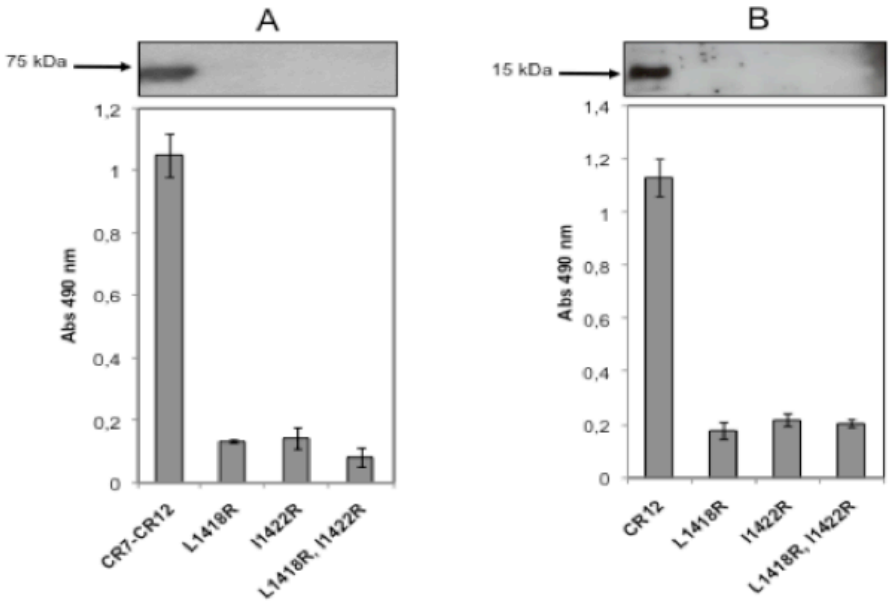

Figura 11. Unión de la toxina Cry1Ac a los fragmentos mutantes CR7-CR12 (Panel A) y CR12 (Panel B). En la parte superior de cada panel se muestran los resultados del ensayo de unión por Far-western y en la gráfica de barras los obtenidos por ELISA.

VI.2 La formación de oligómeros correlaciona con el aumento de la actividad de las toxinas Cry1A's

Previamente se reportó que la presencia del CR12-MPED de *M. sexta* incrementa la actividad de las toxinas Cry1A's hacia varias especies de insectos Lepidópteros (Chen, et al., 2007), en ese trabajo los autores esperaban que la presencia del CR12 compitiera la interacción de la toxina con Bt-R1 y de esta manera inhibir su toxicidad; sin embargo el efecto fue contrario a lo esperado ya que la actividad se incrementó.

En este trabajo realizamos un bioensayo con larvas de *M. sexta* utilizando una LC_{10} de 0.5 ng/cm^2 de las toxinas Cry1A's en presencia o ausencia de varias versiones truncadas del receptor Caderina en una proporción de 1:100 p/p. Utilizamos tres péptidos recombinantes CR7, CR11 y CR12, que contienen los sitios de unión para las asas α-8, 2 y 3 de las toxinas Cry1A's, respectivamente.

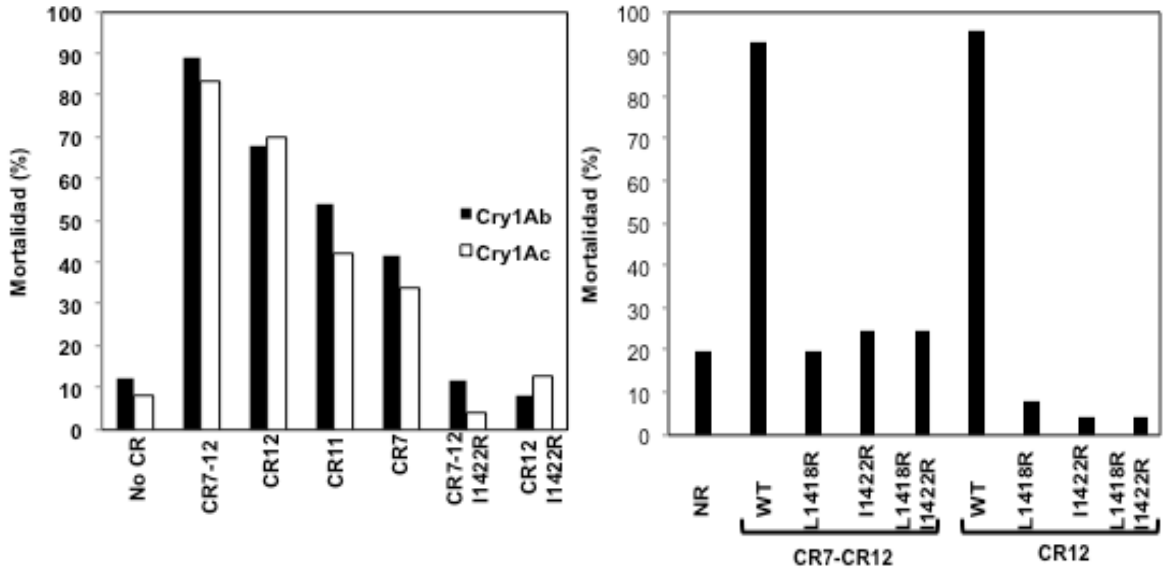

Figura 12. Fragmentos del receptor Caderina aumentan la actividad de las toxinas Cry1A's en *M. sexta*. Porcentaje de mortalidad obtenido en bioensayos utilizando una LC_{10} de las toxinas Cry1A's en presencia o ausencia de los CR's silvestres o mutantes. En el gráfico de la derecha se utilizó la toxina Cry1Ac.

Adicionalmente también utilizamos el CR7-CR12, que contiene los tres sitios de unión. Los resultados obtenidos mostraron que el fragmento CR7-CR12 silvestre aumenta 7 veces la actividad de las toxinas Cry1A's (Figura 12), el mismo efecto se pudo observar al utilizar los péptidos que comprenden únicamente el CR7, CR11 y CR12, aunque el aumento de actividad se vio en menor proporción (2, 2.5 y 6 veces, respectivamente). De igual manera se analizó el efecto de las mutaciones generadas en el CR7-CR12 y CR12 en el aumento de toxicidad en *M sexta*. Como ya se había observado, la presencia del CR7-CR12 y el CR12 aumentó la mortalidad de un 10% hasta un 90%, en contraste con los fragmentos que contienen las mutaciones en el CR12, las cuales afectan de manera importante la unión de las toxinas Cry1A's, no se observó un aumento significativo en la mortalidad hacia *M. sexta* (Figura 12).

Los resultados obtenidos indican que la unión de las toxinas Cry1A's a los fragmentos recombinantes de Caderina es necesaria para que haya un incremento de su actividad hacia el insecto blanco. En el laboratorio hemos observado que el CR12 favorece la formación del oligómero más que cualquier otro CR's de Caderina que contenga algún sitio de unión, esto pudiera explicar que el efecto sinérgico del CR12

se debe a un mayor rendimiento en la formación de oligómero. Previamente se estandarizó la obtención de oligómeros en condiciones *in vitro* utilizando el anticuerpo scFv73 como mimetizante del receptor Caderina (Gómez, et al., 2002). El ensayo consiste en hacer una simulación de las condiciones físico-químicas del intestino del insecto, es decir un pH alcalino, una fuente de proteasas (en este caso jugo digestivo del insecto), receptor y toxina, posteriormente se realizó Western-blot para comprobar la presencia de oligómeros. Al analizar la formación de oligómero utilizando los fragmentos CR7, CR11 y CR12 en lugar del scFv73, observamos que el CR12 tiene una mayor eficiencia para inducir la oligomerización de la toxina Cry1Ab en comparación con el CR7 y CR11 (Figura 13). Al analizar la formación de oligómeros de la toxina en presencia de los fragmentos mutantes en el CR12 observamos un bajo rendimiento en comparación con el CR12 silvestre. Estos datos muestran una directa correlación entre la capacidad de oligomerizar a la toxina Cry1Ab y el aumento de su actividad en *M. sexta.*

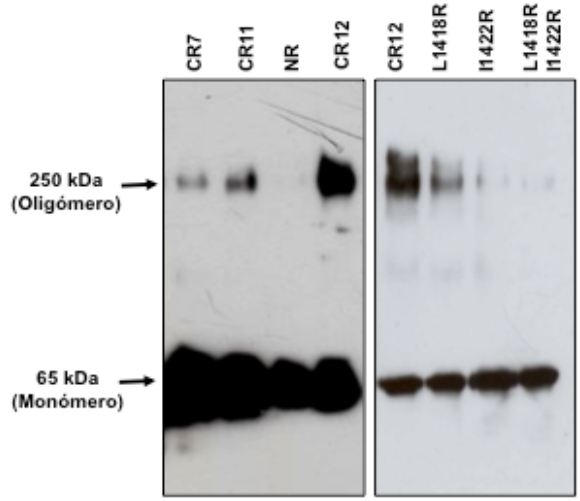

Figura 13. Oligomerización de la toxina Cry1Ab utilizando los fragmentos de la Caderina como receptores. NR, sin receptor.

VI.3 Mutaciones en el asa 3 de la toxina Cry1Ab afectan su unión a VMMA de *M. sexta* y correlaciona con la pérdida de actividad.

Las toxinas Cry1A's de Bt son proteínas que ejercen una actividad insecticida en varios Lepidópteros y el mecanismo por el cual estas toxinas matan a los insectos

es mediante la formación de poros en las membranas del intestino, esta característica implica una transición de un estado soluble a una entidad hidrofóbica que se inserta en las membranas celulares para formar un poro. Para llevar a cabo este cambio estructural estas proteínas oligomerizan una vez que reconocen al receptor Caderina. La unión de la toxina en un estado soluble (monómero) a Caderina involucra la participación de las asas 2 y 3 del Dominio II, sin embargo una vez que la toxina oligomeriza estas regiones sufren cambios estructurales cuya función aun es poco conocida (Gómez, et al., 2006). Esta parte del trabajo se enfoca principalmente en estudiar las implicaciones que tienen estos cambios en el modo de acción de la toxina Cry1Ab en el Lepidóptero *M. sexta.*

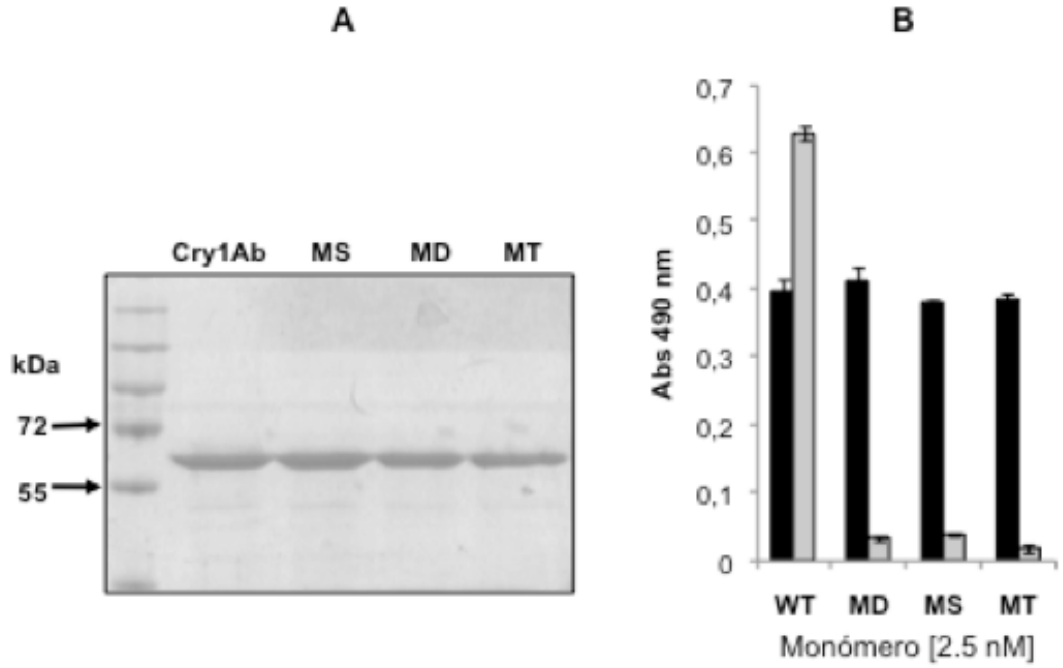

Figura 14. Proteólisis de las mutantes en el asa 3 de la toxina Cry1Ab y análisis de unión con anticuerpos monoclonales. (A) Tinción con Azul de Coomassie de las mutantes MS, MD y MT en el que se observa el procesamiento de la toxina a su forma activa con un peso molecular de 65 kDa. (B) Unión de las toxinas Cry1Ab silvestre y mutantes en el asa 3 a los anticuerpos anti-asa 2 (barras negras) y anti-asa 3 (barras grises), scFv73 y scFv3L3, respectivamente.

Para estudiar la participación del asa 3 de la toxina Cry1Ab se diseñaron mutantes en esta región. Se construyeron 3 mutantes: S441R:N442V, S446V y S441R:N442V:S446V, las cuales se nombraron MD, MS y MT, respectivamente. Estas mutaciones se diseñaron para interrumpir la complementariedad hidropática entre los epítopes de unión de la toxina y el receptor mediante un programa computacional. La cepa Bt 407 acristalífera se transformó con las toxinas mutantes y

se crecieron en medio de cultivo LB hasta esporulación para obtener los cristales. Para evaluar si las mutaciones generadas no afectaron la estabilidad de las proteínas, los cristales de las mutantes se solubilizaron y activados con tripsina al igual que la toxina silvestre. Posteriormente la integridad de las proteínas se analizó mediante SDS-PAGE observando un procesamiento similar al de la toxina Cry1Ab silvestre (Figura 194-A).

Además, estas mutaciones no alteran la estructura de regiones del Dominio II importantes para la interacción con el receptor Caderina, esto fue observado al utilizar los anticuerpos monoclonales scFv73 y scFv3L3 que reconocen específicamente el asa 2 y 3 de la toxina Cry1Ab, respectivamente (Gómez, et al., 2006). Mediante un ensayo de ELISA se observó que el scFv73 reconoce de igual forma a las toxinas mutantes con respecto a la silvestre, por otra parte el anticuerpo scFv3L3 sólo reconoce a la toxina silvestre (Figura 14-B). Estos resultados indican que las mutaciones no afectaron la conformación del asa 2 de la toxina y que las mutaciones generadas afectan el reconocimiento del asa 3 por el anticuerpo scFv3L3.

Una vez obtenidas las toxinas realizamos un experimento de unión en solución a vesículas de microvellosidad media apical (VMMA) de *M. sexta*. Este experimento consiste en incubar la toxina con VMMA obtenidas de intestinos de *M. sexta*, posteriormente la toxina no unida se separa mediante centrifugación y la toxina unida se separa del complejo por SDS-PAGE y se transfiere a una membrana de nitrocelulosa para realizar un experimento de Western-blot, la toxina unida es visualiza utilizando un anticuerpo anti-Cry1Ab. Los porcentajes de unión se calcularon utilizando un programa que estima la intensidad de las manchas en el Western-blot considerando un 100% la unión de la toxina Cry1Ab silvestre. Los resultados mostraron que las mutantes unen en menor proporción que la toxina silvestre, estando más afectada la mutante MT hasta en un 95% y las mutantes MS y MD en un 85% (Figura 15).

Adicionalmente se realizó un experimento de competencia de unión de la toxina silvestre a las VMMA utilizando como competidor las toxinas mutantes. Para ello la toxina Cry1Ab silvestre se marcó con biotina y posteriormente se incubó con las VMMA en presencia de diferentes excesos de las toxinas MS, MD y MT como competidores, posteriormente la toxina marcada es revelada con estreptavidina

acoplada a peroxidasa. En este experimento observamos que la toxina Cry1Ab silvestre no marcada desplaza la unión de la toxina marcada, lo cual indica una competencia de unión por los receptores presentes en las vesículas, en el caso de la competencia con las mutantes MS y MD observamos un mínimo desplazamiento de unión y no así con la mutante MT (Figura 15). Este resultado confirma que las mutantes en el asa 3 no unen de manera eficiente a los receptores presentes en las VMMA de *M. sexta*.

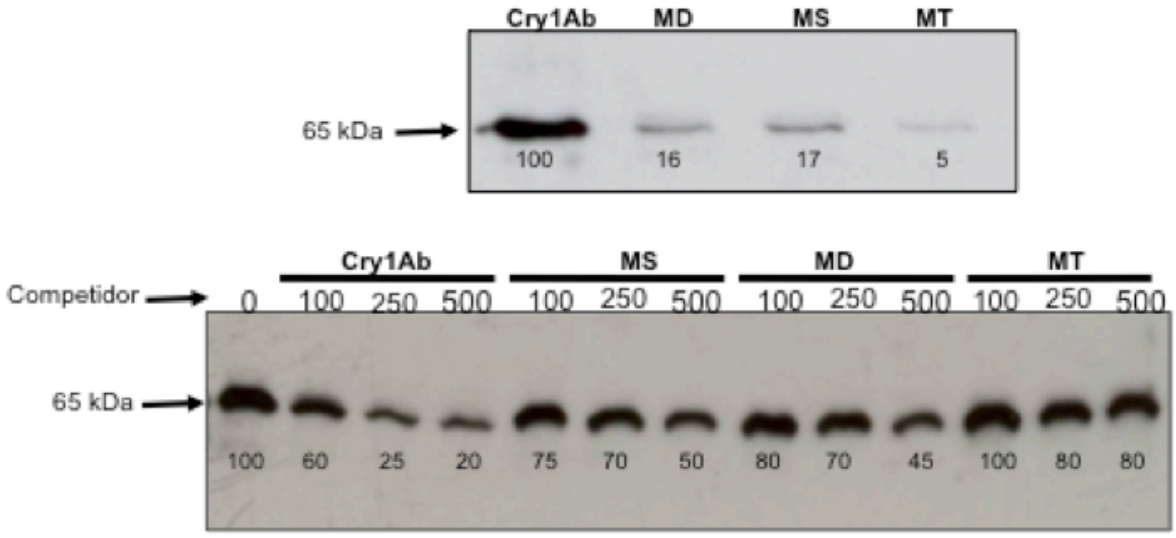

Figura 15. Análisis de unión de las toxinas monoméricas Cry1Ab silvestre y mutantes en el asa 3 a VMMA de *M. sexta*. La figura de arriba representa la unión de 5 nM de la toxina a las VMMA mientras que la de abajo muestra la unión de 5 nM de la toxina Cry1Ab en presencia de 0, 100, 250 y 500 excesos molares del competidor. Los números bajo cada banda representan el porcentaje de toxina unida obtenido por densitometría.

El hecho de que las mutaciones afectaran la unión, indica que su toxicidad debe disminuir, por lo que a continuación se evaluó su actividad en el insecto *M. sexta* utilizando diferentes concentraciones de toxina para estimar la concentración letal media. En este ensayo observamos que las mutantes generadas en el asa 3 afectan de manera importante su toxicidad (Tabla 4). Este resultado correlaciona con los experimentos de unión a VMMA, ya que la mutante MT que esta considerablemente afectada en unión, resultó 70 veces menos tóxica y 9 veces las mutantes MS y MD.

Tabla 4. Concentración letal media (LC_{50}) de las toxinas monoméricas Cry1Ab y mutantes en el asa 3 en *M. sexta*. (ND), no determinado.

Toxina (Monómero)	LC_{50} (ng/cm^2)	$LC_{50(mut)}/LC_{50(wt)}$
Cry1Ab	10.3 (8.9-11.9)	ND
MD	91.7 (75.7-113.7)	8.9
MS	94.7 (73-119.7)	9.19
MT	723 (341.1-2148.9)	70.12

VI.4 El asa 3 de la toxina Cry1Ab en su conformación oligomérica tiene un papel importante para conferir toxicidad

En el modo de acción de las toxinas Cry1A's se ha establecido que la interacción con los receptores es secuencial y diferencial, es decir el monómero interacciona con la Caderina y posteriormente éste oligomeriza e interacciona con la APN. Hasta ahora, hemos trabajado la interacción de esta proteína en forma monomérica hacia VMMA, las cuales contienen ambos receptores. Como se mencionó antes, este experimento únicamente nos permite analizar la unión de manera cualitativa hacia las vesículas aisladas a partir de intestinos de *M. sexta*; para obtener datos más finos sobre la interacción de la toxina Cry1Ab fue necesario oligomerizar la toxina y obtener los receptores purificados y así probar la unión tanto del oligómero como del monómero a los receptores APN y Caderina.

Los cristales de las toxinas, tanto silvestres como mutantes, se utilizaron para realizar ensayos de oligomerización utilizando el anticuerpo scFv73 como mimetizante del receptor Caderina. En la Figura 16 se muestra un Western-blot de la eficiencia de oligomerización de las mutantes MS, MD y MT con respecto a la toxina Cry1Ab silvestre, se puede observar que la MT oligomeriza en menor proporción con respecto a las mutantes MS y MD, lo cual podría tener una correlación con los resultados de unión y toxicidad obtenidos previamente.

Una vez obtenidos los oligómeros, estos se purificaron mediante cromatografía por FPLC utilizando una columna de exclusión de peso molecular (Figura 16-B), analizamos su efecto *in vivo* mediante bioensayos con larvas de *M. sexta* utilizando una sola dosis de 2 ng/cm^2 de oligómero de la toxina Cry1Ab. Esta dosis fue suficiente para matar el 95% de la población de insectos probados, mientras que los

oligómeros de las toxinas mutantes no lo hacen (Tabla 5). Este resultado muestra que las mutaciones localizadas en el asa 3 del Dominio II de la toxina Cry1Ab afectan de manera importante la toxicidad del oligómero.

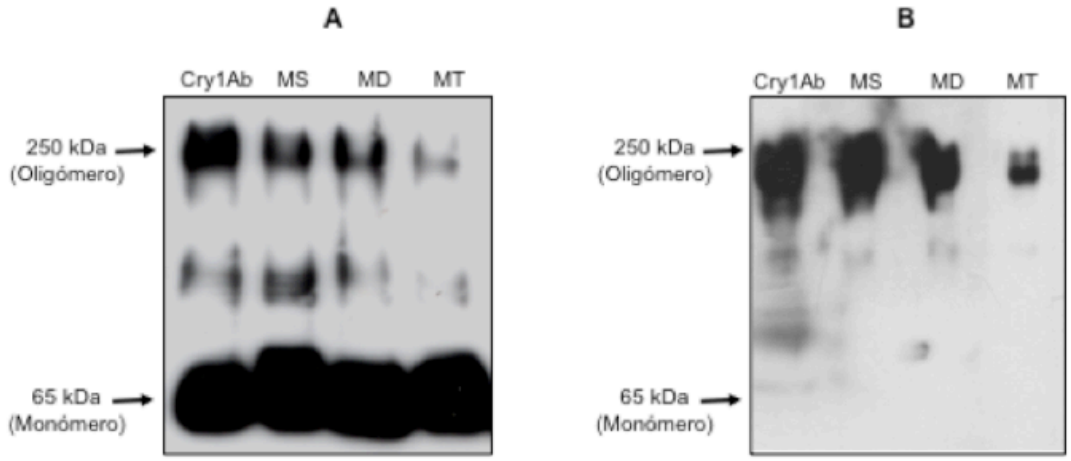

Figura 16. Oligomerización de las mutantes en el asa 3 de la toxina Cry1Ab. (A) Rendimiento de oligomerización de las toxinas utilizando el anticuerpo scFv73 como receptor. (B) Oligómeros purificados por exclusión de peso molecular.

Tabla 5. Bioensayos con *M. sexta* en el que se compara la actividad de la toxina Cry1Ab y de las mutantes en el asa 3 en su forma monomérica y oligomérica.

Monómero (25 ng/cm^2)	Mortalidad (%)	Oligómero (2 ng/cm^2)	Mortalidad (%)
Cry1Ab	95	Cry1Ab	95
MD	5	MD	5
MS	5	MS	5
MT	0	MT	0

VI.5 El asa 3 de la toxina Cry1Ab reconoce diferencialmente a los receptores Caderina y APN de *M. sexta* dependiendo de su forma monomérica u oligomérica

En los experimentos anteriores se observó que el asa 3 del Dominio II de la toxina Cry1Ab tiene un papel importante en su toxicidad, ya sea en monómero u oligómero. Se sabe que mutaciones en el asa 3 de las toxinas Cry1A's afectan la unión a la APN en *L. dispar* (Jenkins, et al., 2000), sin embargo no se ha analizado el

efecto que pudieran tener dichas mutaciones con el receptor Caderina. Por otra parte, la unión del oligómero de las toxinas Cry1A's hacia ambos receptores aun no se conoce a detalle, aunque se sabe que la afinidad por un extracto de proteínas de VMMA de *M. sexta* ancladas por GPI, en el que se encuentran la APN y ALP, tienen mayor afinidad hacia el oligómero (K*d*=0.75 nM) que al monómero (K*d*=175 nM) (Bravo, et al., 2004).

En este trabajo se analizó la unión de las mutantes MS, MD y MT de la toxina Cry1Ab con la APN de *M. sexta* y con los fragmentos de Caderina CR7-CR12 y CR12. Los resultados obtenidos muestran que las mutaciones en el asa 3 no tienen algún efecto significativo con la unión hacia el CR7-CR12. Aunque se ha reportado que el asa 3 de las toxinas Cry1A's está involucrada en la unión con el CR12 de la Caderina, también se sabe que el asa 2 y α-8 reconoce dos sitios de unión localizados en el CR7 y CR11, respectivamente, los cuales están presentes en el fragmento CR7-CR12, esto pudiera explicar que las mutantes en el asa 3 siguen uniendo al CR7-CR12 por reconcer estos dos epítopes, ya que el asa 2 y α-8 son silvestres en las mutantes MS, MD y MT. Al analizar la unión de las toxinas con el fragmento CR12 observamos que las mutaciones generadas en el asa 3 disminuyen de manera importante la unión (Figura 17). Las constantes de afinidad se calcularon haciendo curvas de unión a saturación por ELISA y posteriormente los datos se transformaron con la ecuación de Scatchard para linearizar la curva, el valor de la K*d* corresponde al inverso negativo de la pendiente de la recta obtenida con la ecuación (Figura 17). Estos resultados revelaron que la afinidad de las mutantes MS, MD y MT por el fragmento CR7-CR12 es de aproximadamente 0.2 nM, este valor es igual al obtenido con la toxina Cry1Ab silvestre, en cambio las afinidades hacia el fragmento CR12 disminuyeron de 9.5 nM hasta 820 y 700 nM con las toxinas MS y MD; y de 2,400 nM para la mutante MT. Aunque la afinidad que ha sido reportada hacia el receptor Caderina es de 1 nM, en estos ensayos obtuvimos un incremento de la afinidad de 5 veces para el CR7-CR12, de cualquier manera estos experimentos muestran claramente que las mutaciones generadas en el asa 3 no afectan la unión con el CR7-CR12 y sí con en CR12.

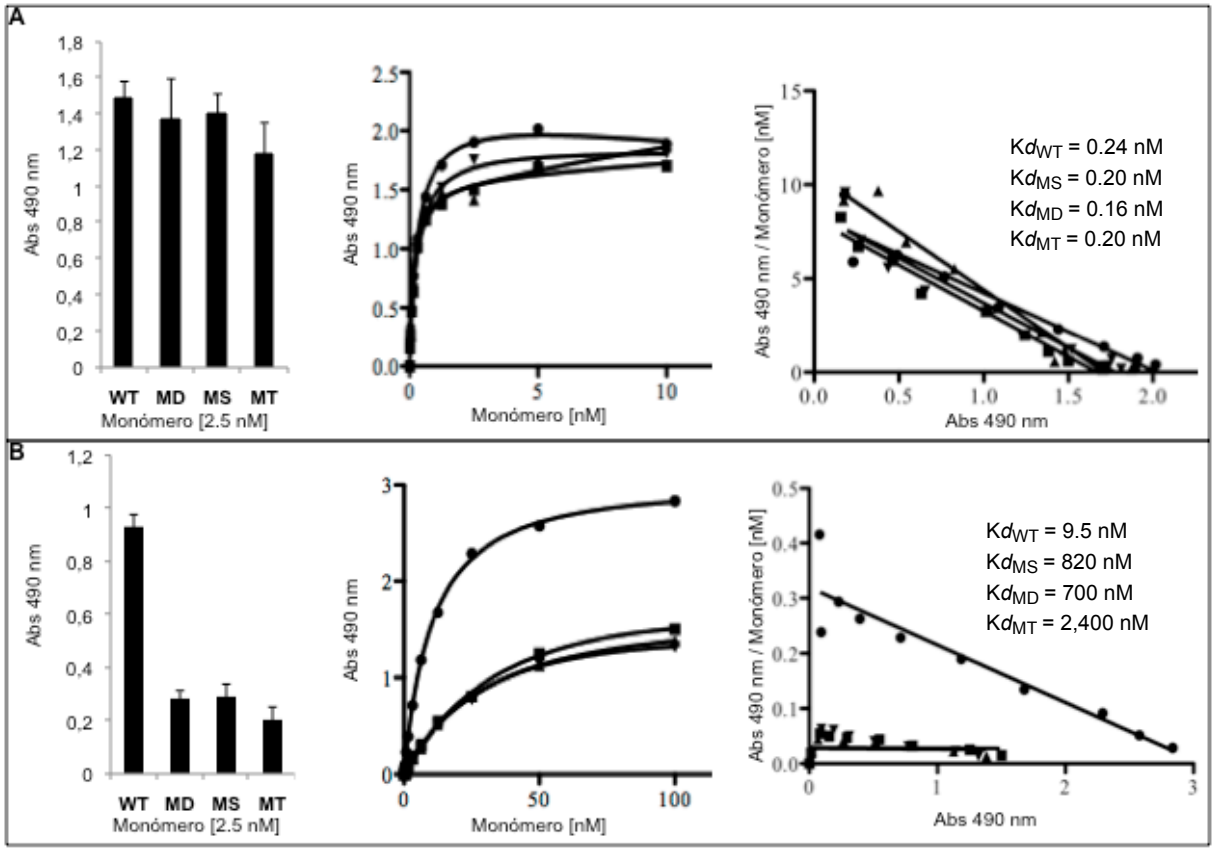

Figura 17. Análisis de unión de las mutantes en el asa 3 de la toxina Cry1Ab en su conformación monomérica a los fragmentos de la Caderina CR7-CR12 (A) y CR12 (B) de *M. sexta*. La gráfica de barras sólidas representa la unión de la toxina a una concentración no saturable. En el gráfico del centro de cada panel se muestran las curvaa de unión a saturacion de las toxinas Cry1Ab (●), MS (■), MD (▲) y MT (▼); la gráfica de la izquierda es la linearización de las curvas con la ecuación de Scatchard (ver Anexo 5).

En la Figura 18 se muestra el análisis de unión mediante un ensayo de ELISA del monómero de la toxina Cry1Ab a la APN de *M. sexta,* y se observa que la toxina se une a este receptor a través del asa 3, ya que las mutantes MS, MD y MT disminuyen su unión hasta en un 75%. La afinidad reportada de las toxinas Cry1A's por la APN es de 100 nM, en este ensayo obtuvimos una afinidad de 85 nM, en cambio para las mutantes MS, MD y MT resultaron de 2.4, 1.2 y 2.0 μM, respetivamente, por lo tanto esta región de las toxinas Cry1A's esta involucrada en el reconocimiento de la APN (Figura 18).

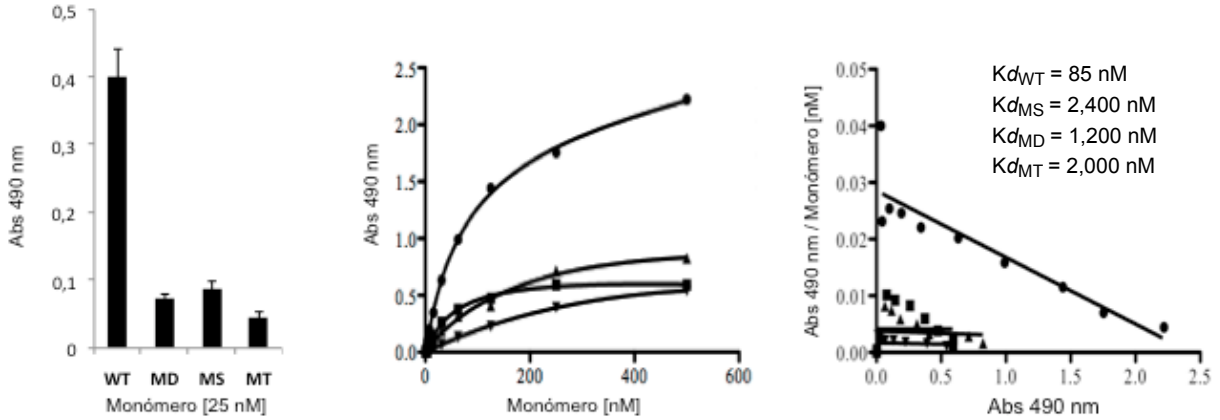

Figura 18. Análisis de unión de las mutantes en el asa 3 de la toxina Cry1Ab en su conformación monomérica al receptor APN de *M. sexta*. Cry1Ab (●), MS (■), MD (▲) y MT (▼).

Por otra parte realizamos ensayos de unión por ELISA del oligómero de las mutantes MS, MD y MT con ambos receptores, previamente se realizó una curva de unión a saturación con el oligómero de la toxina Cry1Ab para trabajar a una concentracion de toxina no-saturable. Los resultados obtenidos revelaron que el oligómero de Cry1Ab se une al receptor Caderina exclusivamente por el asa 3, ya que las mutaciones en al asa 3 afectan la unión tanto al fragmento CR7-CR12 como al CR12. Al analizar la unión de las mutantes MS, MD MT con el la APN se obervó que se unen de manera similar a la toxina silvestre (Figura 19).

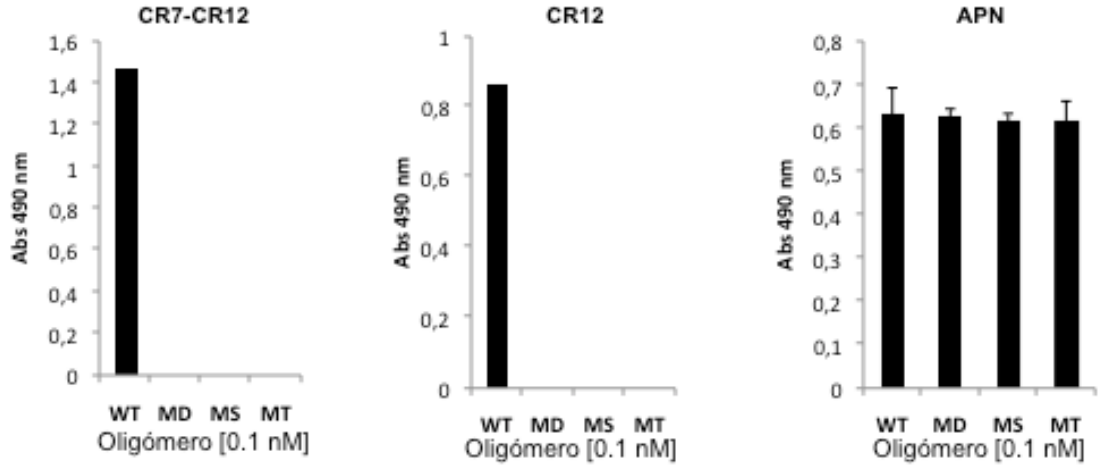

Figura 19. Análisis de unión por ELISA de las mutantes en el asa 3 de la toxina Cry1Ab en su conformación de oligómero a los receptores de *M. sexta*.

VII. DISCUSIÓN

La interacción huésped-patógeno involucra la participación de moléculas que determinan el reconocimiento de la célula blanco, en muchos casos un solo receptor es suficiente para modular esta interacción, sin embargo en algunas ocasiones la participación de más de una molécula es necesaria. Las toxinas Cry de Bt son proteínas formadoras de poros que requieren la participación de dos receptores que son reconocidos de manera secuencial para ejercer su actividad (Bravo, et al., 2002). Durante el proceso de intoxicación en los insectos susceptibles, las toxinas Cry1A's reconocen a una proteína de tipo Caderina, esta interacción promueve el corte proteolítico del α-hélice 1 del Dominio I y favorece un proceso de oligomerización (Gómez, et al., 2002). La estructura oligomérica es una molécula hidrofóbica capaz de insertarse en la membrana, esta inserción es favorecida por la unión a un segundo receptor que puede ser una Aminopeptidasa-N (APN) o Fosfatasa alcalina (ALP) (Pardo-López, et al., 2006). Para que haya una transición de unión de un receptor a otro, debe involucrar la participación de diferentes regiones de la toxina que reconozcan los epítopes de los receptores o bien que haya cambios conformacionales en estas regiones que permitan una unión diferencial.

Previamente se ha establecido que la interacción de las toxinas Cry1A's con la Caderina involucra la participación de varios epítopes. Las asas α-8, 2 y 3 del Dominio II reconocen tres epítopes en el receptor Caderina localizados en el CR7, CR11 y CR12 (Gómez, et al., 2002; Gómez, et al*.,* 2003, Xie, et al., 2005). Por otra parte la unión de la toxina Cry1Ab con la APN de *M. sexta* es mediada por una región del Dominio III, esta región corresponde a la lámina β-16 (Atsumi, et al., 2005, Gómez, et al., 2007). Interesantemente esta región es completamente diferente en la toxina Cry1Ac, con la que comparte un 98% de identidad, sin embargo la interacción con la APN es mediada por una serie de aminoácidos localizados en la misma región que presentan un motivo que reconoce, de manera específica, el azúcar GalNac del receptor (Knowles, et al., 1991). Además también hay evidencia que el Dominio II tiene un papel importante en la unión con la APN, como es el caso de mutaciones en el asa 2 y 3 que afectan la unión a la APN del lepidóptero *L. dispar* (Jenkins, et al., 2000). Sin embargo todos los reportes hechos se han realizado utilizando la toxina en forma monomérica, además la mayor parte de los experimentos realizados ha sido utilizando VMMA de insectos, lo que limita establecer conclusiones sobre a cual de los

dos receptores afecta la unión las mutaciones generadas en las toxinas Cry. Por otra parte no se ha caracterizado la consecuencia de la mutaciones en el proceso de oligomerización ni los patrones de unión del oligómero a los receptores Caderina y APN. En un trabajo previo se observó que dos anticuerpos monoclonales que reconocen las asas 2 y 3 de la toxina Cry1Ab unen de manera preferencial al monómero que al oligómero, lo cual sugiere que, después de la oligomerización, estas regiones importantes para la interacción con el receptor primario sufren cambios conformacionales (Gómez, et al., 2007). Estos datos experimentales marcaron las primeras evidencias sobre la transición de unión de la toxina Cry1Ab hacia ambos receptores.

En este trabajo generamos mutaciones en el asa 3 de la toxina Cry1Ab y analizamos su unión en ambos receptores, probando la unión tanto del oligómero como del monómero. Nuestras mutantes en esta región afectan la unión hacia la APN de *M. sexta* en su forma monomérica, pero no en su conformación oligomérica. En contraste con la unión a la Caderina se observó un comportamiento contrario, el monómero de la mutantes en el asa 3 unen a la Caderina pero no el oligómero. En los experimentos *in vivo* con *M. sexta* revelaron que estas mutaciones afectan la actividad de la toxina en ambas conformaciones, monómero y oligómero, lo cual sugiere que el hecho de afectar su unión a los receptores tiene una consecuencia directa en su actividad *in vivo*.

Las toxinas Cry1A's que carecen del α-hélice 1 del Dominio I diseñadas mediante ingeniería genética (Cry1A'sMod) son capaces de oligomerizar en ausencia del receptor Caderina, y además pueden matar insectos resistentes, cuya tolerancia esta asociada a mutaciones en la Caderina (Soberón, et al., 2007). Estos datos sugieren que en el modo de acción de las toxinas Cry1A'sMod no es necesaria la participación del receptor Caderina, sino que oligomerizan de manera espontánea en el intestino medio de los insectos una vez que son procesadas por las proteasas y entonces el oligómero se une a la APN y ejerce su actividad lítica. Bajo estas observaciones esperábamos que las mutaciones en el asa 3 en el oligómeros de la toxina Cry1Ab afectaran la unión con la APN y con ello su actividad, sin embargo la única diferencia, comparada con la toxina Cry1Ab silvestre, es que ésta última sigue uniendo al receptor Caderina, esta observación es interesante ya que se había sugerido que una vez que se forma el oligómero pierde afinidad por Caderina y la

incrementa por la APN (Bravo, et al., 2004), nosotros observamos que el oligómero sigue unido al primer receptor exclusivamente a través del asa 3. Previamente se observó que podría formarse un complejo formado por Caderina, toxina y APN, ya que cuando se purificaban balsas lipídicas de VMMA de *M. sexta* en presencia de la toxina, podían observarse las tres moléculas en las misma zona de la membrana y no así en ausencia de la toxina, en la que sólo se observa la presencia de la APN (Bravo, et al., 2004). Estos datos sugieren que una vez que la toxina se une a la Caderina es movilizada hacia las balsas lipídicas en donde se encuentra la APN, que finalmente es el sitio donde se inserta para formar un poro, estos datos coinciden con nuestras observaciones de que el oligómero permanece unido a la Caderina, sin embargo no concuerdan con las proteínas Cry1A'sMod, ya que la ausencia de Caderina es irrelevante para ejercer su acción tóxica, por lo que no descartamos que mutaciones en el asa 3 pudiera repercutir en eventos "post-unión".

Varios reportes coinciden en que mutaciones en el asa 3 de las toxinas Cry1A's afectan su actividad *in vivo*, lo cual correlaciona con los resultados obtenidos en este trabajo, por otra parte la unión de la toxina Cry1Ab se ve considerablemente disminuida hacia VMMA de *M. sexta* que previamente fueron tratadas con Fosfolipasa-C para eliminar la APN, aunque el receptor Caderina se encuentra presente, sugiriendo que la unión del monómero a la APN tiene un papel importante en eventos previos a la unión con la Caderina (Bravo, et al., 2004). Por su parte el oligómero se une a la APN posiblemente por una región del Dominio III, que podría ser la región β-16 del Dominio III, o bien por alguna otra región del Dominio II diferentes al asa 3. Aunque no se descarta que se expongan nuevas regiones del Dominio II o III que estén participando en este reconocimiento una vez que la toxina oligomeriza.

Los resultados de este trabajo muestran una compleja interacción secuencial de la toxina Cry1Ab con los receptores Caderina y APN, dependiendo de su estado monomérico u oligomérico, y muestra datos sobre la participación del asa 3 durante esta transición de unión. Puede estar ocurriendo un mecanismo de unión tipo "ping-pong" en el que la primera interacción de la toxina Cry1Ab es con un receptor de baja afinidad, pero muy abundante en las membranas de la célula blanco, como lo es la APN (Anexo 2). Este mecanismo de unión previamente fue propuesto para la toxina Aerolisina producida por *Aeromona spp*., cuyo receptor es el fosfolípido GPI de

proteínas localizadas en la membrana basal de la célula blanco, sin embargo previamente reconoce, con baja afinidad, azúcares de la superficie que le permite localizarse y moverse sobre la membrana debido a su baja constante de disociación (Abrami, et al., 2003). Una vez que la toxinas Cry se localizan sobre la membrana por el reconocimiento de la APN, después se une al receptor Caderina con alta afinidad, esta unión involucra la participación de otras regiones además del asa 3, tales como las asas α-8 y 2, y entonces se desencadena el proceso de oligomerización. Posteriormente el oligómero incrementa su afinidad por la APN, pero permanece unido a la Caderina formando un complejo entre la toxina y los dos receptores cuya función aun es desconocida, finalmente se inserta en la membrana formando poros que lisan a las células del intestino y en consecuencia el insecto muere.

Por otra parte, en estos experimentos resalta el hecho de que una mutación puntual en el receptor Caderina de *M. sexta* tiene como consecuencia la pérdida de unión y por tanto la oligomerización de las toxina Cry1A's. Este efecto ya se había observado en fragmentos recombinantes del receptor Caderina de *H. virescens,* en el que la mutación L1425R abate la unión de la toxina Cry1Ac, esta mutación es consecuencia de un sólo cambio de nucleótido (CTG →CGG) (Xie, et al., 2005). Aunque ya se han seleccionado insectos resistentes con defectos en el gen de la caderina, se ha especulado que el costo adaptativo de estos insectos es alto, probablemente debido a la pérdida de función de esta proteína (Carriere, et al., 2001). Estos insectos contienen codones de paro prematuros en el gen que interrumpen la síntesis de la proteína, o bien deleciones que afectan la integridad y función del receptor (Gahan, et al., 2001; Morin, et al*.,* 2003). Mutaciones puntuales, como las generadas en los fragmentos recombinantes CR7-CR12 de este trabajo, que reduzcan la unión de las toxinas Cry pero que no comprometen su función o estabilidad, podrían conducir al desarrollo de insectos resistentes con proteínas receptoras completamente funcionales que le permitan al insecto sobrevivir en la naturaleza.

En el presente trabajo abordamos este caso estandarizando un sistema de despliegue en fagos de toxinas Cry. Inicialmente el fago M13 se utilizó para desplegar las toxinas Cry1A's por otros grupos y posteriormente se intentó hacerlo en el bacteriófago λ (Marzari, et al., 1997; Kasman, et al., 1998, Vilchez, et al., 2004; Nathan, et al., 2006). En este trabajo desplegamos la toxina Cry1Ac sobre el fago

lítico T7, el éxito de este sistema radicó principalmente en que el gen de la toxina Cry1Ac se insertó dentro del genoma del virus, haciendo una fusión con la proteína de la cápside viral 10B, a diferencia del sistema de despliegue en fagos λ en donde la expresión de la proteína de fusión gpD-Cry1Ac se realizó de manera heteróloga en un vector de expresión (Vilchez, et al., 2004). La toxina Cry1Ac desplegada es completamente funcional, ya que es capaz de reconocer a sus receptores de manera específica y conservan su actividad contra el lepidóptero *M. sexta* (Pacheco, et al., 2006).

Otra parte del proyecto se enfocó en caracterizar los fragmentos recombinantes del receptor Caderina CR7-CR12 y CR12 que contienen mutaciones las cuales afectan su reconocimiento por las toxinas Cry1A's. La importancia *in vivo* de este receptor se ha estudiado en varios insectos mediante ensayos de protección, los cuales consisten en alimentar a los insectos con la toxina en presencia de moléculas que compiten la unión por este receptor y de esta manera inhiben la toxicidad, como pueden ser anticuerpos, fago-péptidos, o bien fragmentos del mismo receptor (Gómez, et al., 2002; Gómez, et al*.,* 2003, Fernández, et al., 2005, Xie, et al., 2005). Sin embargo su papel como receptor es indiscutible por la generación de insectos resistentes debido a mutaciones en esta proteína (Gahan, et al., 2001, Morin, et al., 2003).

Recientemente se publicaron datos que ponen en controversia la utilización de fragmentos del receptor Caderina para proteger a los insectos de la actividad de las toxinas Cry. En estos trabajos se observó que hay un efecto contrario, la presencia de un fragmento recombinante del receptor Caderina de *M. sexta* (CR12-MPED) incrementó la actividad de las toxinas Cry en varios Lepidópteros, argumentando que dichos fragmentos aumentan los sitios de unión a la toxina (Chen, et al., 2006), posteriormente también se reportó que este fenómeno de sinergismo entre las toxinas Cry y fragmentos del receptor Caderina también se presenta en Dípteros y Coleópteros (Hua, et al., 2008; Park, et al., 2009). Cabe señalar que la proporción toxina-receptor es diferente en los trabajos realizados previamente, en donde ven un fenómeno de protección. En la realización de este proyecto observamos que, efectivamente, este aumento de la actividad de las toxinas Cry1A's es dependiente de la proporción Cry1A's-CR's. En proporciones mayores a 1:500 se observa un fenómeno de protección y no así con 1:100, en donde observamos incremento de

actividad. Para ir más allá del simple hecho de que este fenómeno se debe al incremento de sitios de unión, exploramos la posibilidad de que estos fragmentos aumentan el rendimiento de oligomerización. Al analizar la eficiencia de oligomerización de los fragmentos de Caderina de *M. sexta* CR12, CR11 y CR7 observamos una correlación con el aumento de actividad de las toxinas Cry1Ab y Cry1Ac.

Además generamos mutaciones en el epítope de unión localizado en el CR12 y analizamos sus implicaciones en la actividad de las toxina Cry1A's. Como era de esperarse, las mutaciones generadas no aumentaron la actividad de las toxinas Cry1Ab y Cry1Ac hacia *M. sexta* y los fragmentos mutantes del CR12 disminuyen de manera importarte su capacidad de oligomerizar la toxina Cry1Ab, este resultado concuerda con lo reportado por Chen y col. en el que una deleción del epítope de unión en el fragmento CR12-MPED tiene el mismo efecto (Chen, et al., 2007).

El sinergismo entre las toxinas de Bt es una de las estrategias de esta bacteria para incrementar la actividad de sus toxinas Cry y para sobrellevar y evitar el desarrollo de resistencia de insectos. Un ejemplo de esto es la toxina Cyt1Aa, producida por Bt subsp. *israelensis,* la cual sinergiza la actividad de Cry11A, producida por la misma cepa, en Dípteros como *Culex quinquefasciatus* y *A. aegypti* (Wirth, et al., 1997). Este fenómeno se debe a que la toxina Cyt1A funciona como receptor de la toxina Cry11A proporcionando un mayor número de sitios de unión en el insecto; y además favorece la oligomerización de la toxina Cry11A, en consecuencia a ello existe un sinergismo entre ambas toxinas (Pérez, et al., 2005, Pérez, et al., 2007). Resulta atractivo el uso de los CR's de *M. sexta* como potenciadores de la actividad de las toxinas Cry1A's, sin embargo la dependencia de la relación toxina-receptor es una limitante importante.

VIII. CONCLUSIONES

Los receptores descritos para las toxinas Cry1A's son la Caderina y dos proteínas ancladas a la membrana por GPI, que son Aminopeptidasa-N y Fosfatasa alcalina. A estas proteínas se les ha asignado un papel secuencial en el mecanismo de acción, siendo la Caderina el receptor primario y posteriormente la toxina se une a los receptores anclados por GPI. Se ha sugerido que el reconocimiento de ambos receptores es por dos estados estructurales de la toxina; una forma monomérica reconoce al receptor primario y bajo esta interacción, las toxinas Cry1A's son capaces de oligomerizar y entonces se unen al receptor secundario. Sin embargo, la unión diferencial entre ambos receptores hasta ahora había sido poco estudiada.

En este trabajo obtuvimos resultados que muestran que el asa 3 del Dominio II de la toxina Cry1Ab tiene una papel importante en el reconocimiento diferencial de sus receptores, y esta unión es dependiente del estado estructural de la toxina. Mutaciones en esta región afectan su actividad biológica, tanto en su estado monomérico u oligomérico; y correlaciona con la pérdida de unión a los receptores Caderina y Aminopeptidasa-N. Estas mutaciones afectan la unión a la APN y no a la Caderina en su forma de monómero, mientras que en el estado oligomérico afectan la unión con la Caderina y no con la APN. Dado este fenotipo de las mutantes en el asa 3, atribuimos a esta región un papel fundamental en los eventos de unión inicial y tardía en la microvellosidad del intestino medio de *Manduca sexta* durante el proceso de intoxicación.

Adicionalmente, confirmamos que el CR12 de la Caderina de *Manduca sexta* contiene un sitio de unión para el asa 3 de las toxinas Cry1A's, ya que mutaciones en esta región del receptor afectan su interacción con la toxina, y por otra parte mutaciones en el asa 3 de las toxinas Cry1A's afectan su unión al CR12. También demostramos que fragmentos recombinantes del receptor Caderina, que contienen al menos un sitio de unión para las toxinas Cry1A's, incrementan su actividad insecticida; y observamos que este aumento de toxicidad en *Manduca sexta* por las versiones truncadas del receptor, se debe a que incrementan el rendimiento de oligomerización. Con base a las observaciones que se hicieron en este trabajo, proponemos un modelo de acción de las toxinas Cry1A's en el Lepidóptero *Manduca sexta* el cual se esquematiza en el Anexo 2 de este escrito.

IX. PERSPECTIVAS

- Construcción de bibliotecas de variantes de toxinas Cry1A's
- Selección de variantes versus receptores

X. BIBLIOGRAFÍA

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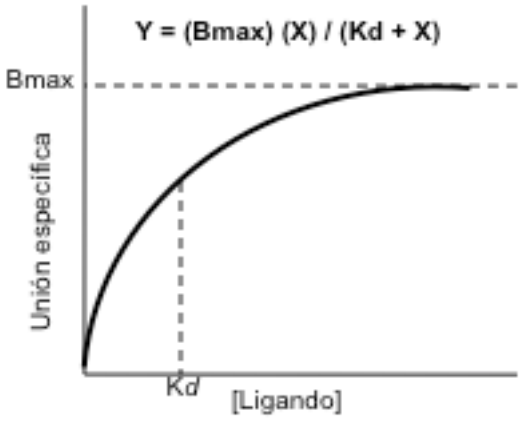
XI. ANEXOS

Anexo 1: Determinación de constantes de afinidad

En un experimento de unión a saturación por ELISA generalmente se varía la concentración del ligando y se mide su unión a un receptor fijo sobre la placa. La finalidad es determinar la K*d* (concentración de ligando necesaria para ocupar la mitad de los sitios de unión del receptor en el equilibrio) y la Bmax (máximo número de sitios de unión). Al graficar los puntos con una función no-lineal obtenemos una hipérbola rectangular en la que el valor de Y = unión específica (Absorbancia, radioactividad, fluorescencia, etc) y X = concentración del ligando:

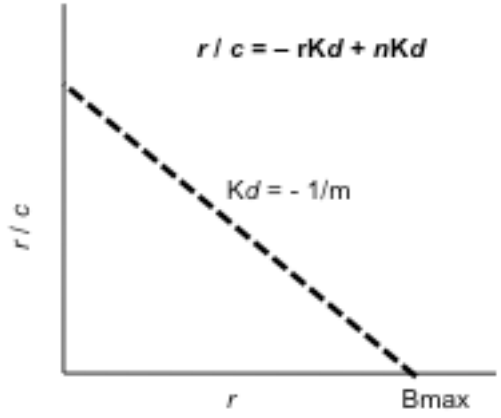


Sin embargo, estimar la EC50 (K*d*) del ligando a partir de una hipérbola resulta difícil y en ocasiones confuso, por lo que se recurre a linearizarla transformando los datos con la ecuación de Scatchard: ***r / c = – rKd + nKd***, donde:

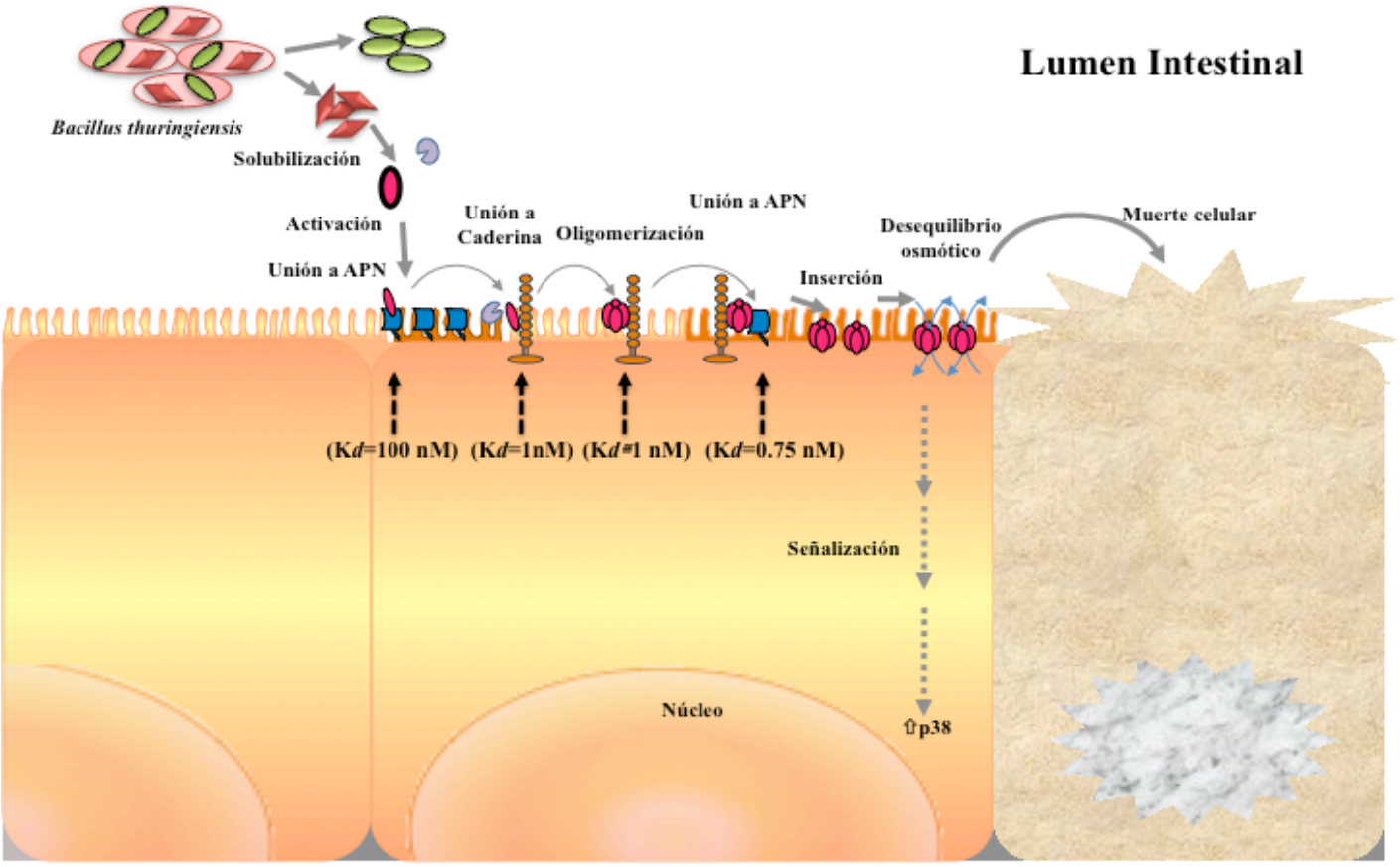
r = Unión específica (Abs)
c = Ligando no unido
n = número de sitios de unión por molécula

y tomando en cuanta que cuando: Y = 0, X = Bmax; y X = 0, Y = *n*K*d*.

La gráfica que se obtiene es una línea recta, donde la K*d* = -1/m:



Anexo 2: Mecanismo de acción de la toxina Cry1Ab en el Lepidóptero *M. sexta*: unión tipo "Ping-Pong" con los receptores APN y Caderina.



Anexo 3: Publicaciones como primer autor en revistas arbitradas

Sabino Pacheco, Isabel Gómez, Ivan Arenas, Gloria Saab-Rincón, Claudia Rodríguez-Almazán, Sarget S. Gill, Alejandra Bravo and Mario Soberón. Domain II loop 3 of *Bacillus thuringiensis* Cry1Ab toxin is involved in a "ping-pong" binding mechanism with *Manduca sexta* aminopetidase-N and cadherin receptors. The Journal of Biological Chemistry. 2009. Nov 20;287(47):32750-7.

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Anexo 4: Publicaciones como co-autor en revistas arbitradas

Angeles Cancino-Rodezno, Cynthia Alexander, Roberto Villaseñor, **Sabino Pacheco**, Helena Porta, Pauchet Yannik, Mario Soberon, Sargeet S. Gill and Alejandra Bravo. The mitogen-activated protein kinase p38 is involved in insect defense against Cry toxins from *Bacillus thuringiensis*. Insect Biochemistry and Molecular Biology. Insect Biochemistry and Molecular Biology. 2010. Jan; 40 (1). 58-63.

Claudia Rodríguez-Almazán, Luis E. Zavala, Carlos Muñoz-Garay, Nuria Jiménez-Juárez, **Sabino Pacheco**, L. Masson, Mario Soberón and Alejandra Bravo. Dominant negative mutants of *Bacillus thuringiensis* Cry1Ab toxin function as anti-toxins: demonstration of the role of oligomerization in toxicity. PLoS ONE. 2009; 4 (5): e5545.

Luisa E. Fernández, Isabel Gómez, **Sabino Pacheco**, Ivan Arenas, Sarget S. Gill, Alejandra Bravo and Mario Soberón. Employing phage display to study the mode of action of *Bacillus thuringiensis* Cry toxins. Peptides. 2008. Feb; 29 (2): 324-329.

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Domain II Loop 3 of *Bacillus thuringiensis* Cry1Ab Toxin Is Involved in a "Ping Pong" Binding Mechanism with *Manduca sexta* Aminopeptidase-N and Cadherin Receptors*

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Bacillus thuringiensis **Cry toxins are used worldwide as insecticides in agriculture, in forestry, and in the control of disease transmission vectors. In the lepidopteran *Manduca sexta*, cadherin (Bt-R_1) and aminopeptidase-N (APN) function as Cry1A toxin receptors. The interaction with Bt-R_1 promotes cleavage of the amino-terminal end, including helix α-1 and formation of prepore oligomer that binds to APN, leading to membrane insertion and pore formation. Loops of domain II of Cry1Ab toxin are involved in receptor interaction. Here we show that Cry1Ab mutants located in domain II loop 3 are affected in binding to both receptors and toxicity against *Manduca sexta* larvae. Interaction with both receptors depends on the oligomeric state of the toxin. Monomers of loop 3 mutants were affected in binding to APN and to a cadherin fragment corresponding to cadherin repeat 12 but not with a fragment comprising cadherin repeats 7–12. In contrast, the oligomers of loop 3 mutants were affected in binding to both Bt-R_1 fragments but not to APN. Toxicity assays showed that either monomeric or oligomeric structures of Cry1Ab loop 3 mutations were severely affected in insecticidal activity. These data suggest that loop 3 is differentially involved in the binding with both receptor molecules, depending on the oligomeric state of the toxin and also that possibly a "ping pong" binding mechanism with both receptors is involved in toxin action.**

Bacillus thuringiensis is a bacterium that produces crystalline inclusions formed by insecticidal proteins, called Cry toxins, during the sporulation phase of growth. Cry toxins are toxic to different insect orders as well as to other invertebrates, such as nematodes, mites, and protozoa (1). Cry toxins have been used worldwide in the control of insect pests in agriculture, either as transgenic crops or as spray formulations.

The molecular mechanism proposed to describe the action of Cry1A toxins, which are active against different lepidopteran insect species, involves several steps. After larval ingestion of the crystalline inclusions, these are solubilized in midgut lumen and activated by proteases releasing a toxic 65-kDa fragment that binds, in a sequential manner, with at least two receptors located in midgut microvilli. The first interaction occurs with cadherin protein (Bt-R_1[2] in the case of *Manduca sexta*). This interaction promotes further proteolytic processing of the N-terminal end, including helix α-1 of the toxin, resulting in the formation of a prepore oligomeric structure (2). The oligomer has higher affinity to secondary receptors, which are anchored by glycosylphosphatidylinositol, such as aminopeptidase-N (APN) or alkaline phosphatase in the case of *M. sexta* or *Heliothis virescens*, respectively (3, 4). Glycosylphosphatidylinositol-anchored receptors are located in specific membrane regions called lipid rafts, where the oligomer inserts into the membrane-forming pores, disrupting the osmotic equilibrium and leading to cell death (1, 5). Although this mechanism of action is generally accepted, it may involve additional binding molecules, such as glycolipids, or more than one glycosylphosphatidylinositol-anchored receptor (5, 6). Furthermore, it was shown that Bt-R_1 that is normally not located in lipid rafts changes its location to lipid rafts after treatment of *M. sexta* microvilli membranes with Cry1Ab protoxin, suggesting that it remains attached to toxin oligomer after binding to APN (3).

The three-dimensional structures of several Cry toxins with different insect specificity have been solved, including Cry1Aa, Cry2Aa, Cry3Aa, Cry3Ba, Cry4Aa, and Cry4Ba (for a review, see Ref. 1). Overall, these proteins show a similar organization in three different domains, suggesting a conserved mode of action as follows. Domain I contains seven α-helixes and is involved in oligomer formation, insertion into the membrane, and pore formation (1, 7); domain II consists of three antiparallel β-sheets, and its structure is the most variable of three domains and is implicated in receptor recognition; and domain III is composed of two antiparallel β-sheets and is also involved in receptor binding (1).

Cry1A toxins bind to cadherin proteins of at least six lepidopteran species, *M. sexta*, *Bombyx mori*, *H. virescens*, *Helicoverpa armigera*, *Pectinophora gossypiella*, and *Ostrinia nubilalis* (8–13). Insect cadherins are composed of an ectodomain

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[2] The abbreviations used are: Bt-R_1, cadherin receptor; APN, aminopeptidase-N; CR, cadherin repeat; BBMV, brush border membrane vesicle(s); ELISA, enzyme-liked immunosorbent assay; scFv, single-chain Fv; MS, single mutant; MD, double mutant; MT, triple mutant; PBS, phosphate-buffered saline; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

formed by 11 or 12 cadherin repeats (CRs), a transmembrane domain, and an intracellular domain (14). The exposed loops of Cry1A domain II have been directly involved in binding with cadherin in *M. sexta*, *H. virescens*, and *B. mori* (15–18). Three Cry1Ab binding sites were mapped in CR7, CR11, and CR12 of the *M. sexta* Bt-R_1 (15, 19). The interaction between Cry1Ab/Cry1Ac toxins and *H. virescens* cadherin was analyzed, showing that loop 3 of domain II binds to CR12 of this receptor (17). Accordingly, loop 3 of Cry1Aa binds the corresponding site of *B. mori* cadherin (Bt-R_{175}) (18). Cry1Ab loop α-8 and loop 2 bind with *M. sexta* Bt-R_1 CR7 and CR11 epitopes (15).

Regarding interaction of Cry1A toxins with the second receptor, Cry1Ac toxin binds to APN by means of domain III, which recognizes *N*-acetylgaloctosamine moieties in the receptor (20). Also, recent reports indicate that the region $\beta16-\beta22$ located in domain III of Cry1Aa and Cry1Ab toxins is involved in the interaction with *B. mori* and *M. sexta* APNs, respectively (16, 21). In addition, domain II is involved in Cry1A toxin interaction with APN because mutations in domain II loop regions that affected toxicity were shown to affect binding of Cry1Ac or Cry1Ab to *M. sexta* and *Lymantria dispar* APNs (22, 23). It is important to mention that these binding studies were performed with monomeric toxins, and these mutations could also affect binding to the cadherin receptor. In another study, scFv antibodies that bind specifically to Cry1Ab domain II loop 2 or loop 3 or to domain III ($\beta16-\beta22$ region) were used to show that these domain II loop regions, in contrast to domain III, undergo a conformational change upon oligomerization, suggesting that this structural change may be involved in the sequential interaction of the Cry1Ab toxin with cadherin and APN receptors (16).

To determine the role of domain II loop 3 residues in the sequential interaction of Cry1Ab toxin with cadherin and APN receptors, we characterized Cry1Ab domain II loop 3 mutants. We analyzed the ability of either monomers or oligomers of these mutants to bind Bt-R_1 and APN and analyzed their effect on toxicity. Our data show that mutations in domain II loop 3 differentially affect the binding with both receptors, depending on the oligomeric state of the toxin. We propose that possibly a "ping pong" binding mechanism with both receptors is involved in toxin action.

MATERIALS AND METHODS

Site-directed Mutagenesis—pHT315-Cry1Ab was used as a template for site-directed loop 3 mutagenesis using QuikChange® Multi following the manufacturer's instructions (Stratagene).

Purification and Activation of Cry1Ab Toxins—The acrystalliferous *B. thuringiensis* strain 407$^-$ was transformed with pHT315-Cry1Ab or the same plasmid containing loop 3 substitutions. *B. thuringiensis* transformant strains were grown for 3 days at 30 °C in LB medium supplemented with 10 μg/ml erythromycin. After sporulation, crystals were purified by sucrose gradients. For monomer production, Cry1Ab crystals were solubilized in alkaline buffer and activated by trypsin as reported previously (24). In order to obtain oligomeric structure, the crystals were activated with 5% *M. sexta* midgut juice in the presence of scFv73 antibody as described (16). The oligomers were purified by size exclusion chromatography with a Superdex 200HR 10/30 (Amersham Biosciences) fast protein liquid chromatography system. Protein concentration was determined by the Bradford assay, using bovine serum albumin as a standard and the extinction coefficient method, where E_M^{280} = 82,280 M^{-1}/cm^{-1} for Cry1Ab toxin.

Expression and Purification of Cadherin Proteins—Fragments of cadherin protein (CR7–CR12 or CR12) were cloned into pET22b vector as reported previously (7, 25, 26). These constructions were transformed into *Escherichia coli* ER2566 cells; protein expression was induced by the addition of 1 mM isopropyl-thio-β-galactopyranoside. Proteins expressed as inclusion bodies were solubilized with 8 M urea solution. Bt-R_1 peptides were purified with nickel affinity columns according to the manufacturer's instructions (Qiagen) and eluted with 250 mM imidazole in PBS buffer to eliminate urea.

Secondary Structure Analysis of CR12 and CR7–CR12 by CD Spectroscopy—CD spectra were recorded with a JASCO model J-715 spectropolarimeter equipped with a Peltier temperature control supplied by Jasco. Spectra were collected from 200 to 240 nm. Buffer conditions were 10 mM potassium phosphate, pH 7.6, and 25 °C. Eight replicate spectra were collected on each sample to improve signal/noise ratio. The final purified protein (CR12 or CR7–CR12) concentration was 0.3 mg/ml, and spectra were collected in a 0.01-cm path length cell. The secondary structure prediction was performed using the CDSSTR algorithm, which requires data from 200 to 240 nm (27–29).

Expression and Purification of scFv Antibodies—The soluble scFv73 and scFv3L3 antibodies were expressed in *E. coli* BL21 (DE3) cells and purified (16, 24).

Preparation of BBMV—Insect midguts of fourth instar *M. sexta* larvae were dissected and used to prepare brush border membrane vesicles by differential precipitation using $MgCl_2$ (30) and stored at −70 °C until use.

Toxin Overlay Assay—One hundred micrograms of BBMV protein were loaded in a single long lane, separated by 9% SDS-PAGE, and electrotransferred to polyvinylidene difluoride membrane (Amersham Biosciences). Single gel blots were incubated with different Cry1Ab proteins using the PR 150 Mini Deca-Probe (Amersham Biosciences) that was designed to incubate each lane of the blot in different conditions, avoiding the need of cutting lanes for different conditions. Ten parallel troughs milled in one side of the upper plate become individual incubation chambers when the unit is assembled. After blocking with PBS-M (PBS, 5% skim milk), the membrane was incubated with 5 nM wild type or mutant Cry1Ab toxins. Unbound toxins were removed by washing the membrane with PBS-T (PBS, 0.1% Tween 20). The membrane was then incubated with rabbit anti-Cry1Ab antibody followed by secondary anti-rabbit antibody conjugated with horseradish peroxidase (Amersham Biosciences). Blots were visualized using luminol (ECL™, Amersham Biosciences).

Binding Assay of Cry1Ab Toxins with BBMV—For binding assays of wild type and mutant Cry1Ab toxins to BBMV, 10 μg of BBMV protein were incubated in binding buffer (PBS, 0.1% bovine serum albumin, 0.1% Tween 20) with 5 nM Cry1Ab or loop 3 mutants. The unbound toxin was removed by centrifugation (10 min at 14,000 rpm). BBMV were washed three times with binding buffer and suspended in 10 μl of PBS, 3 μl of

Laemmli sample buffer 4× (0.125 M Tris/HCl, 4% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.01% bromphenol blue). The samples were separated by 10% SDS-PAGE and electrotransferred to polyvinylidene difluoride membrane. The membrane was revealed using rabbit anti-Cry1Ab (1:5000) and secondary anti-rabbit (1:5000) conjugated with horseradish peroxidase antibodies.

For competition binding assays, Cry1Ab toxin was labeled with biotin using biotinyl-*N*-hydroxysuccinimide ester according to the manufacturer's instructions (Amersham Biosciences). Ten micrograms of BBMV protein were incubated in binding buffer (PBS, 0.1% bovine serum albumin, 0.1% Tween 20) with 5 nM biotinylated Cry1Ab toxin in the presence or absence of a severalfold molar excess of unlabeled wild type or Cry1Ab-activated mutants. The unbound toxin was removed by centrifugation (10 min at 14,000 rpm). BBMV were washed three times with binding buffer and suspended in 10 μl of PBS, 3 μl of Laemmli sample buffer 4×. The samples were separated by 10% SDS-PAGE and electrotransferred to polyvinylidene difluoride membrane. The membrane was incubated with streptavidin-peroxidase conjugate (Amersham Biosciences) for 1 h, and blots were visualized using luminol (Amersham Biosciences).

APN Purification—Aminopeptidase-N was purified from BBMV of fifth instar *M. sexta* larvae as reported (31). BBMV were incubated in solubilization buffer (1% CHAPS, 5 mM EDTA, 0.1 M NaCl, 1 mM phenylmethylsulfonyl fluoride, 20 mM Tris/HCl, pH 8.5) for 2 h at 4 °C. After centrifugation, supernatant was recovered and dialyzed in buffer A (2 mM $MgCl_2$, 2 mM KCl, 20 mM Tris/HCl, pH 8.5). The sample was loaded into an ion exchange column (Mono-Q) connected to a fast protein liquid chromatography system, APN was eluted using a 0–1 M NaCl gradient, and finally the eluted APN was visualized by SDS-PAGE and Western blot. The activity of purified APN was determined using L-leucine-*p*-nitroanilide as substrate as reported previously (32).

ELISA Binding Assay of Cry1Ab Toxins—ELISA plates were coated with 1 μg of either cadherin protein fragment (CR7–CR12 or CR12), APN from *M. sexta*, or scFv antibodies (scFv73 or scFv3L3) in 100 ml of PBS/well overnight at 4 °C. The plates were washed three times with PBS, blocked with 200 μl/well PBS-M for 2 h at 37 °C, and washed three times with PBS. Different non-saturated concentrations of monomeric or oligomeric Cry1Ab were used in the different binding assays as indicated throughout. The unbound toxins were removed by three washes with PBS-T and three washes with PBS. The bound toxins were detected using rabbit anti-Cry1Ab and anti-rabbit conjugated with horseradish peroxidase antibodies. Finally, *ortho*-phenylenediamine (Sigma) plus H_2O_2 was used as substrate for detection. The reaction was stopped adding 50 μl of 1 M H_2SO_4 and measured at 490 nm using an ELISA microplate reader. Data were analyzed using GraphPad Prism software (version 5.0b), and data curves were transformed by the Scatchard equation for obtaining relative binding affinities (K_d).

Insect Bioassay—Bioassays were performed with *M. sexta* neonate larvae by the surface contamination method (2). Different doses of toxin-activated proteins (from 0.1 to 2000 ng/cm^2) were applied onto the diet surface contained in 24-well polystyrene plates (Cell Wells, Corning Glass). A total of 24 larvae/plaque were fed with different doses of trypsin-activated toxins. The plates were incubated at 28 °C with 65 ± 5% relative humidity and a light/dark photoperiod of 16/8 h. Mortality was recorded after 7 days, and the 50% lethal concentration (LC_{50}) was analyzed with Probit software. For single dose assays, 2 ng/cm^2 of pure oligomer or 25 ng/cm^2 of monomer samples were used with 24 larvae per concentration, and mortality was recorded after 5 days.

RESULTS

Construction of Cry1Ab Loop 3 Mutants—Previously, we reported that loop 3 of Cry1Ab toxin showed a hydropathic complementary pattern with the binding epitope of *H. virescens* cadherin, suggesting a putative interaction through a complementary hydropathic profile (17). Here we introduce amino acid substitutions in loop 3 of Cry1Ab toxin designed to disrupt the hydropathic profile of this region. Three mutants were constructed, a single point mutant S446V (named MS), a double mutant S441R/N442V (MD), and the combination of these substitutions in a triple mutant S441R/N442V/S446V (named MT). After mutagenesis, plasmids were sequenced and transformed into an acrystalliferous *B. thuringiensis* strain, and cells were sporulated to produce crystal proteins. The crystalline inclusions produced by the loop 3 mutants, MS, MD, and MT, and the Cry1Ab toxin were solubilized and activated with trypsin. Fig. 1*A* shows that trypsin activation of the loop 3 mutants produced a 65-kDa protein similar to the Cry1Ab toxin. To determine whether these mutations affect oligomer formation we activated mutant protoxins with *M. sexta* midgut juice in the presence of scFv73 antibody. Antibody scFv73 was previously shown to mimic a cadherin binding region and facilitates formation of the oligomer structure when the Cry1Ab protoxin was proteolytically activated in the presence of this molecule (2, 24). Fig. 1*B* shows the Cry1Ab oligomers revealed by Western blot using a Cry1Ab antibody that recognizes both the monomeric and the oligomeric structures. The MS and MD mutants produced 40% lower yields of oligomer than the Cry1Ab protein, as revealed by scanning the optical density of the 250-kDa oligomer band (Fig. 1*B*). However, the MT mutant produced significantly lower levels of oligomer than Cry1Ab or MS and MD proteins (Fig. 1*B*).

To determine if loop 3 mutations affect the structure of other regions of domain II, we analyzed the binding of two anti-domain II antibodies (scFv73 and scFv3L3) that recognize either domain II loop 2 or loop 3, respectively, and compete with binding of Cry1Ab with Bt-R_1 in toxin overlay assays (16, 24, 33). Anti-loop 2 scFv73 antibody bound to the trypsin-activated mutants and to Cry1Ab toxin, indicating the same toxin conformation in this binding epitope (Fig. 1*C*). In contrast, the anti-loop 3 scFv3L3 antibody only recognized the Cry1Ab toxin, indicating that loop 3 mutations specifically affected the binding capacities of the loop 3 amino acid region (Fig. 1*D*).

Binding Assays of Cry1Ab Loop 3 Mutants with M. sexta BBMV Proteins—To determine the effect of loop 3 mutations in binding to both cadherin and aminopeptidase receptors, toxin overlay assays were performed. In these assays, BBMV proteins were separated by SDS-PAGE and blotted to polyvinylidene

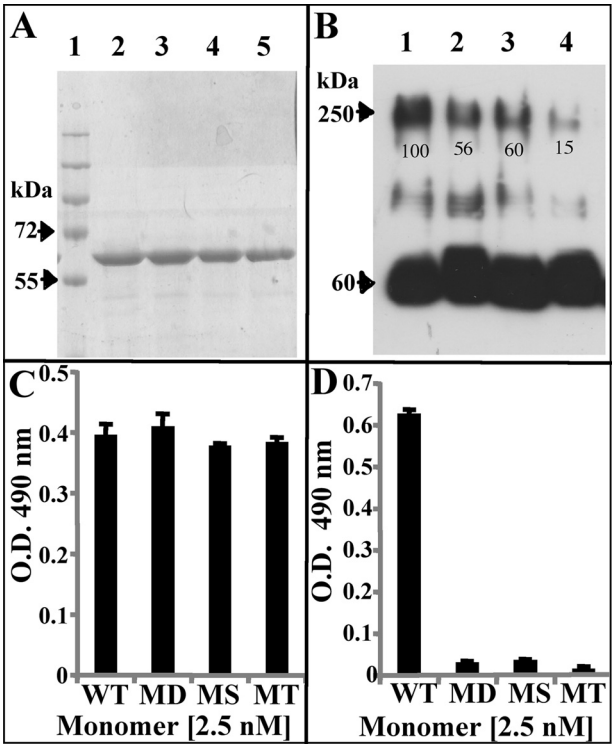


FIGURE 1. **Cry1Ab domain II loop 3 mutants are structurally stable.** *A*, SDS-PAGE electrophoresis pattern of trypsin-activated Cry1Ab (*lane 2*), MS (*lane 3*), MD (*lane 4*), and MT (*lane 5*) toxins. *Lane 1*, molecular weight marker. *B*, Western blot of toxin-activated samples in the presence of scFv73 antibody, Cry1Ab (*lane 1*), MS (*lane 2*), MD (*lane 3*), and MT (*lane 4*). The 250-kDa oligomer and 60-kDa monomer bands are shown. *Numbers below* the 250 kDa band represent the percentage of oligomer concentration after scanning this band. *C* and *D*, ELISA binding analysis of 2.5 nM trypsin-activated Cry1Ab loop 3 mutants to anti-loop 2 scFv73 antibody (*C*) or anti-loop 3 scFv3L3 (*D*).

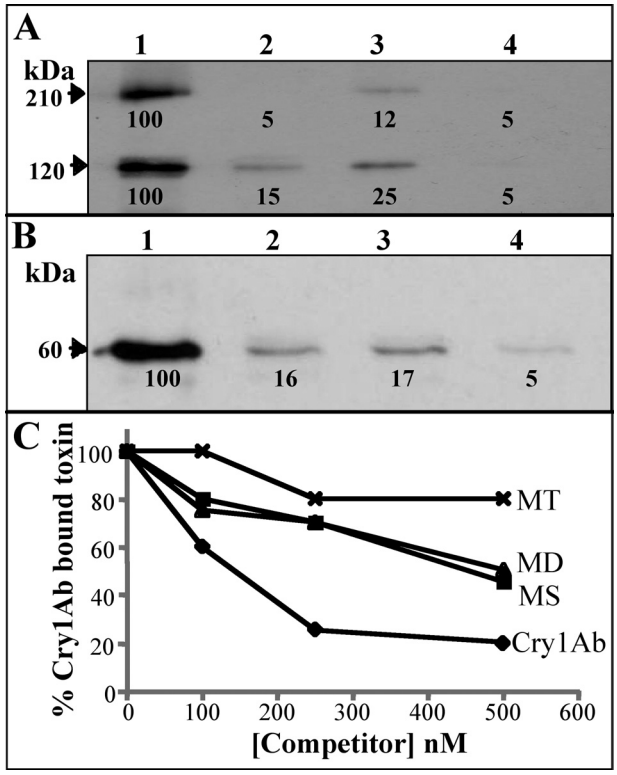
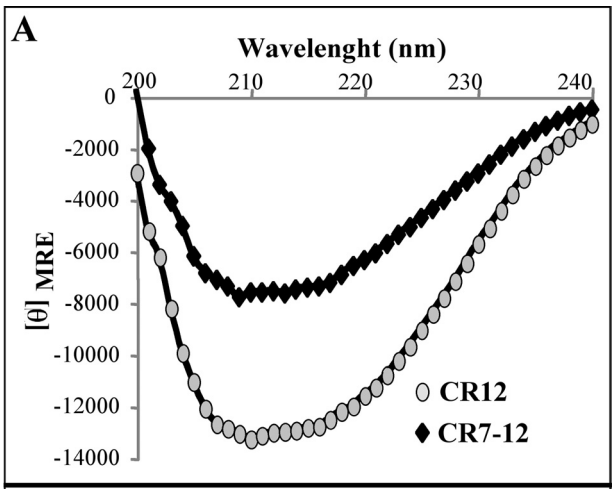


FIGURE 2. **Cry1Ab loop 3 mutants are affected in binding to *M. sexta* BBMV proteins.** *A*, toxin overlay binding assays of Cry1Ab (*lane 1*), MS (*lane 2*), MD (*lane 3*), and MT (*lane 4*) toxins to blotted *M. sexta* BBMV proteins. The 210-kDa protein corresponds to Bt-R_1, and the 120-kDa protein corresponds to APN. The *numbers* represent the percentage of binding after scanning bands. *B*, binding assays of 10 nM Cry1Ab (*lane 1*), MS (*lane 2*), MD (*lane 3*), and MT (*lane 4*) toxins with *M. sexta* BBMV. The 65-kDa protein corresponds to bound toxins recovered after centrifugation of BBMV samples and revealed with antiCry1Ab antibody as described under "Materials and Methods." The *numbers* represent the percentage of binding after scanning bands. *C*, binding competitions of biotinylated Cry1Ab toxin to BBMV using different excess of unlabeled Cry1Ab (♦), MS (■), MD (▲), and MT (×) proteins.

difluoride membranes. BBMV proteins that bind monomeric Cry1Ab toxin were identified after incubation with Cry1Ab proteins and revealed with a polyclonal anti-Cry1Ab antibody. Fig. 2*A* shows that the Cry1Ab monomeric toxin bound with 210- and 120-kDa BBMV proteins that were previously identified as Bt-R_1 and APN, respectively (8, 24, 31). In contrast, loop 3 mutants were affected in binding both 210- and 120-kDa BBMV proteins (Fig. 2*A*). The MT mutant was the most affected because it barely bound to Bt-R_1 or APN (Fig. 2*A*, *lane 4*). These results indicate the loop 3 mutations affect the binding of Cry1Ab to both Bt-R_1 and APN receptor molecules.

To determine the effect of Cry1Ab loop 3 mutations on the binding to *M. sexta* BBMV, Cry1Ab and loop 3 mutant proteins were incubated with BBMV, and bound labeled proteins were observed using an anti-Cry1Ab polyclonal antibody. Fig. 2*B* shows that the three loop 3 mutants were affected in binding to BBMV. Binding competition experiments of biotinylated Cry1Ab to BBMV using Cry1Ab or loop 3 mutants as unlabeled competitors show that the three mutants did not compete with the binding of Cry1Ab, suggesting that they bound BBMV with lower affinity than Cry1Ab and that the MT mutant was the most affected in binding (Fig. 2*C*).

Binding Assays of Cry1Ab Loop 3 Mutants with Bt-R_1 and APN—Toxin overlay assays determined binding of Cry toxins to partially unfolded proteins after SDS-PAGE. To analyze the effect of loop 3 mutations on toxin interaction with Bt-R_1 and APN in native conformation, we performed ELISA binding saturation assays of the activated Cry1Ab, MS, MD, and MT proteins to two cadherin fragment proteins produced in *E. coli* and to APN protein purified from *M. sexta* midgut tissue. Previously, it was shown that the apparent binding dissociation constant of Cry1Ab monomeric toxin with Bt-R_1 was in the range of 1 nM (8). Also, it was shown that binding with APN was in the range of 100 nM (20). We previously cloned and produced in *E. coli* two cadherin fragments corresponding to CR7–CR12 (residues Met^{810}–Ala^{1485}) and to CR12 (residues Gly^{1370}–Ala^{1485}) of *M. sexta* Bt-R1 (7, 25, 26). It was reported that Cry1Ab bound to a cadherin peptide (CR12-MPED) similar to CR12 with a binding affinity of 9.5 nM (34). Fig. 3 shows the analysis of folding of cadherin fragments CR7–CR12 and CR12 by circular dichroism spectra, indicating that both polypeptides have a similar content of α-helices and β-strand structures, with the β-fold structure the most abundant secondary structure. Fig. 4 shows that the Cry1Ab loop 3 mutants were not affected in binding to CR7–CR12 cadherin fragment but were affected in binding to CR12 and APN. Calculation of apparent binding affinities by Scatchard plots (not shown) revealed that Cry1Ab and the three loop 3 mutants bound CR7–CR12 with very high binding affinity (K_d = 0.2 nM). In the case of CR12, the



B

	helix	β-strand	turn	unordered
CR12	18%	31%	26%	21%
CR7-CR12	14%	36%	23%	27%

FIGURE 3. **Analysis of secondary structure composition of cadherin fragments by CD spectra.** *A*, CD spectra of cadherin fragments CR12 (♦) and CR7–CR12 (●). *MRE*, mean residue ellipticity. *B*, prediction of content in percentage of different secondary structures.

MT mutant showed a 250-fold reduction on binding affinity, whereas MT and MD showed a 70- and 80-fold reduction in binding in comparison with Cry1Ab (K_d = 9.5 nM). Finally, the MS, MD, and MT mutants were severely affected upon binding with APN, showing a 28-, 14-, and 23-fold reduction in binding affinity to APN, respectively, in comparison with Cry1Ab (K_d = 85 nM). The apparent binding affinities obtained by the saturation ELISA binding assays are in the range of those previously reported for cadherin fragment CR12-MPED and APN (20, 34). However, binding to CR7–CR12 showed a very high apparent dissociation of 0.2 nM, 5-fold higher than the reported binding affinity of 1 nM for Bt-R_1 (8). In any case, the data clearly show that Cry1Ab loop 3 mutations affected binding with CR12 and APN but not with CR7–CR12.

Interaction of Cry1Ab Loop 3 Mutant Oligomers with M. sexta Bt-R_1—In the proposed model of the mode of action of Cry1Ab, binding of Cry1Ab to Bt-R_1 and APN depends on the oligomeric state of the toxin (3). To determine the effect of the loop 3 mutations characterized here on the binding of the oligomeric structure with both receptors, we performed oligomerization assays of Cry1Ab, MS, MD, and MT proteins and further purified the oligomers by size exclusion chromatography. Despite the lower yields of oligomer formation by the MT mutant (Fig. 1*B*), we obtained sufficient amounts of oligomer proteins (Fig. 5*A*) to perform binding assays with both Bt-R_1 and APN.

We performed ELISA binding assays of both oligomeric and monomeric structures of Cry1Ab proteins with cadherin fragment proteins produced in *E. coli*. Non-saturated concentrations of oligomers were used in the comparison with the binding of the loop 3 mutants as judged by saturation binding assays of Cry1Ab oligomer with both cadherin fragments and APN.[3] The binding of 0.1 nM Cry1Ab and loop 3 mutant oligomers with the cadherin fragments revealed that the loop 3 mutations affected significantly the binding of the oligomer with both CR7–CR12 and CR12 cadherin fragments (Fig. 5, *B* and *C*). As controls, we performed ELISA binding assays using monomers that confirmed that loop 3 mutations affected monomer binding with CR12 but not with CR7–CR12 (Fig. 5, *D* and *E*).

Interaction of Cry1Ab Loop 3 Mutant Oligomers with M. sexta APN—To determine the effect of the loop 3 mutations on the binding of Cry1Ab oligomer with APN, we performed ELISA binding assays with APN purified from *M. sexta* BBMV, using either oligomeric or monomeric structures of each mutant and of Cry1Ab. As above, non-saturated concentrations were used in the ELISA binding assays. Fig. 6*A* shows that 0.1 nM Cry1Ab, MD, MS, and MT oligomers bound similarly to APN protein. In contrast, 25 nM monomeric MD, MS, and MT proteins were affected in binding to APN in contrast to the wild type Cry1Ab monomer (Fig. 6*B*).

Toxicity Effects of Cry1Ab Loop 3 Mutations—We determined the effect of the Cry1Ab loop 3 mutations on toxicity against *M. sexta* larvae. Table 1 shows that the activated MS and MD mutants had a 9-fold reduction in mortality, whereas MT had a 70-fold reduction in mortality when the LC_{50} lethal values were compared with activated Cry1Ab protein. To determine the effect on mortality in the context of both monomeric and oligomeric structures, we performed a single dose toxicity assay of pure monomer or oligomeric structures. Table 1 shows that a single dose of Cry1Ab monomer (25 ng/cm^2) or Cry1Ab oligomer (2 ng/cm^2) resulted in 95% larval mortality. In contrast, the same doses of monomeric and oligomeric structures of the three loop 3 mutants were severely affected in toxicity to *M. sexta* larvae (Table 1).

DISCUSSION

Interaction of pathogens with their target cells involves specific recognition of surface molecules to modulate cell recognition, membrane insertion, or cell internalization. In the case of bacterial pore-forming toxins, the interaction of single receptors seems to be a general strategy, although in the case of several toxins, such as diphtheria, anthrax protective antigen, or aerolysin, more than one surface molecule is involved in the binding and mode of action of these toxins (35–37). With viruses, sequential interaction with several surface molecules is important for infection where structural changes of the viral proteins are involved in target cell interactions (38, 39).

Regarding insecticidal Cry1A toxins, membrane insertion is the result of the sequential interaction with at least two receptor molecules in the lepidopteran *M. sexta*, Bt-R_1 and APN (3). Binding of Cry1Ab toxin with Bt-R_1 facilitates removal of helix α-1, triggering toxin oligomerization (2). The oligomer gains binding affinity to the second receptor, APN, and this interaction enhances insertion of the oligomer into the membrane (3, 40). We previously hypothesized that sequential interaction of the Cry1Ab toxin with the two receptors involves structural changes of binding epitopes upon oligomerization (16). By use

[3] I. Arenas and I. Gómez, unpublished data.

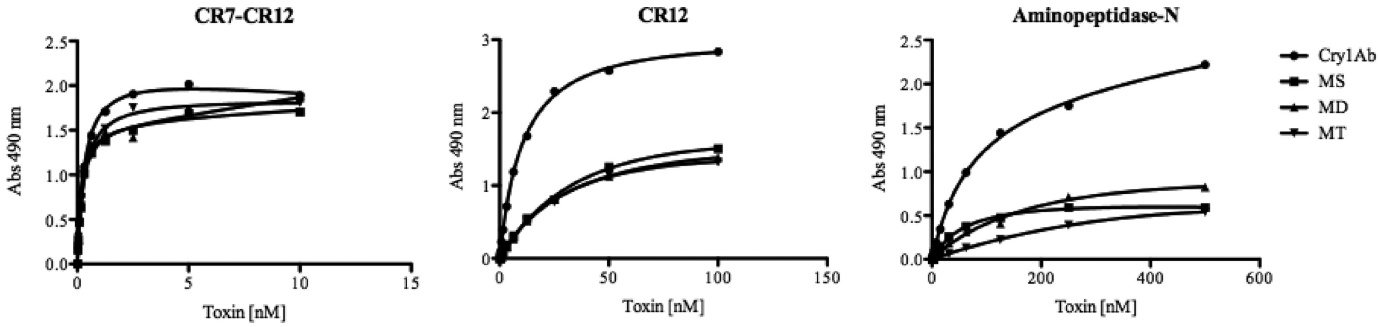


FIGURE 4. **ELISA saturation binding assays of Cry1Ab, MS, MD, and MT toxins to cadherin fragments CR7–CR12, CR12, and APN.**

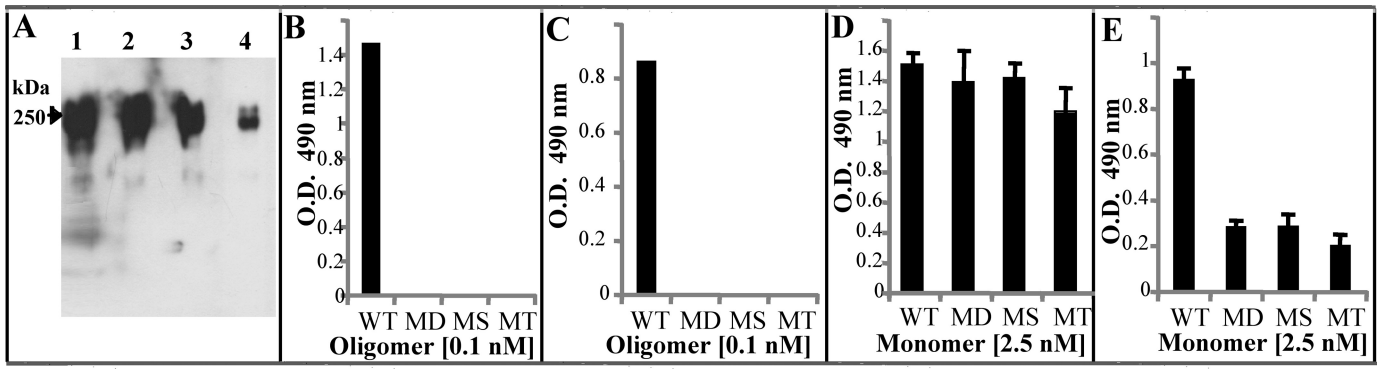


FIGURE 5. **Binding analysis of Cry1Ab and loop 3 mutants to cadherin fragments.** *A*, Western blot of pure oligomer samples obtained after size exclusion chromatography of toxin samples activated in the presence of scFv73 antibody, Cry1Ab (*lane 1*), MS (*lane 2*), MD (*lane 3*), and MT (*lane 4*). *B*, ELISA binding assays of 0.1 nM Cry1Ab (wild type (*WT*)), MS, MD, and MT oligomeric structures to cadherin fragment CR7–CR12. *C*, ELISA binding assays of 0.1 nM Cry1Ab, MS, MD, and MT oligomeric structures to cadherin fragment CR12. *D*, ELISA binding assays of 2.5 nM Cry1Ab, MS, MD, and MT monomeric structures to cadherin fragment CR7-CR12. *E*, ELISA binding assays of 2.5 nM Cry1Ab, MS, MD, and MT monomeric structures to cadherin fragment CR12.

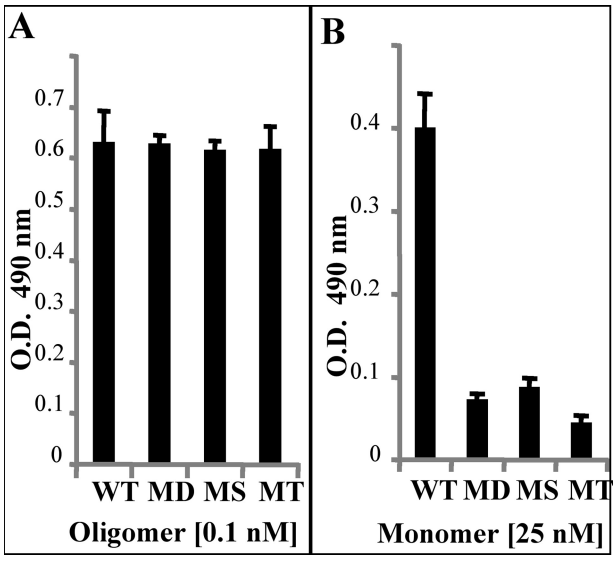


FIGURE 6. **Binding analysis of Cry1Ab and loop 3 mutants to APN.** *A*, ELISA binding assays of 0.1 nM Cry1Ab (wild type (*WT*)), MS, MD, and MT oligomeric structures to APN. *B*, ELISA binding assays of 25 nM Cry1Ab, MS, MD, and MT monomeric structures to APN.

TABLE 1
Toxicity of Cry1Ab toxin to *M. sexta* larvae

	LC_{50}[a]	LC_{50}(mutant)/ LC_{50}(wild type)	Monomer sample (25 ng/cm^2) mortality	Oligomer sample (2 ng/cm^2) mortality
	ng/cm^2		%	%
Cry1Ab	10.3 (8.9–11.9)	ND[b]	95	95
MS	94.7 (73–119.7)	9.19	5	5
MD	91.7 (75.7–113.7)	8.9	5	5
MT	723 (341.1–2148.9)	70.12	0	0

[a] 50% lethal concentration of trypsin-activated Cry1Ab proteins.
[b] ND, not determined.

of scFv antibodies that bind with two different domain II loop regions, loop 2 and loop 3, or with the domain III $\beta16-\beta22$ region, we showed that antibodies to domain II loop recognized preferentially the monomeric structure rather than the oligomer, in contrast to the anti-domain III scFv molecule that recognized equally both structures. This finding suggests a subtle structural change in domain II loop 2 and 3 binding regions upon oligomerization of the toxin (16). Here we show that the loop 3 mutations have a differential effect on binding to both Bt-R_1 and APN, depending on the oligomeric state of the toxin (monomer *versus* oligomer structures). Thus, structural changes that occur upon oligomerization also affect Cry1Ab toxin binding capacities.

In this study, we mutagenized domain II loop 3 to determine its role in the *in vivo* binding to both Bt-R_1 and APN receptors. Published data regarding the role of this binding region in the interaction with both receptor molecules are incomplete because binding of loop 3 mutant oligomers with the two receptor molecules was not previously analyzed. Mutagenesis studies of Cry1Ab and Cry1Ac loop 3 previously performed indicated that this amino acid region was important for binding with *M. sexta* BBMV and toxicity (40, 41). In addition, alanine substitutions of loop 3 residues in Cry1Ab and Cry1Ac toxins showed a correlative effect on APN binding, suggesting that their effects on toxicity were due to defects in this binding (22, 23). However, it was also shown that loop 3 is the cognate bind-

ing region of CR12 in *H. virescens* cadherin and in Bt-R_1 receptors (17) (Fig. 4), suggesting that this toxin region may be involved in the interaction with both Bt-R_1 and APN receptors.

Toxin overlay binding analysis of loop 3 mutants confirmed the hypothesis that this amino acid region may be involved in the interaction with both Bt-R_1 and APN receptors because binding with both of these proteins was greatly reduced, data that correlated with the observed reduced binding with *M. sexta* BBMV. However, binding analysis of monomeric or oligomeric structures to non-denatured Bt-R_1 fragment or APN revealed unexpected results that indicate that the mode of action of Cry1Ab toxin may involve multiple binding interactions with both receptor molecules during the intoxication process. ELISA binding assays showed that loop 3 mutations had a significant effect on the binding of monomeric toxin with APN. In the case of aerolysin, another pore-forming toxin, the first binding event with the target cell involves binding with a high abundance low affinity binding molecule and then with a low abundance but high affinity binding site (37). APN has been shown to be an abundant molecule in *M. sexta* midgut, in contrast with Bt-R_1 that is present in much lower concentrations (42). We therefore speculate that monomeric Cry1Ab binds first with the high abundance low affinity APN site before the high affinity interaction with Bt-R_1.

Regarding the interaction of Cry1Ab monomer with Bt-R_1 receptor, previous work showed that domain II, loop α-8, loop 2, and loop 3 are involved in the binding of Cry1Ab toxin to this receptor (2, 16, 32). Loop 2 was the cognate binding epitope of the CR7 region (^{869}HITDTNNK^{876}) (33); loop α8 and loop 2 interact with the CR11 region (^{1331}IPLPASILTVTV^{1342}) (15), whereas loop 3 binds to the CR12 region of the cadherin receptors in *H. virescens* and *B. mori* (17, 18). Monomeric structures of the three loop 3 mutants characterized here were only affected in the binding with the CR12 fragment of Bt-R_1 and not with a Bt-R_1 fragment corresponding to CR7–CR12 (Fig. 4), confirming that Cry1Ab loop 3 binds CR12 and that binding with the CR7–CR12 fragment also involves loops α-8 and 2.

Loop 3 mutations had no effect on the binding interaction of the Cry1Ab oligomeric structure with APN but had a significant effect on oligomer binding with Bt-R_1 (Figs. 5 and 6). These results suggest that when oligomer is formed after interaction with Bt-R_1, it remains bound with Bt-R_1 through loop 3 and that oligomer interaction with APN involves other regions as previously reported, corresponding to the domain III β16–β-22 region (16, 21). Furthermore, the formation of such a protein complex (Bt-R_1-Cry1Ab-APN complex) was previously suggested because Bt-R_1 was mobilized into lipid rafts after toxin interaction (5). What could be the role of such a protein complex (Bt-R_1-oligomer-APN) in Cry1Ab toxicity? Loop 3 mutations severely affected the toxicity of Cry1Ab oligomer, suggesting that this protein complex may be important for toxicity. However, engineered Cry1Ab- and Cry1Ac-modified toxins (Cry1AbMod and Cry1AcMod) lacking helix α-1 formed oligomers in the absence of cadherin interaction and killed *M. sexta* larvae that were silenced for the cadherin gene, showing that cadherin interaction is not important for toxicity mediated by Cry1Ab oligomer (25). Therefore, the effect of loop 3 mutations on oligomer toxicity should be due to postbinding APN events.

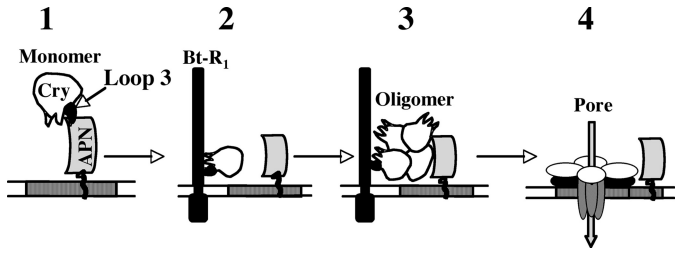


FIGURE 7. **Ping pong binding model of Cry1Ab toxin interaction with APN and Bt-R_1 receptors.** *1*, binding of 65-kDa Cry1Ab monomer to APN through domain II loop 3. *2*, the monomer binds Bt-R_1 through domain II loops α-8, 2, and 3. *3*, formation of a 250-kDa oligomer that binds Bt-R_1 through loop 3 and to APN through domain III. *4*, membrane insertion of the 250-kDa oligomer and pore formation after APN interaction.

Previously, it was shown that the APN binding through *N*-acetylgaloctosamine moieties enhances membrane insertion of Cry1Ac toxin (43). It is possible that domain II loop 3 mutations affect structural changes that are triggered by APN and necessary for membrane insertion of the Cry1Ab oligomer. In this regard, it has been shown that mutations in domain II loop 2 Phe^{371} retain binding with *M. sexta* BBMV but are affected in membrane insertion (44, 45).

The results shown here indicate that Cry1Ab toxin specificity is determined by a complex binding mechanism with two different receptor molecules that depends on the oligomeric state of the toxin. A "ping pong" binding mechanism may occur where domain II loop 3 may be involved in the first binding event with the high abundance low affinity APN receptor. This interaction is followed by a high affinity binding to Bt-R_1 receptor that involves participation of other regions of domain II in addition to loop 3, such as loops α-8 and 2. Interaction with Bt-R_1 triggers cleavage of helix α-1 and oligomer formation. The oligomeric structure gains binding affinity with APN through other regions of the toxin, such as the domain III β16–β22 region, but remains bound with Bt-R_1 through loop 3. Finally, residues in domain II loop 3 may be also involved in post-APN binding events important for toxicity, such as oligomer membrane insertion (Fig. 7).

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Enhancement of insecticidal activity of *Bacillus thuringiensis* Cry1A toxins by fragments of a toxin-binding cadherin correlates with oligomer formation

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ABSTRACT

Cry1A toxins produced by *Bacillus thuringiensis* bind a cadherin receptor that mediates toxicity in different lepidopteran insect larvae. Insect cadherin receptors are modular proteins composed of three domains, the ectodomain formed by 9–12 cadherin repeats (CR), the transmembrane domain and the intracellular domain. Cry1A toxins interact with three regions of the *Manduca sexta* cadherin receptor that are located in CR7, CR11 and CR12 cadherin repeats. Binding of Cry1A toxin to cadherin induces oligomerization of the toxin, which is essential for membrane insertion. Also, it has been reported that cadherin fragments containing the CR12 region enhanced the insecticidal activity of Cry1Ab toxin to *M. sexta* and other lepidopteran larvae. Here we report that cadherin fragments corresponding to CR7 and CR11 regions also enhanced the activity of Cry1Ac and Cry1Ab toxin to *M. sexta* larvae, although not as efficient as the CR12 fragment. A single point mutation in the CR12 region (I1422R) affected Cry1Ac and Cry1Ab binding to the cadherin fragments and did not enhance the activity of Cry1Ab or Cry1Ac toxin in bioassays. Analysis of Cry1Ab *in vitro* oligomer formation in the presence of wild type and mutated cadherin fragments showed a correlation between enhancement of Cry1A toxin activity in bioassays and *in vitro* Cry1Ab-oligomer formation. Our data shows that formation of Cry1A toxin oligomer is in part responsible for the enhancement of Cry1A toxicity by cadherin fragments that is observed *in vivo*.

1. Introduction

Bacillus thuringiensis produces crystalliferous proteins known as δ-endotoxins or Cry toxins with insecticidal activity against different insect orders and nematodes [4]. Different Cry toxins are used worldwide either as sprays or in transgenic plants in the control of insect pests in agriculture and insect vectors of human diseases [4].

The structure of five different Cry toxins and one Cry protoxin – with different insect specificity – belonging to the family of three-domain Cry toxins was solved and shown to share a similar structure [2,3,9,16,20,22]. Domain I is a seven α-helix bundle in which a central helix α-5 is surrounded by six outer helices involved in membrane insertion. Domain II consist of three anti-parallel β-sheets packed around a hydrophobic core in a "beta-prism" and is involved in receptor interaction. Finally, domain III is a β-sandwich of two anti-parallel β-sheets involved in receptor binding [4].

The mode of action of Cry toxins is a multi-step process, involving sequential interaction with at least two different

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receptors and has been studied primarily in lepidopteran insects [5]. Cry1A toxins active against lepidopteran pests are synthesized as 130 kDa protoxins. In order to exert their toxic activity, crystal inclusions are ingested by larvae, dissolved and activated in the midgut lumen by proteases to yield a 60 kDa monomer. The activated toxin binds with high affinity (K_d, 1 nM) to a cadherin receptor (Bt-R_1 in the case of *Manduca sexta*) that is located in the apical membrane of midgut epithelial cells [25]. Binding of Cry1A toxins to cadherin facilitates the proteolytic cleavage from helix α-1 of the N-terminal end of the toxin facilitating the formation a pre-pore oligomeric structure [11]. Engineered Cry1A toxins lacking helix α-1 form oligomers *in vitro* in the absence of cadherin receptor and kill resistant lepidopteran insects that have mutations in the cadherin structural gene [24]. The toxin-oligomer binds to secondary receptors, glycosylphophatydilinositol-anchored proteins such as aminopeptidase-N or alkaline-phosphatase, resulting in the insertion of the pre-pore in membrane lipid rafts leading finally to pore-formation and cell lysis resulting in death of the insect [5,19,23].

A limiting step in the mode of action of Cry1A toxins is the binding to the cadherin receptor. Insect cadherin receptors are modular proteins composed of three domains, the ectodomain formed by 9–12 cadherin repeats (CR) depending on the insect species, the transmembrane domain and the intracellular domain [25]. In different lepidopteran insect colonies that have evolved resistance to Cry1A toxins, the resistance is linked to mutations in the cadherin structural gene [8,21,27,28]. Cry1Ab and Cry1Ac share identical domain II amino acid regions involved in receptor interaction and bind the cadherin receptor by means of domain II loop α-8, loop 2 and loop 3 that interact with amino acid regions of Bt-R_1 receptor located in CR11, CR7 and CR12, respectively [12–14,26]. Of these binding regions, CR12 has been shown to mediate toxicity in cultured insect cells [17]. Interestingly, a CR12 fragment (named CR12-MPED) produced in *E. coli* cells enhanced the activity of Cry1Ab toxin in different lepidopteran insect larvae [6]. It was suggested that the CR12 fragment increases the toxin concentration in the microvillar membrane of the larvae, since this fragment binds this membrane with high affinity (K_d, 32 nM) and was localized in the microvilli of the larvae fed CR12. The Cry toxin would then be able to interact with GPI-anchored Cry1A secondary-receptors [6].

Since binding of Cry1A toxin to cadherin receptor facilitates the formation of the pre-pore oligomer, we hypothesized that enhancement of Cry1A activity by CR12-MPED may be in part due to enhanced oligomer formation. In this article, we analyzed the insecticidal activity of Cry1Ab and Cry1Ac toxin to *M. sexta* larvae in the presence of different cadherin fragments and in mutant cadherin fragments that affected toxin binding. Also we analyzed the *in vitro* oligomer formation in the presence of the different cadherin fragments. Our data shows that the enhanced activity of Cry1A toxin by CR12 correlates with oligomer formation. These data are in agreement with the role of the cadherin receptor in facilitating Cry1A toxin oligomerization and support the hypothesis that the oligomers are essential for toxicity.

2. Methods

2.1. Growth of B. thuringiensis, purification of Cry1A crystal inclusions and toxin activation

Crystal inclusions of Cry1Ab and Cry1Ac were produced in the acrystalliferous Bt strain 407cry$^-$ transformed with the pHT315Ab plasmid harboring the *cry1Ab* gene or with pHT3101 plasmid harboring the *cry*1Ac gene [1] (kindly supplied by Dr. A. Aronson, Purdue University). Bt strains were cultured for 3 days at 29 °C with shaking at 200 rpm in nutrient broth sporulation medium supplemented with 10 μg erythromycin per ml. The crystals of Cry1Ab or Cry1Ac were recovered and purified by sucrose gradients and solubilized in 10 mM carbonate buffer pH 10.5, 0.2% β-mercaptoethanol at 37 °C for 2 h and activated with 1:50 (w/w) TPCK-treated bovine trypsin (Sigma–Aldrich Co.) w/w for 2 h at 25 °C. Protoxin digestion was stopped with 1 mM phenylmethylsulfonyl fluoride (PMSF). Protein concentration was determined using the Bio-Rad Protein assay (Bio-Rad Labs, Hercules, CA).

2.2. Site directed mutagenesis of cadherin fragments

The CR7-12 and CR12 fragments were previously cloned by PCR as described elsewhere [18,24]. Mutagenesis of pET22b plasmids harboring either CR7-12 or CR12 cadherin fragments [18,24] was performed using QuikChange XL-kit (Stratagene, La Jolla, CA). A mutagenic oligonucleotide (5′-CCA CTT CCT GCT TCC CGC CTT ACT GTC ACT GTT AC-3′) was synthesized for I1422R mutant construction. Mutant I1442R was sequenced and transformed into BL21 *E. coli* strain for protein production.

2.3. Expression and purification of cadherin fragments

CR7 and CR11 fragments were amplified by PCR using the pET22b:CR7-12 as template with the following primers which include EcoRI and HindIII restriction sites in 5′ end of the forward or the reverse primers, respectively. For CR7 amplification: CR7For, 5′-TAC AGA ATT CCA TGA TCG ACT TCC TCA CGG GTC AAA TTT CC-3′ and CR7Rev, 5′-TCT TAA GCT TTG CAT AAT TGA TGA CGT AGC TCA C-3′. For CR11 amplification: CR11For, 5′-TAC AGA ATT CAA TGG AAG AGT CTC ACC AAC TTC-3′ and CR11Rev: TCT TAA GCT TTG CAT AAT TGA TGA CGT AGC TCA C-3′. The PCR reactions were performed with Vent-Polymerase (New England BioLabs, Beverly, MA). The PCR products (342 bp for CR7 and 429 bp for CR11) were purified with QIAquick PCR Purification Kit (QIAGEN, Valencia, CA), digested with EcoRI and HindIII (New England BioLabs, Beverly, MA) and ligated into the previously digested vector pET22b (Novagen, EMD Biosciences Inc.). All cadherin fragments including the CR7-12 and CR12 fragments previously cloned [18,24] were purified to homogeneity from BL21 *E. coli* cultures grown at 37 °C in 2× TY (tryptone 1.6% (w/v), yeast extract 1% (w/v) and NaCl 0.5% (w/v), supplemented with 100 μg/ml ampicillin and 0.1% glucose) until they reached an OD of 0.7 at 600 nm. Induction of cadherin fragments was done by adding of 0.5 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) that riggers the *lacZ* promoter, to the culture and grown for 4 h at 25 °C. All cadherin fragments were solubilized from inclusion bodies with 8 M

urea in300 mM Tris–HCl pH 8. The solubilized fragments were applied to a Ni-agarose column (Qiagen, GmbH, Germany), washed with PBS and the cadherin protein fragments eluted with 2 ml of 250 mM imidazole, 0.2% azide in PBS

2.4. Proteolytic activation and oligomer formation assay

For oligomer formation, 1–2 μg of Cry1Ab protoxin was incubated for 1 h with cadherin fragments CR7-12, CR7, CR11 or CR12 (in a mass ratio 1:1), digested 1 h at 37 °C with 5% midgut juice from *M. sexta*. PMSF was added to a final 1 mM concentration to stop proteolysis. The reaction mixture was separated in SDS–PAGE, transferred onto a PVDF nitrocellulose membrane, blocked with skimmed milk (5%) and detected with anti-Cry1Ab polyclonal antiserum (1/30,000; 1 h) and visualized with a goat anti-rabbit antibody coupled with horseradish peroxidase (HR) (Sigma, St. Louis, MO) (1/1000; 1 h), followed by SuperSignal chemiluminescent substrate (Pierce, Rockford, IL) as described by the manufacturers.

2.5. Toxin overlay assays

Toxin overlays to CR7-12 and CR7 (10 μg) loaded in 10% SDS–PAGE were performed as reported [12] using 10 nM biotinylated Cry1Ac or Cry1Ab, and detected with streptavidin-HRP conjugate (1:5000) and SuperSignal chemiluminescent substrate.

2.6. ELISA binding assays

ELISA plates, 96-wells, were incubated 12 h at 4 °C with 0.5 μg/ml of CR7-12 in 50 mM $NaHCO_3$ pH 9.6, followed by five washes with PBS, 0.2% Tween 20. The plates were then incubated with PBS, 0.5% gelatin (BioRad) 0.2%/Tween 20, for 1 h at 37 °C and washed five times with buffer A (PBS, 0.1% Tween 20). The ELISA plates were incubated with 5 nM Cry1Ac for 2 h at 37 °C and washed again with buffer A. The Cry1Ac or Cry1Ab proteins bound to CR7-12 were detected with anti-Cry1Ac or Cry1Ab antibody (1:5000) 2 h at 37 °C, followed by a secondary goat-anti-rabbit-HRP antibody (1:5000) 1 h at 37 °C. The HRP enzymatic activity was revealed with a freshly prepared substrate (40 mg of *o*-phenylenediamine, 18 ml of H_2O_2 in 100 ml of 100 mM NaH_2PO_4, pH 5.0). The enzymatic reaction was stopped with 6N HCl and the absorbance read at 490 nm with a precision microplate reader from Molecular Devices (Sunnyvale, CL).

2.7. Insect bioassays

M. sexta larvae were reared on an artificial diet in 24-well trays (Cell Wells, Corning Glass Works, Corning, NY). Cry1Ac or Cry1Ab activated toxins either at 1 ng or 0.05 ng/cm^2 in the presence of 5 ng/cm^2 of cadherin fragments was applied on the surface of diet and one neonate larvae was placed in each well. Mortality was recorded after 5 days at 28 °C, 65 ± 5% relative humidity and a photoperiod of 16:8 h light:dark. This entire experiment was replicated twice, yielding a total sample size of 48 larvae reared on Cry1Ab or Cry1Ac-treated diet.

3. Results

3.1. Different cadherin fragments enhance Cry1Ac toxicity

Cry1A toxins interact with cadherin through three binding epitopes located in cadherin repeats CR7, CR11 and CR12 [10,12,13,26]. Previously it was demonstrated that a cadherin fragment corresponding to CR12 (CR12-MPED) enhanced the activity of Cry1Ab toxin in different lepidopteran insect larvae [6]. In order to determine if CR7 or CR11 also enhanced Cry1A toxin activity, a bioassay that used sub-lethal doses of Cry1Ab or Cry1Ac (10% mortality) in the presence of three peptides corresponding to these cadherin regions was performed against *M. sexta* larvae. Fig. 1 shows the cadherin protein structure and the cadherin fragments that were characterized in this work. Previously two cadherin fragments, one include from CR7 to CR12 (M810-A1485) and fragment CR12 (G1370-A1485), were cloned into pET22b vector and produced in *E. coli* [18,24]. Using appropriate primers and CR7-12 clone as template, a CR7 (M810-A926) and a CR11 (M1257-S1400) fragments were amplified by PCR and cloned also into pET22b. The four protein fragments, CR7-CR12, CR7, CR11 and CR12, were purified from *E. coli* transformant cells. Fig. 2 shows that all fragments enhanced the activity of Cry1Ab and Cry1Ac toxins. The CR7-12 fragment showed eightfold enhancement of Cry1Ab or Cry1Ac activity. From the three individual fragments we observed that CR12 was the most effective since it enhanced the activity sevenfold followed by CR11 that increased fourfold and CR7 that showed threefold higher activity in response to a sub-lethal dose of both Cry1A toxins.

3.2. A single amino acid change I1422R affects Cry1A binding to cadherin fragments

In order to determine the role of toxin binding in the enhanced activity of Cry1A toxins by cadherin fragments a single point mutation in the CR12 fragment, which affected its interaction with Cry1A was constructed. Previously, extensive mutagenesis of a CR12 amino acid region in the *Heliothis*

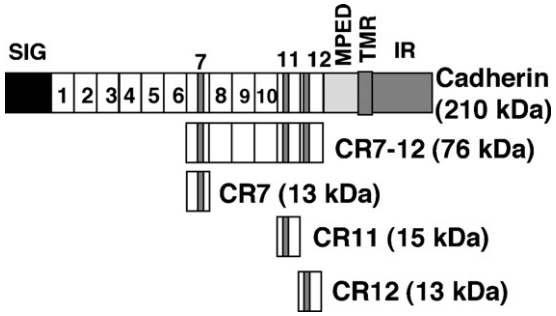

Fig. 1 – Organization of *M. sexta* cadherin Bt-R_1 receptor. Sig is the signal peptide, in numbers are depicted the cadherin repeats (CR) and cadherin repeats 7, 11, 12 show the toxin-binding regions. MPED is the extracellular membrane proximal region, TMR the transmembrane region and IR is the intracellular region. The hatched areas represent the toxin-binding regions. Below the peptides characterized in this work with the molecular weight are shown.

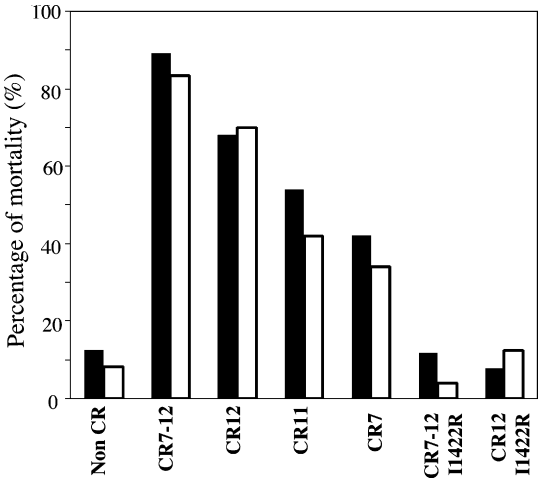

Fig. 2 – Cadherin peptides enhance Cry1Ac (black bars) and Cry1Ab (empty bars) toxins when fed to *M. sexta* larvae. LC10 concentration of Cry1Ac or Cry1Ab toxins (0.05 ng/cm^2 of diet) were mixed with 5 ng/cm^2 of different cadherin peptides corresponding to CR7-12, CR12, CR11, CR7, CR7-12-I1422R and CR12-I1422R. Mortality % of 48 larvae after 5 days in diet. Representative results of two experiments are shown.

virescens cadherin protein revealed a Cry1Ac binding region (^{1423}GVLTLNFQ^{1430}) [26]. Single arginine changes of L1425 and F1429 affected binding of Cry1Ac to a *H. virescens* cadherin fragment containing these mutations [26]. Furthermore a deletion mutant in the *M. sexta* homologous region (^{1416}GVLTNIQ^{1423}) affected binding of Cry1Ab and the synergistic effect of CR12-MPED fragment [6]. We therefore replaced I1422 to arginine in the *M. sexta* CR7-12 and CR12 fragments (see Section 2). Binding analysis of the two cadherin fragments and the corresponding mutant fragments (I1422R) by toxin overlay assays showed that this single amino acid change affected Cry1Ac binding (Fig. 3A and B). ELISA binding assays confirmed that the I1422R mutation affected Cry1Ab and Cry1Ac binding to both cadherin fragments (Fig. 3C).

Bioassays of Cry1Ac toxin in the presence of the mutated cadherin fragments revealed that the I1422R mutant did not enhanced Cry1Ab or Cry1Ac toxicity as the wild type cadherin fragments, CR7-12 (eightfold) or CR12 (sevenfold) (Fig. 2).

3.3. *Oligomerization correlates with synergistic effect of cadherin fragments*

As mentioned previously, binding of Cry1A toxins to cadherin receptors facilitates the formation of a 250 kDa toxin oligomer that is membrane-insertion competent [11]. Cry1Ab toxin oligomerizes *in vitro* when the protoxin is incubated with proteases in the presence of cadherin fragments corresponding to CR7 to CR12, or CR12, while Cry1Ac oligomerization has been observed when activated with midgut proteases in the presence of CR12 only [12–14,23,24]. To determine if oligomer formation correlates with the enhancement of Cry1A toxicity by cadherin fragments, first we compare the *in vitro* oligomerization of Cry1Ab in the presence of each CR7, CR11 and CR12 cadherin protein fragments. Cry1Ab was incubated with *M. sexta* midgut juice as source of proteases and with each of the three cadherin fragments and oligomer formation was observed by Western blot after SDS–PAGE of the activation reactions. Fig. 4A shows that the three cadherin fragments facilitate the formation of the 250 kDa oligomer, although oligomer formation was more efficient in the presence of CR12 than with either CR11 or CR7. Finally we determine if the mutated CR12-I1422R fragment could induce Cry1Ab oligomerization. Fig. 4B shows that a 250 kDa oligomer is observed in the protein sample incubated with the CR12 fragment but not in the sample incubated with the CR12-I1422R protein.

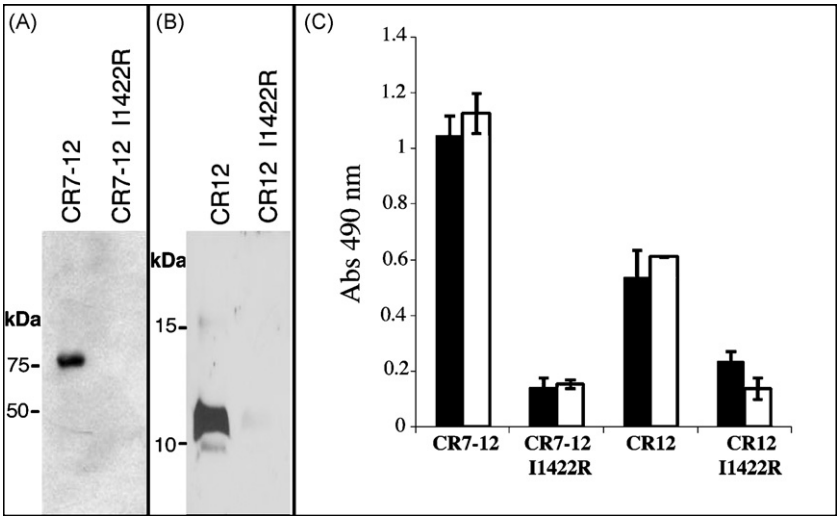

Fig. 3 – Mutation of I1422R in the cadherin fragments affects binding to Cry1Ab and Cry1Ac toxin. (A) Ligand blot of CR7-12 and CR7-12-I1422R peptides with Cry1Ac toxin and revealed with anti-Cry1Ac antibody. (B) Ligand blot of CR12 and CR12-I1423R peptides with Cry1Ac toxin and revealed with anti-Cry1Ac antibody. Similar results were obtained with Cry1Ab toxin (not shown). (C) ELISA binding assay of CR7-12, CR7-12-I1422R and CR12-I1422R with Cry1Ac (black bars) and Cry1Ab (empty bars) toxins revealed with anti-Cry1Ac or anti-Cry1Ab antibodies.

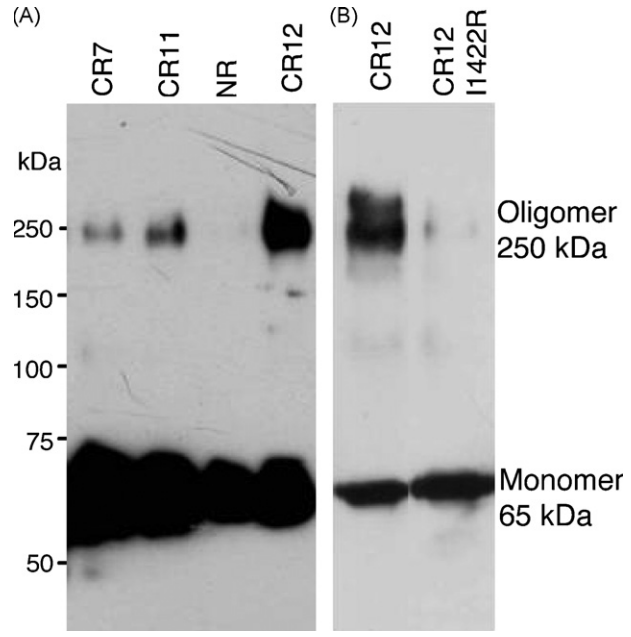

Fig. 4 – Oligomer formation of Cry1Ab protoxin in the presence of different cadherin fragments. (A) Cry1Ab protoxin was incubated with 5% midgut juice in the presence of CR7, CR11 or CR12 and oligomer formation was revealed after SDS–PAGE electrophoresis and Western blot using anti-Cry1Ab antibody. NR is without any CR peptide. (B) Cry1Ab protoxin was incubated with 5% midgut juice in the presence of CR12 and CR12-I1422R and oligomer were revealed as described above.

4. Discussion

Previously, we characterized the Cry1A toxin-binding sites in the *M. sexta* cadherin receptor and found that cadherin fragments corresponding to CR7 (TBR1) and CR11 (TBR2) inhibited Cry1Ab toxin activity in bioassays [13]. Inhibition of Cry1Ab toxicity by TBR1 and TBR2 peptides was achieved using a concentration of the toxin corresponding to a LC90, and 1000–3000 molar excess of cadherin peptides that were purified from soluble fractions [13]. Also, a cadherin peptide containing CR10 to CR12 of the *H. virescens* cadherin inhibited Cry1Ac toxicity in bioassays [26]. Moreover, a *M. sexta* cadherin fragment containing CR11-12 (TBR) was reported to inhibit Cry1Ab activity in bioassays with *M. sexta* [7]. Nevertheless, it was reported that peptide CR12-MPED extracted from *E. coli* inclusion bodies enhanced the activity of Cry1Ab in the same insect species [6]. It was argued that the differences observed in the effect of the TBR peptide could be due to an unfolded nature of proteins that enhance Cry1Ab activity since the proteins were extracted from inclusion bodies and shown to be unfolded in contrast to fragments that are purified from soluble fractions [6]. In the case of the cadherin fragments characterized in this work, all were extracted from inclusion bodies and circular dicroism spectra analysis showed that the CR12 peptides were mostly unfolded (data not shown). Another possibility is that different protein ratios of Cry1A toxins and cadherin protein competitors could have different effects, either inhibiting or enhancing toxicity. In this regard, we found that the CR7-12 peptide did not enhance Cry1Ac toxicity when used at 50 ng/cm^2 (10-fold higher peptide concentration than normal bioassays) was used in the bioassay (data not shown).

Cadherin repeat 12 has been recognized as an important region of cadherin mediating Cry1A toxicity since S2 cultured insect cells expressing cadherin receptor proteins containing CR12 in their surface become sensitive to Cry1Ab toxin [17]. Moreover, a protein fragment corresponding CR12 (CR12-MPED) enhanced activity of Cry1Ab toxin when fed to different insect larvae [6]. Nevertheless, the specific binding site of CR12 that interacts with the Cry1A toxin is in debate since two different regions of this cadherin repeat have been reported. Griko et al. identified a structural binding site in *M. sexta* cadherin formed by the linker region between CR11 and CR12 (amino acids 1349–1374) and the carboxi-terminal part of CR12 (residues 1349–1447) by expressing different protein fragments and showing that both regions are important in binding the Cry1Ab toxin [15]. However, Xie et al. using different cadherin fragments of the *H. virescens* cadherin and single point mutagenesis identified ^{1423}GVLTLNFQ^{1430} as an important Cry1Ac binding epitope [26]. In ageement with Xie et al. [26],a deletion mutant of the corresponding region in the *M. sexta* CR12-MPED fragment (^{1416}GVLTLNIQ^{1423}) did not bind Cry1Ab toxin [6], suggesting that this site is the binding site for Cry1A toxins. In addition, in this work we describe a single point mutation (I1422R) in the *M. sexta* cadherin fragments CR7-12 and CR12 that affects Cry1Ac binding and toxicity, since it did not enhance mortality in response to a sub-lethal dose of Cry1Ac toxin, supporting the ^{1416}GVLTLNIQ^{1423} epitope as a key region in cadherin receptor involved in Cry1Ac binding and toxicity. The CR12 fragment characterized in this study, that binds the Cry1Ab and Cry1Ac toxins and enhanced toxicity, comprised only amino acids G1370–A1485 showing that residues 1349–1369 are not important for Cry1A toxin interaction nor for toxicity. The protein fragments characterized by Griko et al. were fused to the maltose binding protein and this could affect the interaction with Cry1Ab toxin [15]. Although binding to the cadherin receptor is a limiting step en the mode of action of Cry1A toxins in lepidopteran insects, its role in the mode of action of these toxins is still controversial. Two opposing models propose a different role for the cadherin binding in mediating the toxicity of Cry1A toxins. In the pore-forming model, cadherin binding facilitates the proteolytic cleavage of helix α-1 in domain I resulting in the formation of a pre-pore oligomer structure that subsequently binds to GPI-anchored receptors and inserts into membrane causing cell death [5]. In the signaling model, binding of Cry1A to cadherin triggers a signal transduction pathway that leads to oncotic-like cell death [29]. However, recently we demonstrated that engineered Cry1A toxins lacking helix α-1 form oligomers *in vitro* in the absence of cadherin binding and kill resistant insects with either mutations in the cadherin gene or that do not express cadherin protein showing that the main role of cadherin binding is to facilitate oligomer formation [24]. In this article, we show that the enhanced activity of Cry1A toxins in the presence of toxin-binding cadherin fragments correlates with oligomer formation. These data are also consistent with the pore-forming model and argue against the signal transduction model.

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Short communication

Functional display of *Bacillus thuringiensis* Cry1Ac toxin on T7 phage

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Abstract

The Cry1Ac toxin from *Bacillus thuringiensis* was displayed on the surface of T7 phage. The *cry1Ac* gene was fused to the C-terminal end of T7-10B capsid protein and displayed on the surface of T7 phage as revealed by Western blot analysis of the purified phage particles. The T7-Cry1Ac phages retained toxicity against *Manduca sexta* larvae. We demonstrated that the T7-Cry1Ac phage interacts with Cry1Ac receptors present in *M. sexta* BBMV either in solution or in overlay binding assays.

Keywords: Phage-display; Cry-toxins; Insect specificity

Bacillus thuringiensis (Bt) produces insecticidal proteins during sporulation phase as parasporal crystals. These crystals are predominantly comprised of Cry toxin proteins. Cry toxins are specific to their target insect, innocuous to humans, and completely biodegradable. Therefore, Bt is a viable alternative for the control of insect pests in agriculture and against disease vectors of importance in public health.

Numerous Bt strains have been isolated that show activity towards Lepidoptera, Diptera, Coleoptera, Hymenoptera, Homoptera, Orthoptera, and Mallophaga insect orders and to nematodes, mites, and protozoa (Crickmore et al., 1998; de Maagd et al., 2001; Schnepf et al., 1998; Wei et al., 2003). However, there are many insect pests that show no susceptibility to Cry toxins or that are poorly controlled by the available Cry proteins. Also, a major threat for the use of Cry toxins in transgenic plants is the appearance of insect resistance. A single nucleotide change in the *Heliothis virescens* cadherin receptor gene of Cry1A toxins resulted in the lost of toxin binding (Xie et al., 2005). Therefore, the need of genetic evolution of Cry toxins to kill novel targets or to recover toxicity, in the case of the appearance of resistance in the field, is growing.

Cry toxin specificity against different targets has been engineered by two approaches. The first approach was the construction of chimeric genes among different toxins by exchanging domain II or III regions involved in receptor interaction, resulting in chimeric Cry proteins with improved or broader specificity (Bosch et al., 1994; de Maagd et al., 2000; Ge et al., 1989; Lee et al., 1995). The second approach was site-directed mutagenesis that resulted in the production of Cry proteins with improved toxicity to different insects (Rajamohan et al., 1996; Wu et al., 2000). Another alternative is the selection of Cry mutants with improved or changed binding capacities from a pool of variants by phage-display (Kasman et al., 1998; Marzari et al., 1997; Vílchez et al., 2004). Cry1Aa and Cry1Ac toxins, active against lepidopteran insects, have been displayed on filamentous M13 or λ phages. In the case of the M13 studies, the Cry1Aa toxin was not properly displayed resulting in deletions of the fused protein (Marzari et al., 1997). In contrast, the Cry1Ac displayed on M13 retained toxicity to *Manduca sexta* larvae, but did not bind to functional receptors in vitro, suggesting structural constraints of the displayed toxin (Kasman et al., 1998). M13 phage-display system has an intrinsic problem in displaying

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proteins of large size since fusion proteins require to be transported to the periplasm for phage assembly. In contrast, λ phage assembly occurs in the cytoplasm of *Escherichia coli* allowing the display of big proteins. It was demonstrated that the λ-Cry1Ac phage particles were capable to interact with the *M. sexta* aminopeptidase-N receptor (Vílchez et al., 2004). However, the Cry1Ac-gpD capsid fusion protein produced in *E. coli* cells showed 20-fold reduced toxicity probably due to structural constraints of the fusion protein that could affect post-receptor binding steps, as toxin oligomerization and/or toxin membrane insertion (Vílchez et al., 2004).

A T7 phage-display system capable to display proteins of up to 1200 residues was developed (Rosenberg et al., 1996). The T7 Select system (Novagen) uses the T7 capsid proteins (10A and 10B, where 10B is a translational frameshift of 10A) to display proteins on the phage-surface (Rosenberg et al., 1996). T7 phage has some advantages over λ phage since it replicates faster and is stable to harsh conditions that may inactivate other phages, expanding the variety of biopanning selection procedures. Also, the commercial in vitro T7-packaging system (Novagen) produces up to 10^8 recombinant phages per microgram of arms in comparison to 10- to 50-fold lower yield observed with λ phage (Rosenberg et al., 1996). In this work, we show that Cry1Ac toxin could be efficiently displayed on T7 phage surface retaining its capacity to bind to natural receptor molecules and showing toxicity against *M. sexta*. These results show that this system is adequate for the generation of Cry toxin libraries that could be selected by binding procedures.

The *cry1Ac* gene cloned in pHT3101 (Aronson et al., 1995) was used as template for PCR amplification. We used PCR primers containing *Bam*HI and *Not*I restriction sites that amplified a 1.8 kb fragment from residue Gly26 to the end of domain III (Ala614) (Fig. 1 and Table 1), and fused this DNA fragment to 10B gene using T7Select1-1b cloning vector previously digested with *Bam*HI and *Not*I (Rosenberg et al., 1996). The 10B-Cry1Ac construction contained the Cry1Ac amino-terminal trypsin site allowing the release of the Cry1Ac protein by trypsin treatment (Figs. 1 and 2). The ligation mixture was packaged using the commercial packaging extracts (Novagen) and amplified using *E. coli* BLT5403 or BLT5615 strains. These *E. coli* strains produce the capsid 10A protein from an inducible T7 or *lacZ* promoters, respectively, allowing the assembly of phage particles that display the fusion protein in 0.1–1 copy per phage particle (Rosenberg et al., 1996).

The fusion gene *10B-cry1Ac* was detected by PCR using T7 oligonucleotides (Table 1) showing that it has the right size (data not shown). Upon using *E. coli* BLT5615 strain for phage production, a purified phage preparation was obtained after polyethylene glycol precipitation (0.4 M NaCl, PEG 8000 8%, and suspended in 1 M NaCl, 10 mM Tris–HCl, pH 8.0, and 1 mM EDTA). The 100-kDa 10B-Cry1Ac fusion protein was present in the precipitated phages and absent in the remaining supernatant as revealed by Western blot of phage particles after 8% SDS–PAGE using an anti-Cry1Ac polyclonal antibody (1:5000) (Fig. 2A). To further demonstrate that the fusion protein was associated with the phage, the phage particles were purified by CsCl gradient. Western blot of these purified

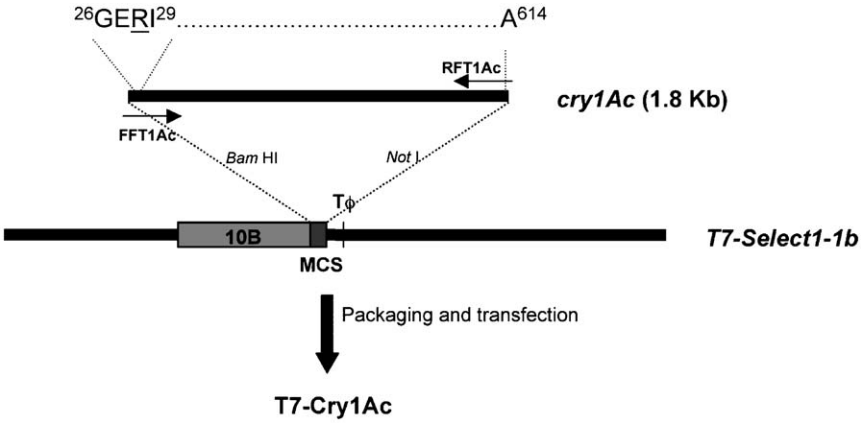

Fig. 1. Cloning strategy for the construction of T7-Cry1Ac phage particles. PCR primers are shown as horizontal arrows. The amino- and carboxy-terminal sequence of Cry1Ac protein is shown above. Underline sequence GERI indicates the trypsin site in the amino-terminal end of Cry1Ac. Restriction sites used for cloning (*Bam*HI and *Not*I) are shown.

Table 1
PCR primers

Primer	Sequence	Reference
FFT1Ac	5′-CGCGGATCCAGGAGAAAGAATAGAAACTGGTTAC-3′	This work
RFT1Ac	5′-$(GA)_{10}$GCGGCCGCAGCCTCGAGTGTTGCAGTAACTG-3′	This work
T7SelectUp	5′-GGAGCTGTCGTATTCCAGTC-3′	Rosenberg et al. (1996)
T7SelectDown	5′-AACCCCTCAAGACCCGTTTA-3′	Rosenberg et al. (1996)

Sequences underlined are restriction sites used for cloning.

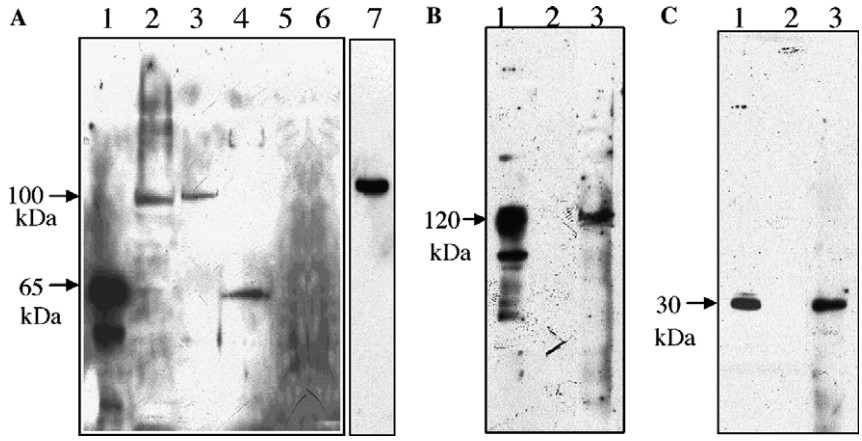

Fig. 2. Display and binding of T7-Cry1Ac phages to Cry1Ac receptors present in *M. sexta*. (A) Immunodetection of Cry1Ac. Lane 1, positive control Cry1Ac toxin; lane 2, T7-Cry1Ac phage after PEG precipitation (2×10^{10} pfu); lane 3, crude lysate of T7-Cry1Ac phage (2×10^{10} pfu); lane 4, trypsin activated T7-Cry1Ac phage particles (2×10^{10} pfu) after PEG purification; lane 5, supernatant of PEG precipitation treatment; lane 6, T7 wt phage particles (2×10^{10} pfu) after PEG precipitation; lane 7, T7-Cry1Ac phage particles (2×10^{10} pfu) after CsCl purification. (B) Overlay binding assays of Cry1Ac toxin and T7-Cry1Ac phage to *M. sexta* brush border membrane vesicles. Lane 1, binding of biotinylated-Cry1Ac toxin revealed with streptavidin; lane 2, binding of T7 wt (5×10^8 pfu) revealed with anti-T7 antibody; lane 3, binding of T7-Cry1Ac (5×10^8 pfu) revealed with anti-T7 antibody. (C) Overlay binding assays of Cry1Ac toxin and T7-Cry1Ac to scFv73 antibody (30 kDa). Lane 1, binding of biotinylated-Cry1Ac toxin revealed with streptavidin; lane 2, binding of T7 wt (5×10^8 pfu) revealed with anti-T7 antibody; lane 3, binding of T7-Cry1Ac (5×10^8 pfu) revealed with anti-T7 antibody. Phage lysates were amplified on *E. coli* BLT5615 strain.

phage also showed the 100-kDa fusion protein using an anti-Cry1Ac antibody (Fig. 2A). Treatment of the T7-Cry1Ac phage particles with trypsin revealed a protein of 65 kDa that corresponds to the size of mature Cry1Ac protein indicating that the trypsin cleavage site between 10B and Cry1Ac is accessible (Fig. 2A). Unexpectedly, when we analyzed the phage lysates from *E. coli* BLT5403 strain, the fusion protein was only observed in the remaining supernatant after phage precipitation with PEG and was not observed in the phage particles fraction (data not shown), suggesting that fusion protein was produced mainly in soluble form. Therefore, all the following experiments were performed with phage lysates amplified in BLT5615 strain.

To determine if the T7-Cry1Ac phage particles were toxic to *M. sexta* larvae, neonate larvae were fed with purified 1.75 pfu $\times 10^{10}$/cm^2 T7-Cry1Ac phage particles or with 1–2 ng/cm^2 Cry1Ac toxin. Phage particles were quantified by plating assays using serial dilutions of the phage lysates and counting plaques on a lawn of BLT5615 in the presence of IPTG. If we assume one copy of Cry1Ac per phage particle, 1.75×10^{10}/cm^2 particles correspond approximately to 2 ng/cm^2 of Cry1Ac toxin. Table 2 shows that T7-Cry1Ac phage preparation was toxic to *M. sexta* similarly as soluble Cry1Ac toxin in contrast to T7 wt phage particles that showed no toxicity. The high toxicity of T7-Cry1Ac particles may be due to the release of mature 65 kDa protein from the phage particles by *M. sexta* midgut proteases.

Table 2
Toxicity of Cry1Ac protein and T7-Cry1Ac phage to *M. sexta* larvae

Treatment	Mortality[a]
Cry1Ac (1 ng/cm^2)	50
Cry1Ac (2 ng/cm^2)	85
T7-Cry1Ac (1.75×10^{10}/cm^2)	100
T7 wt (1.75×10^{10}/cm^2)	0

[a] Percentage of 24 larvae per treatment. Results of three experiment repetitions with a variation of less than 10% are shown.

To analyze the binding of T7-Cry1Ac to receptor molecules, overlay assays were performed. Cry1Ac binds to an aminopeptidase-N (APN) of 120 kDa and to a cadherin-like molecule (Bt-R$_1$) of 210 kDa present in the microvilli of *M. sexta* midgut cells (Knight et al., 1994; Sangadala et al., 1994; Vadlamudi et al., 1995). Brush border membrane vesicles (BBMVs) were prepared as described from isolated midgut cells of fourth instar *M. sexta* larvae (Bravo et al., 2002). Fifty micrograms of protein from the BBMV sample was separated by 8% SDS–PAGE electrophoresis and BBMV proteins were blotted into nitrocellulose membrane. Binding proteins were revealed either by biotinylated-Cry1Ac and streptavidin coupled to peroxidase (1:5000) or with T7-Cry1Ac phage (10^{10} pfu) and anti-T7 antibody coupled to peroxidase (Novagen) (1:5000). Fig. 2B shows that both Cry1Ac toxin and T7-Cry1Ac phage, in contrast to T7 phage particles, bound to the 120 kDa APN protein, in addition Cry1Ac bound to another protein of 75 kDa molecular mass that could be a degradation product of APN. The 210 kDa cadherin receptor protein was not observed in the overlay assays with Cry1Ac toxin, nor with T7-Cry1Ac particles (Fig. 2B). Previously, we characterized an scFv antibody (scFv73) that mimics a Cry1A-binding epitope of the cadherin-like receptor (Gómez et al., 2001). The scFv73 antibody was purified by nickel affinity column as previously described (Gómez et al., 2001), 5 μg of scFv73 was subject to electrophoresis in 8% SDS–PAGE, blotted

Table 3
Recovery of bound phages to *M. sexta* BBMV[a]

Phage	BBMV	BBMV + GalNAc (250 mM)	BBMV + Cry1Ac (1 μM)
T7-Cry1Ac	1.4×10^4	3.5×10^2	3×10^2
T7 wild type	1×10^2	ND	ND

[a] pfu recovered after incubating 10^6 pfu of a T7-Cry1Ac or T7 lysate with 50 μg BBMV. ND, not determined. Results of three experiments with a variation of less than 10% are shown.

to nitrocellulose membrane, and revealed either by biotinylated-Cry1Ac toxin or with T7-Cry1Ac phage (10^{10} pfu) as described above. Fig. 2C shows that biotinylated-Cry1Ac toxin or T7-Cry1Ac phage bound to the 30 kDa scFv73 antibody in contrast to T7 phage particles. These results suggest that the Cry1Ac toxin displayed on T7 phage is able to bind to the natural receptor molecules, APN and cadherin.

In the case of Cry1Ac displayed on λ phage, it was not possible to observe differential binding of λ-Cry1Ac and λ-wild-type phage to *M. sexta* BBMV suggesting non-specific interaction of λ phage with BBMV (Vílchez et al., 2004). Binding of λ-Cry1Ac phage to membrane proteins was demonstrated after solubilization of BBMV proteins and coating ELISA plates (Vílchez et al., 2004). To determine if T7-Cry1Ac phage particles could recognize the receptor molecules in the presence of membranes, binding of T7-Cry1Ac phage to *M. sexta* BBMV was performed in solution. BBMVs (50 μg protein) were incubated 1 h with 10^6 pfu in washing buffer (PBS, Tween 0.1%, and 1.5 M NaCl), centrifuged, washed five times with washing buffer and two times with PBS, and used for infection of *E. coli* BLT5615 cells. In the case of T7-Cry1Ac phage, 1.4×10^4 phage particles were recovered in contrast to 100 phage particles with T7 wild-type phage (Table 3). However, non-specific binding of T7 to BBMV was observed when binding was performed without NaCl in the washing buffer (1.5×10^3 pfu for T7 and 1×10^3 pfu for T7-Cry1Ac). The binding of λ-Cry1Ac phage to 300 μg of *M. sexta* BBMV proteins in ELISA plates yielded only 3×10^2 phage particles (Vílchez et al., 2004). It should be interesting to compare T7-Cry1Ac and λ-Cry1Ac particles in similar binding conditions, like binding to BBMV in the presence of 1.5 M NaCl. Finally, to determine the specificity of binding, binding of 10^6 T7-Cry1Ac pfu to BBMV was competed with Cry1Ac toxin or with *N*-acetylgalactosamine (GalNAc) that has been previously shown to be a binding determinant of Cry1Ac domain III to APN (Burton et al., 1999). Table 3 shows that binding of T7-Cry1Ac to *M. sexta* BBMV was competed with Cry1Ac or with GalNAc.

T7 seems to be an efficient vector for displaying Cry1A toxins. T7 in vitro packaging system gives high yields of phage particles, and we showed that T7 phage could be used in the presence of BBMV isolated from *M. sexta* larvae without non-specific binding. Additionally, the availability of commercial cloning and packaging systems makes T7 phage a good choice for displaying different Cry proteins. This system may allow the identification of novel Cry toxins with desirable binding properties from a pool of variants of the toxin displayed on the phage particles.

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The mitogen-activated protein kinase p38 is involved in insect defense against Cry toxins from *Bacillus thuringiensis*

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ABSTRACT

The insecticidal Cry toxins are pore-forming toxins produced by the bacteria *Bacillus thuringiensis* that disrupt insect-midgut cells. In this work we analyzed the response of two different insect orders, the Lepidopteran *Manduca sexta* and Dipteran *Aedes aegypti* to highly specific Cry toxins, Cry1Ab and Cry11Aa, respectively. One pathway activated in different organisms in response to a variety of pore-forming toxins is the mitogen-activated protein kinase p38 pathway (MAPK p38) that activates a complex defense response. We analyzed the MAPK p38 activation by immunodetection of its phosphorylated isoform, and the induction of p38 by RT-PCR, real-time PCR quantitative assays and immunodetection. We show that MAPK p38 is activated at postraductional level after Cry toxin intoxication in both insect orders. We detected the p38 induction at the transcriptional and traductional level, and observed a different response. In these three levels, we found that both insects respond to Cry toxin action but *M. sexta* responses more strongly than *A. aegypti*. Gene silencing of MAPK p38 *in vivo*, resulted in both insect species becoming hypersensitive to Cry toxin action, suggesting that the MAPK p38 pathway is involved in insect defense against Bt Cry toxins. This finding may have biotechnological applications for enhancing the activity of some Bt Cry toxins against specific insect pests.

1. Introduction

Pore-forming toxins (PFT) are important virulent factors produced by several pathogenic bacteria. Attack by PFT represents a fundamental threat to the host after infection and consequently general host defense responses have evolved against PFT. Among them, the mitogen-activated protein kinase (MAPK) p38 pathway has been recognized as an important player, triggering survival responses in several cell types after treatment with different PFT. MAPK p38 activation after treatment with Cry (a PFT) was first described in *Caenorhabditis elegans* treated with the *Bacillus thuringiensis* (Bt) Cry5B toxin (Huffman et al., 2004). Several works in other studied mammalian models showed that other PFT such as aerolysin (Huffman et al., 2004), pneumolysin (PLY), streptolysin O (SLO), α-hemolysin (Hla), and anthrolysin O produced by different bacteria, when assayed at low doses in cultured-epithelial cell lines induced the activation of MAPK p38 pathway (Ratner et al., 2006). The pore-formation activity of PFT seems to play an important role since toxin deficient mutants or single-point mutations in toxin regions essential for pore-formation activity were unable to induce the MAPK p38 response, suggesting that the observed phosphorylation of MAPK p38 protein correlated with formation of – at least – few pores in the membrane (Ratner et al., 2006). Recently, it was shown that loss of K^+ ions is likely involved in inducing activation of MAPK p38 as a response to α-toxin, *Vibrio cholera* cytolysin (VCC), SLO or *Escherichia coli* hemolysin (HlyA) (Kloft et al., 2009).

Regarding downstream responses induced after activation of MAPK p38, it was described that one of the targets of MAPK p38 in the nematode *C. elegans* was *ttm-1* gene, an orthologue of a human divalent cation transporter, suggesting that up regulation of an efflux transporter may be important in removing cytotoxic cations from the cytosol (Huffman et al., 2004). Later, it was shown that the endoplasmic reticulum stress response to unfolded proteins (UPR) was also induced in *C. elegans* and in HeLa cells, as a downstream

Abbreviations: PFT, Pore-forming toxins; MAPK, mitogen-activated protein kinase; PLY, pneumolysin; SLO, streptolysin O; Hla, α-hemolysin; UPR, endoplasmic reticulum stress response to unfolded proteins; LLO, listeriolysin; TNF-α, tumor necrosis factor; LC_{50}, 50% lethal concentration; RNAi, RNA interference.

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response induced after activation of MAPK p38 by two different PFT. This pathway protects cells from accumulation of unfolded proteins and increases phospholipid biogenesis to defend cells against these toxins (Bischof et al., 2008). In mast-cells, low doses of SLO or listeriolysin (LLO) activates the MAPK p38 pathway resulting in up regulation of cytokines mRNA expression such as tumor necrosis factor alpha (TNF-α). This cytokine plays an important role in host defense in the murine model, recruiting inflammatory cells critical for innate and adaptive immunity (Gekara et al., 2007; Stassen et al., 2003).

Most of these studies were done in cultured cells not in complete animals, the only exception being studies performed with *C. elegans*. In insects, the responses to PFT Cry toxins produced by *B. thuringiensis* and specifically the participation of MAPK p38 pathway during Cry toxin intoxication have not been described. The mechanism of action of Cry1A toxins in insect larvae involves sequential interaction with several receptors, toxin oligomerization and pore formation in the apical membrane of larval midgut cells causing osmotic shock, cell lysis and insect death (Bravo et al., 2004, 2007). Since it was shown that Cry5B toxin induced a defense response in the nematode *C. elegans* (Huffman et al., 2004), we hypothesized that other Cry toxins may induce a similar response in insects. Therefore, we analyzed the response of two different insect orders, *Manduca sexta* as a model of Lepidopteran insects and *Aedes aegypti* as a model of Dipteran insects, after intoxication with specific Cry toxins. We set up conditions for an effective RNA interference analysis by feeding dsRNA to larvae and demonstrated that the MAPK p38 pathway plays a protective role *in vivo* against Cry toxins action in both insect orders.

2. Materials and methods

2.1. Cry toxin purification

Bt strains harboring pHT315-Cry1Ab [8] or pCG6-Cry11Aa (Chang et al., 1993; Wu et al., 1994) plasmids were grown at 30 °C in nutrient broth sporulation medium with 10 μg/ml erythromycin until complete sporulation (Meza et al., 1996). Crystal inclusions were observed under phase contrast microscopy and purified by sucrose gradients (Thomas and Ellar, 1983). As control we used Cry1Ab-R99E (Jiménez-Juárez et al., 2007) and Cry11Aa-R90E (Muñoz-Garay et al., 2009), two different helix α-3 point mutants that were non-toxic to their corresponding insect-targets and were reported to be affected in oligomerization and pore-formation activity.

2.2. Bioassays

For bioassays using *M. sexta* larvae spore-crystals suspensions of wild type and mutant Cry1Ab (from 0 to 2000 ng/cm^2) were applied onto the diet surface in 24-well plates as described (Gómez et al., 2002). For clarity each well in the plate has a surface of 2 cm^2, we applied a volume of 35 μl per well containing the different toxin concentrations and wait until the surface is complete dry, then one larva was added per well and 24 larvae per dose. Mortality was recorded after seven days and lethal concentration (LC_{50}) estimated by Probit analysis (Polo-PC LeOra Software). Protein concentration of spore-crystal preparation was determined by the Bradford assay. For bioassays using *A. aegypti* larvae wild type and mutant Cry11Aa spore-crystal suspensions (from 0 to 10,000 ng/ml) were directly added to 100 ml H_2O. Ten fourth instar larvae were added. Mortality was recorded after 24 h and LC_{50} values and protein concentration were determined as above.

For insect intoxication assays we used second instar *M. sexta* larvae fed with different concentrations (from 0 to 20 ng/cm^2) of Cry1Ab crystal-spore suspension for different times (from 30 min to 7 days). For *A. aegypti* experiments, 4th instar larvae were feed with 0–2500 ng/ml Cry11Aa crystal-spore suspension during different times (from 30 min to 24 h). Larval midguts were dissected from survivors to obtain total RNA and protein samples for RT-PCR or Western blot assays, respectively.

2.3. Western blot assays

Total protein samples were prepared from isolated *M. sexta* midguts. Midguts were homogenized in CelLytic M Cell Lysis reagent (Sigma) supplemented with protease inhibitors (Complete; Roche) and 1 mM NaVo, 20 mM NaF phosphatase inhibitors (Sigma) at 4 °C. Total protein extracts from *A. aegypti* midguts were suspended and homogenized in lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 40 mM DTT) supplemented with protease complete inhibitors and phosphatase inhibitors as described above at 4 °C. 20 μg of protein sample from *M. sexta* or 25 μg of protein sample from *A. aegypti* larvae were boiled 5 min in Laemmli sample loading buffer, separated in SDS-PAGE and electrotransferred onto a nitrocellulose membrane. The proteins were detected by Western blot using commercial antibodies: monoclonal antibody M8432 to non activated MAPK p38 (dilution 1:15,000) (Sigma); monoclonal antibody M8177 to activated p38 MAPK (phosphorylated) (dilution 1:5000) (Sigma); anti phosphorylated p38 MAPK (Thr180/Tyr 182) antibody 9215 (Dilution 1:500) (Cell Signaling); mouse anti-tubulin 180092 antibody (dilution 1:3000) (Zymed). HRP-coupled rabbit anti-mouse IgG (dilution 1:15,000) (Zymed) or goat anti-rabbit IgG (dilution 1:10,000) (Invitrogen) were used as secondary antibodies. Results are representative experiments of three repetitions. Blots were revealed with luminol (ECL; Amersham Pharmacia Biotech) as described by the manufacturers. Molecular weight markers used in all SDS-PAGE were precision pre-stained plus standards all blue (BioRad, Hercules CA).

2.4. RT-PCR and real-time PCR

Total RNA was isolated using the RNeasy Kit (Qiagen). One microgram of total RNA was used for RT-PCR amplification using a First Strand cDNA Synthesis Kit for RT-PCR (AMV, Roche). Specific oligonucleotides (Table 1) were designed to amplify *M. sexta* MAPK p38 (Ms-p38-F and Ms-p38-R) based on larval midgut ESTs generated using 454 pyro-sequencing (Pauchet et al., 2009). Specific oligonucleotides for MAPK p38 from *A. aegypti* (Ae-p38-F and Ae-p38-R) were designed based on the genome sequence accession number AAEL008379. Actin and RPS3 genes from *M. sexta* (accession numbers L13764 and U12708, respectively) were amplified as controls using Ms-actin and Ms-RPS3 primers described in Table 1. Four replicates were performed with independent biological samples. Real-time PCR was performed by Eurofins MWG GmbH Germany, on an ABI 7000 Instrument using Amplifluor Detection System (Serologicals, Clarkston, GA). *M. sexta*

Table 1
Sequences of oligonucleotides used for transcript amplification.

	Oligonucleotide sequence
Ms-p38-F	5′ CCC AAG CTT CGG CCA TAG ACA CTT TGC ATA ATA TG
Ms-p38-R	5′ CCG GAA TTC CCG ATT ACA TTT TCA TGG TTC
Ae-p38-F	5′ GAA GAT CTT CGG AAA TCA TGC TCA ACT
Ae-p38-R	5′ GAA GAT CTT CGG GAC GAA ATT AAG TAC
Ms-actin-F	5′ GTC GAC AAT GGC TCC GGC ATG TGC A
Ms-actin-R	5′ TCT GGG TCA TCT TTT CCC TGT TGG CCT TGG GGT
Ms-RPS3-F	5′ CCG ATC GGA GAT CAT CAT CAT GGC C
Ms-RPS3-R	5′ CAT CCG CGA GTT GAC TTC GGT CGTC

were fed 2.5 ng/cm^2 toxin during different times from 30 min to 7 days. Actin and RPS3 were used as real-time PCR normalization controls. Quadruplicate rounds of quantitative real-time PCR were performed.

2.5. RNA interference (RNAi) assays

MAPK p38 cDNA fragments were amplified from *M. sexta* and *A. aegypti* as stated above and cloned into a TOPO cloning vector using TOPO TA cloning kit (Invitrogen) and subcloned into pLitmus28i vector (HiScribeTM, New England Biolabs, Beverly, MA) containing two T7 promoters flanking the multi-cloning site. These promoters enabled amplification of the cloned fragment by using a T7 oligonucleotide. The PCR product was purified with QIAquick PCR purification kit (Qiagen Valencia, CA). *In vitro* transcription of both DNA strands of the insert was performed with T7 RNA polymerase using the HiScribe RNAi Transcription Kit (New England Biolabs) as reported by the manufacturer, yielding dsRNA.

One hundred *M. sexta* neonates were fed with a single dose of 1 μl drop per larvae containing 5 μg Ms-p38 dsRNA. Each neonate *M. sexta* was located just in front of the drop. We were careful to observe that each larva ingested the μl drop, and then they were transferred to artificial diet until they reached third instar for midgut dissection.

For gene silencing in *A. aegypti*, 200 neonates were fed for 16 h with 200 μg of dsRNA previously encapsulated in Effectene transfection reagent (Qiagen). For encapsulating the dsRNA in Effectene, 200 μg of dsRNA in a final volume of 4 ml of DNA-condensation buffer (EC buffer Qiagen), were mixed with 0.8 ml of Enhancer buffer (Qiagen) by vortexing and incubated 5 min at room temperature. Then the sample was mixed with 1.3 ml of Effectene by vortexing and incubated 10 min at room temperature. This sample was diluted in dechlorinated water to a final volume of 10 ml where 200 larvae were added and incubated for 16 h. After dsRNA feeding, the mosquito larvae were transferred to clean water and fed with regular diet, ground brewers yeast, lactalbumin and rat food Chow (1:1:1 ratio) (Bayyareddy et al., 2009) daily, until they reached fourth instar when bioassays were performed or guts were dissected for analysis by RT-PCR and Western blot. The Effectene-liposomes may be slightly toxic since feeding *A. aegypti* larvae with Effectene-liposomes causes mortality depending on time of exposure and concentration. In the conditions described here, RNA-free Effectene-liposomes caused 5% mortality.

3. Results

To evaluate the consequence of PFT action on insect larvae we analyzed the effect of two different Cry toxins that are specifically active against two different insect orders. Cry1Ab toxin is highly active against larvae of the lepidopteran *M. sexta*, while Cry11Aa toxin is active against dipteran *A. aegypti* larvae. Using a dose-dependent toxicity assay, a lethal concentration at which 50% of the larvae die (LC_{50}) was obtained for both insect models. Cry1Ab bioassays were performed using *M. sexta* neonates by applying various concentration of the spore-crystal preparation at the surface of artificial diet, and mortality was recorded after seven days of toxin exposure. For Cry11Aa bioassays, fourth instar *A. aegypti* larvae were exposed to various concentrations of spore-crystal sample in dechlorinated water and mortality was recorded after 24 h of toxin exposure (Mulla et al., 1985; Pérez et al., 2005). As controls we included spore-crystal samples containing two non-toxic mutants, Cry1Ab-R99E for *M. sexta*, and Cry11Aa-R90E for *A. aegypti*. These two mutants are unable to oligomerize properly which affects their pore-formation activity (Jiménez-Juárez et al., 2007; Muñoz-Garay et al., 2009). The LC_{50} values obtained for spore-crystal samples of both wild type and mutant toxins on their corresponding target insect are summarized in Table 2.

3.1. Activation of MAPK p38 pathway in insects after intoxication with Cry toxins

We decided to investigate the activation of MAPK p38 pathway by analyzing the induction of MAPK p38 protein phosphorylation by Cry toxins. Phosphorylation of MAPK p38 was analyzed by Western blot experiments using an anti-p38 antibody that specifically recognizes the phosphorylated isoform of MAPK p38. In *M. sexta* larvae, phosphorylation of MAPK p38 was observed at short time incubation, we fed the larvae during 30 min with spore-crystal suspension at LC_{50} and observed that the phosphorylation of this protein increased even more after 1 h of treatment (Fig. 1A). Phosphorylation of MAPK p38, in *M. sexta*, is dose-dependent since one-hour treatment with 2 ng of Cry1Ab spore-crystal per cm^2, corresponding to the LC_{50}, resulted in a lower activation than a treatment with 20 ng of Cry1Ab spore-crystal per cm^2. A treatment of 1 h with the spore-crystal suspension of non-active mutant Cry1Ab-R99E did not induce any activation of MAPK p38 protein (Fig. 1A), suggesting that phosphorylation of MAPK p38 is an *in vivo* response of larval midgut cells to intoxication by Cry1Ab toxin. Similar results were observed in *A. aegypti* larvae (Fig. 1B). Phosphorylation of MAPK p38 was observed after 30 min treatment with Cry11Aa and increased further after 1 h of toxin ingestion (LC_{50}). In comparison, no activation of MAPK p38 was observed when mosquito larvae were fed the spore-crystal suspension of the non-toxic Cry11Aa-R90E mutant (Fig. 1B).

In order to determine the effect of Cry toxins on MAPK p38 expression in both insects, the p38 protein level was analyzed by Western blot using an anti-p38 antibody after long time of intoxication with the spore-crystal suspensions. In *M. sexta* larvae, we observed an increase in MAPK p38 protein level after seven days of spore-crystal exposure, with LC_{50}. If the larvae were fed with a higher dose of spore-crystal corresponding to LC_{90} concentration, the amount of MAPK p38 was higher than the control without toxin but lower than the LC_{50} dose; this is probably due to the fact that LC_{90} is a high dose where most of the larvae are death after seven days. (Fig. 2A). Similarly, RT-PCR analysis showed a clear induction by Cry1Ab of MAPK p38 transcript level detected after 24 h and up to seven days when larvae were fed with a dose of LC_{50} (Fig. 2B). The mRNA levels of MAPK p38 in *M. sexta* larvae were low without induction with Cry1Ab toxin (see control lane in Fig. 2B) only after 24 h there is a clear increase in RNA levels. These data were confirmed by quantitative real-time PCR experiments, indicating that MAPK p38 transcript level increases 0.53, 1.83, 3.08 and 7.42 fold after 30 min, 24 h, 48 h and seven days respectively (Fig. 2C).

Table 2
Toxicity of spore-crystal suspensions of wild type and mutated Cry toxins against *Manduca sexta* and *Aedes aegypti* larvae.

δ-endotoxin	Insect	LC_{50} (95% fiducial limits)	Slope (error value)	Fold increase in susceptibility
Cry1Ab	*M. sexta*	2 (0.8–4)[a]	2.5 (0.5)	1
Cry1Ab	p38-silenced *M. sexta*	0.25 (0.15–0.4)[a]	1.8 (0.3)	8
Cry1Ab-R99E	*M. sexta*	>2000[a]	ND[c]	ND
Cry11Aa-R90E	*A. aegypti*	>10,000[b]	ND	ND
Cry11Aa + Effectene	*A. aegypti*	30 (25–36)[b]	5.0 (0.5)	1
Cry11Aa + Effectene	p38-silenced *A. aegypti*	3 (2–4)[b]	4.2 (0.4)	10

[a] LC_{50} ng/cm^2.
[b] LC_{50} ng/ml.
[c] Not determined.

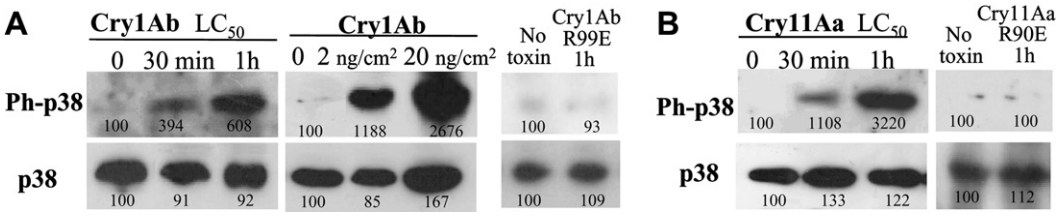

Fig. 1. Phosphorylation of MAPK p38 in insects after intoxication with Cry toxins. Panel A, first instar *Manduca sexta* larvae were intoxicated with 2 ng/cm^2 (LC50) or 20 ng/cm^2 Cry1Ab for up to 1 h. *M. sexta* larvae were also treated 1 h with the non-toxic mutant Cry1Ab-R99E affected in oligomerization and pore formation. Panel B, fourth instar *Aedes aegypti* larvae were fed an LC50 of Cry11Aa toxin or the non-active mutant Cry11Aa-R90E. The presence of phosphorylated and total MAPK p38 proteins was analyzed by western blot using specific antibodies. The blots presented here are representative figures of three independent experiments. Numbers under the blots are percentage in relation to the control band (no toxin or time 0, which was considered as 100%), after scanning the bands. Ph-p38, phosphorylated MAPK p38; p38, total MAPK p38.

In contrast, very small or no changes in protein or transcript levels were observed for MAPK p38 in *A. aegypti* larvae exposed to Cry11Aa toxin (Fig. 2A and B).

3.2. Silencing of MAPK p38 by RNAi in M. sexta and A. aegypti

In order to determine the role of MAPK p38 pathway in protection against Cry toxins in both insect orders, we set up conditions for silencing by RNAi their corresponding MAPK p38 transcript. Previously, we demonstrated that injecting dsRNA into *M. sexta* larvae resulted in specific silencing of the Cry1Ab cadherin receptor (Soberón et al., 2007). However, it was recently reported that RNAi was efficiently induced in both Lepidopteran and Coleopteran insect orders by delivering dsRNA by feeding (Baum et al., 2007; Mao et al., 2007). In this work we set up the conditions to efficiently deliver dsRNA by feeding both *M. sexta* and *A. aegypti* larvae (Materials and methods). In the case of *M. sexta*, dsRNA was offered to starved and water deprivated neonates and after eating the dsRNA, these exposed larvae were returned to artificial diet until they reached third instar. The MAPK p38 transcript and protein levels were then determined in third instar *M. sexta* larvae by RT-PCR and Western blot (Fig. 3). The *M. sexta* larvae that were fed with p38-dsRNA showed undetectable levels of MAPK p38 protein and transcript in contrast with larvae fed with water with an 80% silencing efficiency (Fig. 3). As a control we analyzed expression of tubulin protein and actin and RPS3 transcripts that remained unchanged after MAPK p38 dsRNA feeding. In *A. aegypti* larvae, the silencing of gene expression by RNAi was not a straightforward process, since mosquito larvae are filter-feeding insects. Therefore, we fed the larvae either with high amounts of dsRNA directly added to a small volume of water, or with transformed *E. coli* bacteria that were activated for expression of the dsRNA. None of these treatments resulted in any silencing of the MAPK p38 gene (data not shown). Finally we fed first instar larvae with MAPK p38-dsRNA encapsulated in Effectene-liposomes (see Materials and methods). After feeding the mosquito larvae with Effectene-liposome encapsulated dsRNA, larvae were transferred to clean water with regular diet until they reached fourth instar where MAPK p38 protein and RNA levels were analyzed by Western blot and RT-PCR respectively. Similarly as in lepidopteran *M. sexta*, the MAPK p38 transcript and MAPK p38 protein were down regulated in contrast to tubulin protein and to actin transcript that were not modified by MAPK p38-dsRNA treatment (Fig. 3).

3.3. Role of MAPK p38 response in defense mechanisms of M. sexta and A. aegypti against Cry toxins

Finally, to determine the effect of silencing of MAPK p38 on Cry toxin susceptibility in both insect species, we analyzed the insecticidal activity of Cry1Ab and Cry11Aa spore-crystal suspension in MAPK p38-silenced and in control larvae. Both *M. sexta* and *A. aegypti* p38-silenced larvae became hypersensitive to the insecticidal action of Cry toxins (Table 2). For the p38-silenced *M. sexta* larvae, we assayed a range of Cry1Ab spore-crystal concentrations that range from 0 to 10 ng/cm^2 of diet. We observed an 8 fold decrease of the LC_{50} of the *M. sexta* silenced larvae when compared with the normal *M. sexta* larvae, indicating higher susceptibility to the toxin. In *A. aegypti* larvae, RNA-free Effectene-liposomes caused a decrease of the Cry11Aa LC_{50} in compared to untreated larvae. However, when MAPK p38-silenced *A. aegypti*

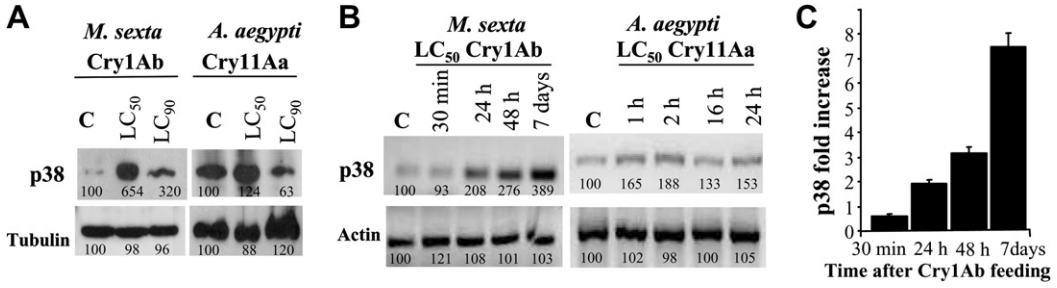

Fig. 2. Activation of MAPK p38 protein synthesis in insects after intoxication with Cry toxins. Panel A, Analysis of the MAPK p38 protein levels in *Manduca sexta* or in *Aedes aegypti* larvae by Western blot experiments using an anti-p38 antibody at various times after intoxication. First instar *M. sexta* larvae were treated with LC_{50} or LC_{90} Cry1Ab toxin concentrations and analyzed after seven days of intoxication. Fourth instar *A. aegypti* larvae were also treated with corresponding LC_{50} or LC_{90} Cry11Aa toxin concentrations and analyzed after 24 h. Detection of tubulin protein was determined as control protein. The blots presented here are representative figures of three independent experiments. Numbers under the blots are percentage in relation to the control band, after scanning the bands. The control band corresponds to no toxin, which was labeled with a C and was considered as 100%. Panel B, Analysis of the expression of MAPK p38 RNA by RT-PCR assays in *M. sexta* or in *A. aegypti* after treatment with the corresponding Cry1Ab or Cry11Aa toxins at different times. Expression of actin gene was used as control in these assays. The results presented here are representative figures of four independent experiments. Numbers under the bands are percentage in relation to the control band, after densitometry analysis. The control band corresponds to time 0, which was labeled with a C and was considered as 100%. Panel C, Regulation of MAPK p38 gene in *M. sexta* larvae after intoxication with LC_{50} of Cry1Ab toxin analyzed by quantitative RT-PCR assays.

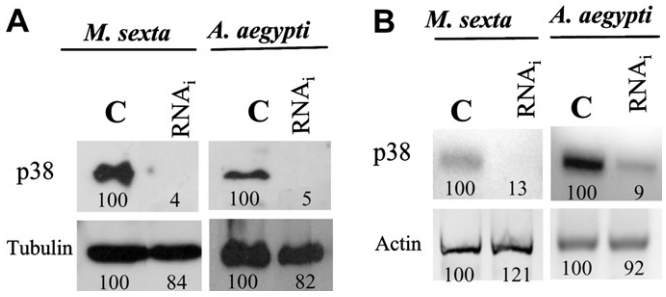

Fig. 3. Silencing of MAPK p38 by RNAi in *Manduca sexta* and *Aedes aegypti* larvae. MAPK p38 expression was silenced by feeding dsRNA to *M. sexta* and *A. aegypti* larvae. Panel A, The presence of total MAPK p38 protein was analyzed by western blot assays using specific antibodies. Panel B, The expression of MAPK p38 gene was analyzed by RT-PCR assays. Numbers under the bands are percentage in relation to the control band, after densitometry analysis. The control bands correspond to non-silenced larvae, which were labeled with a C and were considered as 100%.

larvae were tested for their susceptibility to Cry11Aa a 10-fold increase in susceptibility to the toxin, in comparison to larvae treated with RNA-free Effectene-liposomes, was observed (Table 2).

Therefore, our results suggest that the MAPK p38 pathway has a protective function against the PFT Cry1Ab and Cry11Aa toxins, in both lepidopteran and dipteran insects.

4. Discussion

Understanding the mechanism of action of PFT as well as host responses to these toxins would provide ways to deal with different pathogens and to improve the action of toxins that have biotechnological applications. Eukaryotic cells have developed a variety of defense mechanisms against PFT. Epithelial cells have evolved cellular non-immune defense mechanisms to deal with membrane impairment in their membrane permeability, such as changes in osmotic pressure, ion composition and changes in intracellular calcium concentration induced by PFT (Aroian and van der Goot, 2007). One of these early responses involves activation of the MAPK p38 pathway. It was demonstrated that osmotic stress produced after formation of a few pores by different PFT in target cells, induces a MAPK p38-phosphorylation-response that is a crucial switch for a survival response (Huffman et al., 2004; Ratner et al., 2006; Bischof et al., 2008; Husmann et al., 2006; Aroian and van der Goot, 2007). However, most of these studies were done in cultured cells not in complete animals. The only exceptions were the reports done with the nematode *C. elegans*, intoxicated with Cry5B toxin. It is important to mention that the mechanism of action of Cry5B in the nematode is not completely understood and may have significant differences in relation of the mode of action of other Cry toxins that are specific against insects. The main difference is that protein receptors have not been described in nematodes and that pore-forming activity of Cry5B or oligomerization of this toxin has not been demonstrated. In this work we focused to analyze the cellular response regarding to MAPK p38 activation presented by two insect species after intoxication with specific insecticidal PFT such as the Bt Cry toxins.

Here we describe intracellular effects induced by Cry toxins in insects. We demonstrate that in two different insect orders MAPK p38 pathway was specifically activated at postraductional level by phosphorylation when insect larvae were exposed to active Cry toxins at short times and that exposure to point-mutant toxins affected in oligomerization and pore formation were unable to induce this response. However, the induction response of MAPK p38 kinase in both insects differ since in *M. sexta* the transcript and protein levels were induced when larvae were exposed to Cry1Ab toxin, but in *A. aegypti* larvae no induction of MAPK p38 transcript or protein levels were observed after exposure to Cry11Aa toxin.

To analyze the role of the MAPK p38 pathway as a response to insecticidal activity of Cry toxins the expression of MAPK p38 pathway was inhibited by dsRNA. Recently it was reported that delivering of dsRNA by feeding is an efficient way for specific gene silencing in lepidopteran (Mao et al., 2007) and coleopteran species (Baum et al., 2007). Also that transgenic plants expressing these dsRNA could be used in the control of insect pests (Baum et al., 2007). Here, we show that delivering dsRNA by feeding is also an efficient way for gene silencing in another insect order, the dipteran *A. aegypti* larvae. However effective silencing was only observed when dsRNA was previously encapsulated in liposomes. To our knowledge this is a novel method to silence genes in the larval stage of an important mosquito vector of human diseases. We also silenced expression of MAPK p38 in the lepidopteran *M. sexta* and demonstrated that in both insect species the MAPK p38 plays a central role in defense responses *in vivo* against Cry toxins, since elimination of MAPK p38 protein leads to hypersensitivity to the PFT Cry1Ab or Cry11Aa.

The MAPK p38 pathway is a highly conserved signaling module known to participate in stress responses. Therefore its down regulation may compromise the insects' ability to respond against environmental stress. In this work we show that insects become hypersensitive to Cry toxin action after silencing MAPK p38 expression. Pore formation induced by Cry toxins probably perturbs both ionic homeostasis and intracellular ATP pool. Therefore it is crucial that target cells possess mechanisms to heal membrane lesions in order to survive. It has been shown that aerolysin treatment of CHO cells results in the activation of the SREBP pathway (Gurcel et al., 2006). Also it was reported that MAPK p38 is involved in activation of the *ire-1-xbp-1* branch of the UPR pathway in HeLa cells after aerolysin treatment (Bischof et al., 2008). In both cases the final outcome is an increase in phospholipids biogenesis, suggesting that target cells may respond to PFT by increasing membrane biogenesis in order to repair the membrane lesions (Gurcel et al., 2006). Alternatively another way to repair cell surface lesions is their internalization into endosomes. Supporting this scenario, Husmann et al. (2008) reported that *Staphylococcus aureus* α-toxin is internalized by endocytosis during cell recovery. Also, Idone et al. (2008) showed that Ca^{+2} dependent endocytosis is involved in membrane resealing and cellular recovery after SLO exposure. Interestingly, it was shown that MAPK p38 could modulate endocytosis by regulation of Rab5 cycle and recruitment of its effectors (Cavalli et al., 2001; Mace et al., 2005). The role of endocytosis, SREBP and UPR responses in defense of Cry toxin intoxication in insects, as well as the role of genes that were induced during Cry toxin intoxication remains to be analyzed.

It is clear that eukaryotic cells may have multiple mechanisms to respond against different stress situations. Valaitis (2008) reported that in *Lymantria dispar* larvae the treatment with active Cry1A toxins induce shedding of two glycosyl phosphatidylinositol (GPI)-anchored receptors (aminopeptidase N (APN) and alkaline phosphatase). It was proposed that receptor shedding might be a defense mechanism against Cry toxins, since ectodomains of receptors may function as competitive inhibitors preventing toxin interaction with cell surface receptors (Valaitis, 2008). The APN-shedding could be inhibited by different molecules such as cyclic AMP; inhibitors of the MEK/ERK pathway such as PD98059 and U1026; suramin, a broad-spectrum antagonist of cell surface receptors and piceatannol, a potent inhibitor of the Syk tyrosine kinase. However, inhibition of the shedding of GPI-anchored receptors by cAMP did not affect Cry1A toxicity (Valaitis, 2008), suggesting that shedding of APN may not be involved in the defense to Cry toxin action.

Finally, Loeb et al. (2001) reported that treatment of *Heliothis virescens* cultured midgut cells with low doses of Cry1Ac toxin stimulated the proliferation of stem cells and induced their differentiation. These data represent a complex response; since stem cell proliferation and differentiation is orchestrated by multiple feedback controls involving hormones, grow factors, smaller regulatory molecules and transcription factors. However, the role of MAPK p38 in this effect remains to be analyzed.

Our results show that insects respond to PFT by activation of the MAPK p38 transduction pathway that results in defense mechanism to Cry toxin action. Still remains to be determined the specific defense cellular responses induced by the MAPK p38 pathway. Proteomic analysis of cellular responses in silenced and non-silenced MAPK p38 larvae could reveal the proteins involved in defense to Cry toxins. However, our results show that it is possible to enhance Cry toxin action by inhibiting a specific signal transduction pathway. This may have biotechnological applications for enhancing the activity of some Bt Cry toxins against specific insect pests. Several insect pests are poorly controlled by Cry toxins and in these cases it may be feasible to enhance Cry toxin activity by feeding at the same time the specific MAPK p38 dsRNA and the Cry toxin by co-expressing both factors in the same transgenic plant.

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Dominant Negative Mutants of *Bacillus thuringiensis* Cry1Ab Toxin Function as Anti-Toxins: Demonstration of the Role of Oligomerization in Toxicity

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Abstract

Background: *Bacillus thuringiensis* Cry toxins, that are used worldwide in insect control, kill insects by a mechanism that depends on their ability to form oligomeric pores that insert into the insect-midgut cells. These toxins are being used worldwide in transgenic plants or spray to control insect pests in agriculture. However, a major concern has been the possible effects of these insecticidal proteins on non-target organisms mainly in ecosystems adjacent to agricultural fields.

Methodology/Principal Findings: We isolated and characterized 11 non-toxic mutants of Cry1Ab toxin affected in different steps of the mechanism of action namely binding to receptors, oligomerization and pore-formation. These mutant toxins were analyzed for their capacity to block wild type toxin activity, presenting a dominant negative phenotype. The dominant negative phenotype was analyzed at two levels, *in vivo* by toxicity bioassays against susceptible *Manduca sexta* larvae and *in vitro* by pore formation activity in black lipid bilayers. We demonstrate that some mutations located in helix α-4 completely block the wild type toxin activity at sub-stoichiometric level confirming a dominant negative phenotype, thereby functioning as potent antitoxins.

Conclusions/Significance: This is the first reported case of a Cry toxin dominant inhibitor. These data demonstrate that oligomerization is a fundamental step in Cry toxin action and represent a potential mechanism to protect special ecosystems from the possible effect of Cry toxins on non-target organisms.

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Introduction

Bacillus thuringiensis (Bt) bacteria produce crystal proteins (denominated also Cry toxins) that have insecticidal activity. One of the most successful applications of Cry proteins has been their expression in transgenic crops resulting in their effective protection from insect damage and lowering the use of chemical insecticides [1]. Extensive studies show that Cry toxins used in transgenic crops are safe to the environment and non-toxic to other organisms [2–4]. Nevertheless, there are still concerns related to the possible impact of by products from transgenic Bt crops as Bt-cotton and Bt-corn on non-target organisms in ecosystems adjacent to agricultural fields [5–9].

Pore-forming toxins are important virulent-factors in different diseases induced by several mammalian-pathogenic bacteria [10]. Based on an understanding of their mechanism of action, different strategies have been proposed to neutralize their action [11]. Among these strategies, the use of neutralizing antibodies that recognize toxin regions involved in receptor binding or the use of fragments of toxin-receptors were shown to efficiently protect the cells from intoxication [12,13]. In addition, dominant negative (DN) inhibitors which are inactive mutant-toxins, able to form oligomer structures but affected in their pore formation activity, work as powerful inhibitors since they are able to co-assemble into hetero-oligomers together with the wild type toxin resulting in an effective inactivation of pore formation and toxicity [14–16].

Cry toxins produced by Bt are pore-forming toxins [1]. Their mechanism of action is complex and involves several steps. In the case of Lepidopteran-active Cry1A proteins, the binding to a primary receptor, the cadherin protein, induces the cleavage of an amino-terminal helix α-1 leading to toxin oligomerization [17,18]. Then the Cry oligomer binds to a second receptor. Second receptors such as aminopeptidase N or alkaline phosphatase are anchored to the membrane by a glycosylphosphatidylinositol-anchor, and are localized within lipid rafts [18,19]. The oligomeric toxin inserts into the membrane forming ionic pores causing osmotic lysis of midgut epithelial cells and insect death [1,18].

Although it has been recognized for decades that Cry toxins exert their toxic effect by forming pores into the midgut cells of their target insect, recently an alternative and opposing model was

proposed. The alternative model proposed that after the monomeric Cry toxin binds cadherin, a Mg^{+2}-dependent adenylyl cyclase/PKA-signaling pathway is activated leading to cell death [20]. In this alternative model, neither oligomerization or pore formation are involved in Cry toxicity.

We hypothesized that mutants of Cry toxins affected in pore formation might work as DN inhibitors. The Domain I of Cry toxins is involved in pore formation [21–25]. In this work we analyzed several mutations in helix α-4, in helix α-3 or in domain II-loop 3. These mutants were affected in pore formation, toxin oligomerization and receptor binding, respectively. We found that DN phenotype is linked to mutations affected in pore formation but that are still able to form oligomeric structures with the wild type toxin resulting in a complete inhibition of its insecticidal activity.

The fact that DN mutations blocked toxicity of wild type Cry toxin, supports the concept that oligomerization is a fundamental step in Cry toxin mode of action in agreement with the pore formation model of Cry toxin action.

Results

Cry1Ab mutant characterization

We isolated and characterized Cry1Ab mutants affected at different steps of their mode of action, namely receptor binding, oligomerization and pore-formation to determine if any of them showed a DN phenotype. First, we constructed a Cry1Ab mutant G439D located in loop 3 of domain II. We selected this mutation since a similar mutant, previously characterized in another Cry toxin, the Cry1Ac [26], was shown to have reduced toxicity toward *M. sexta*, reduced binding to BBMV and because the loop 3 region is important for binding with cadherin receptor [26–28]. Secondly, we used a previously described Cry1Ab mutant R99E, located in helix α-3 that showed impaired toxin oligomerization [21]. Finally, we constructed several point mutations in helix α-4 of Cry1Ab such as E129K, N135C, D136N, A140K, T142C, T143D, and T143N, that in the context of Cry1Aa toxin showed to be affected in pore formation and toxicity [22,23]. We also constructed two double mutants, the D136N/T143D and E129K/D136N. Binding analysis with *P. xylostella* BBMV, were reported only for E129K and D136N mutants, revealing no effects on binding of these two mutants, and suggesting that loss of binding was not the reason for the loss of toxicity in these Cry1Aa mutants [25]. However, the characterization of these mutants was partial since the binding to *M. sexta* membranes, as well as the oligomerization process was not analyzed.

All of the Cry1Ab mutants analyzed in this work produce bipyramidal crystal inclusions similar to the wild type toxin with exception of mutant T143N that was not further analyzed. With the exception of two mutants, all other mutant toxins showed severe reductions in toxicity when tested against *M. sexta* larvae (Table 1). The two toxins that retain activity corresponds to mutants D136N and A140K, located in helix α-4, that showed a reduction of two- and four-fold in their insecticidal toxicity when compared with the wild type toxin, respectively. The crystal inclusions produced by Cry1Ab mutants were purified and protoxins were activated with trypsin; all proteins produced a similar 60 kDa activated toxin fragment, indicating no major effects on toxin structure that would result in enhanced susceptibility to protease action (data not shown).

To determine if the Cry1Ab mutants had altered receptor binding, trypsin activated proteins were labeled with biotin and their binding to *M. sexta* BBMV was analyzed (Fig. 1). All mutants except G439D toxin, bound specifically to BBMV as shown in the homologous binding competition assay. The exception was mutant G439D that showed reduced binding to BBMV as was previously reported in the context of Cry1Ac toxin [26]. Figure 1 shows that the rest of biotinylated toxins bind to BBMV membranes isolated from *M. sexta* larvae when assayed in the absence of competitor (lanes marked −). In contrast, in the presence of 500-fold molar excess of unlabeled toxin competitor (lanes marked +) the binding of biotinylated toxin is competed.

Table 1. Toxicity of wild type and mutated Cry1Ab toxins against *Manduca sexta* larvae.

δ-endotoxin	LC_{50} ng/cm² (95% fiducial limits)	Location of mutated residues
Wt Cry1Ab	1.3 (0.9–1.7)	
R99E	>2000	Helix α-3 of Domain I
E129K	>2000	Helix α-4 of Domain I
N135C	16.4 (10.9–22.7)	Helix α-4 of Domain I
D136N	2.8 (2.2–3.8)	Helix α-4 of Domain I
A140K	5.3 (2.8–8.2)	Helix α-4 of Domain I
T142C	34.9 (28.3–41.7)	Helix α-4 of Domain I
T143D	>2000	Helix α-4 of Domain I
D136N, T143D	>2000	Helix α-4 of Domain I
E129K, D136N	>2000	Helix α-4 of Domain I
G439D	>2000	Loop 3 of Domain II

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We then analyzed the ability of the mutant proteins to oligomerize. In this assay the Cry1Ab mutant-protoxins were proteolytically activated with *M. sexta* midgut proteases in the presence SUV liposomes and the antibody scFv73 that mimics an epitope of the cadherin receptor that interacts with loop 2 of domain II [17,18,21]. The oligomeric structure was observed as a low mobility 250-kDa band in a Western blot assay using a specific anti-Cry1Ab antiserum. As shown in Figure 2, only mutant R99E, located in helix α-3 was affected in oligomerization as previously reported [21]. The oligomeric structure of wild type Cry1Ab toxin was mainly found inserted into the membrane pellet, in contrast with the helix α-4 mutants, that remained in the soluble fraction

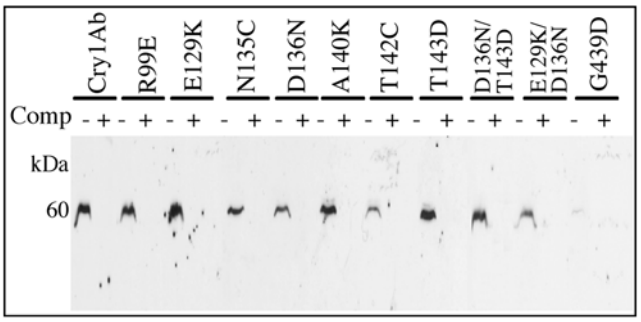

Figure 1. Binding competition assays of Cry1Ab mutants to BBMV of *Manduca sexta* larvae. Binding of biotin labeled toxins was analyzed in the absence (lanes −) or in the presence (lanes +) of 500-fold molar excess of unlabeled toxin. The biotinylated toxins bound to the vesicles, were visualized with streptavidin-HRP conjugate. The Cry1Ab and all mutants located in domain I (helices α-3 or α-4) bound specifically to BBMV only mutant G439D was affected in binding to the *M. sexta* BBMV.

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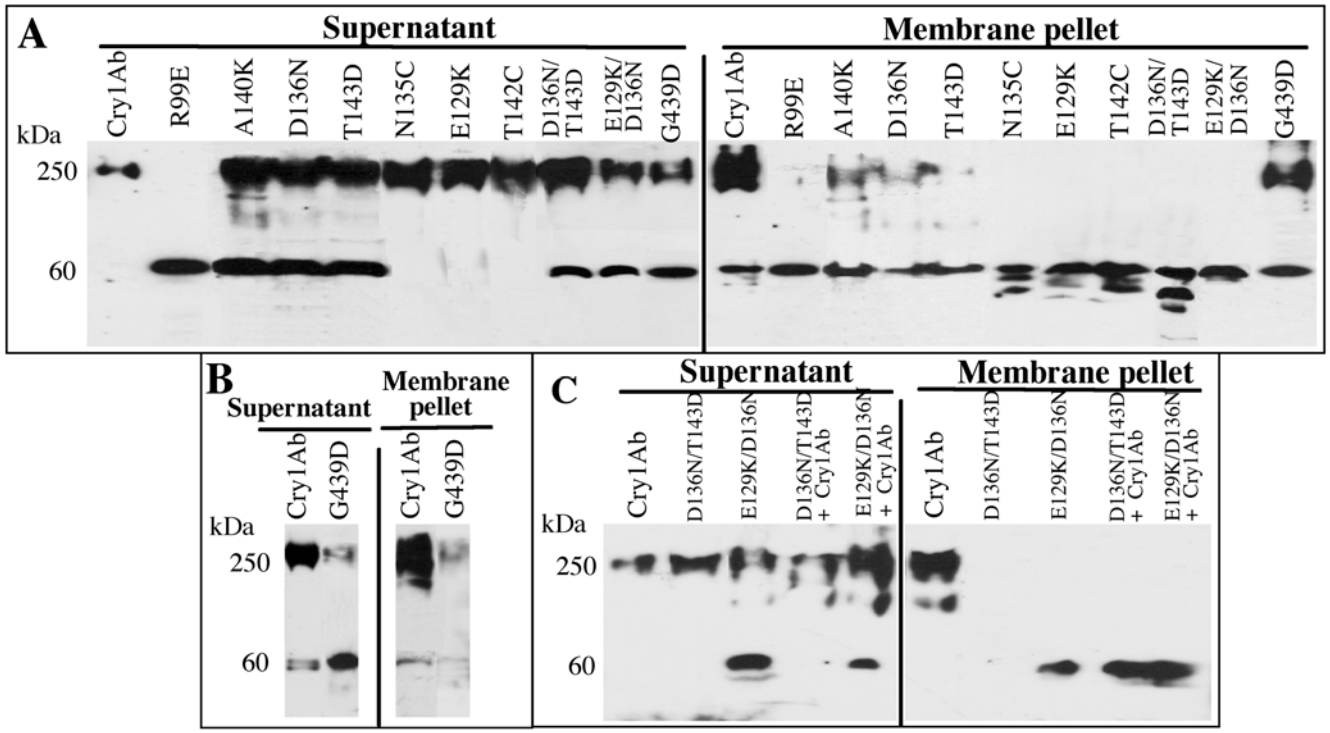

Figure 2. Oligomerization of Cry1Ab proteins. Panel A, Cry1Ab and mutant protoxins were proteolytically activated with *M sexta* midgut proteases in the presence of SUV liposomes and scFv73 antibody. Membrane pellets were recovered by centrifugation and the toxin detected by Western blot using an anti-Cry1Ab antibody in the supernatant and in the membrane fraction. The oligomeric structure of 250-kDa of the Cry1Ab is observed inserted into the membrane pellet, in contrast with the helix α-4 mutants, that remains in the soluble fraction. The mutant R99E, located in helix α-3 did not form oligomeric structures. Panel B, Oligomerization of Cry1Ab and mutant G439D proteins performed as above but in the presence of the cadherin CR12 fragment instead of scFv73 antibody. Under these conditions the oligomerization of the Cry1Ab wild type is observed inserted into the membrane and oligomerization of G439D mutant was severely reduced. Panel C, Oligomerization of the mixtures of 1:1 Cry1Ab: Mutant proteins performed as in Panel A. The oligomer of double mutants or in the 1:1 mixture of Cry1Ab with the double mutants is observed in the soluble fraction.
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suggesting that helix α-4 mutants were affected in membrane insertion (Fig. 2A). Finally, the G439D mutant, located in domain II loop 3, also showed an oligomeric structure that was mainly found inserted into the membrane.

Since our oligomeric assay utilizes the scFv73 antibody that mimics the cadherin repeat 11 (CR11) region of the cadherin receptor which recognizes loop 2 in domain II and considering that the G439D mutation is located in a toxin region which interacts with a different region of the cadherin receptor, i.e. the CR12 fragment [28,29], we repeated the oligomerization assay of G439D using a purified CR12 fragment from cadherin receptor, instead of the scFv73 antibody. Under these conditions the oligomerization of the G439D mutant was severely reduced when compared with the wild type toxin (Fig 2B).

In vivo inhibition of toxin insecticidal activity

To compare the potency of the mutants as DN inhibitors, we tested their ability to inhibit the toxicity of Cry1Ab to *M. sexta* larvae. We fed the larvae with different mixtures of wild type and mutant toxins. We used an equimolar ratio (1:1) as well as a lower ratio (0.25:1 of mutant: wild type). Figure 3A shows that some mutants located in helix α-4 completely blocked toxin action even at sub-stoichiometric ratios. Mutants D136N and A140K did not show DN phenotype because they were not severely affected in toxicity (Table 1), showing an increase in mortality when mixed with the wild type toxin at 1:1 ratio. The higher activity is due to the fact that we used 2 ng/cm^2 of each toxin, one being wild type

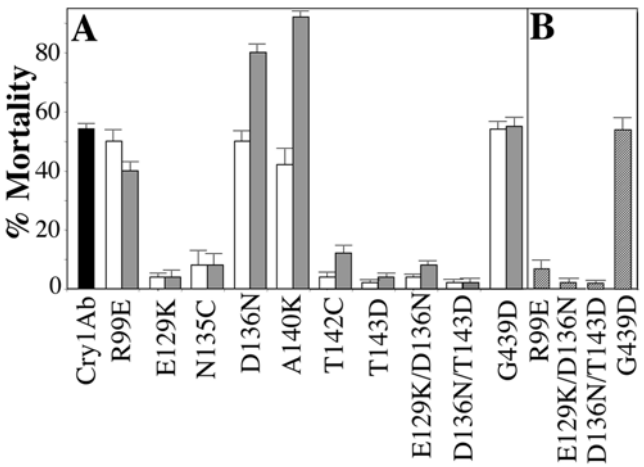

Figure 3. *In vivo* analysis of the Dominant Negative phenotype of Cry1Ab mutants. Panel A, Toxicity assays against *M sexta* larvae with Cry1Ab at 2 ng/cm^2 of diet (black bar) or with a mixture of the same concentration of Cry1Ab wild type with the mutant proteins at two different ratios, 0.25:1 mutant:wild type (white bars) or 1:1 (grey bars). Some mutants of helix α-4 show a clear DN phenotype. Panel B, Toxicity assays against *M sexta* larvae as panel A but at 10:1 mutant:Cry1Ab ratio (dashed bars). R99E reduce toxicity of wild type under this condition in contrast mutant G439D did not affect toxicity of the wild type toxin.
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and the other being either the D136N or A140K mutant that showed reduced toxicity but remain active (Table 1). This mixture represents, therefore, the additive mortality of the two toxin proteins. In contrast, helix α-3 R99A and domain II-loop 3 G439D mutants did not show a DN phenotype. The R99A mutant, showed a competition phenotype since only a high ratio of 10:1 reduced the toxicity of Cry1Ab. In contrast, the G439D mutant showed no effect on Cry1Ab toxicity even at a 10:1 ratio (Fig. 3B).

In vitro inhibition of toxin pore formation activity

To determine if pore formation inhibition by the DN mutants depends on the ability to form hybrid complexes with wild type toxin, we produced homo- and hetero-oligomers and measured their ability to form conductive ion channels in black lipid bilayers. Wild type Cry1Ab or the D136N/T143D and E129K/D136N double mutants were activated in the presence of SUV liposomes and scFv73 antibody as described above to produce oligomeric structures. The hetero-oligomers were prepared by mixing the DN mutants with the wild type in a 1:1 ratio during activation under similar conditions described above. We analyzed oligomer formation in the supernatant and pellet fractions, after centrifugation of the activation reaction to separate toxin inserted into liposomes from soluble proteins. Figure 2C shows that the 250-kDa oligomer was observed mainly in the pellet in the case of Cry1Ab. Nevertheless, in the case of the D136N/T143D and E129K/D136N double mutants or in the 1:1 mixture of Cry1Ab with the double mutants, the 250-kDa oligomers were observed in the soluble fraction (Fig 2C). The soluble and membrane pellet fractions of activation reactions were used to assay pore formation activity in black lipid bilayer system as described previously [21]. The results indicated that oligomers produced by the mutant toxins were severely affected in their pore formation activity when compared with wild type toxin. The hetero-oligomers formed by a mixture of wild type and mutant proteins were also inactive in pore formation. Figure 4A shows representative traces of the activity of Cry1Ab, the mutant E129K/D136N and the mixture of these two proteins in lipid bilayers. Similar data were obtained with the mutant D136N/T143D (data not shown). Current-voltage curves are presented in figure 4B, showing that only wild type Cry1Ab toxin has pore formation activity. These results are consistent with the notion that DN mutants inactivate the wild type toxic action *in vivo* by forming inactive hetero-oligomers unable to insert into the membrane.

Discussion

The helix α-4 mutations analyzed in this study do not impair toxin assembly in a pre-pore structure, but rather block an essential conformational transition of the assembled complex necessary for membrane insertion and pore formation. The helix α-4 mutations that resulted in loss of toxin action act as DN antitoxins blocking toxicity and pore formation of wild type toxin. These data strongly indicate that oligomerization and pore formation are necessary steps in the mode if action of Cry toxins. In contrast, the helix α-3 R99A mutant that is affected in the process of oligomerization but retain binding capacities to membrane receptors, displayed competitive binding for the receptor at 10:1 ratio (mutant: wild type). Finally a mutant in domain II, G439D, with altered binding interaction with the BBMV and the cadherin receptor, did not compete with Cry1Ab for binding and neither showed a DN phenotype.

These data are similar to some reported mutants of the anthrax toxin; a mutant affected in its activation by furin, was unable to undergo oligomerization, yet still bound to, and competed receptor binding causing a competitive inhibition of toxin action only at high at 10:1 ratios [30,31]. In another report an anthrax mutant affected in toxin oligomerization did not show a DN phenotype since it was unable to form hetero-oligomers with the wild type toxin [32]. Finally, an anthrax mutant with altered receptor binding did not compete for receptor binding and neither affected wild type activity [32].

The molecular mechanism observed in DN phenotype involves toxin oligomerization between different Cry toxin-monomers forming hetero-oligomeric structures between mutant and wild type monomers. The hetero-oligomer that is formed with the double mutants and the wild type Cry1Ab toxin was severely affected in membrane insertion and pore formation activity suggesting a problem in the transition from pre-pore to pore as was previously proposed for anthrax DN mutants [16].

If the assembly of the Cry toxin oligomeric structure is an stochastic procedure, then at a 1:1 ratio the probability to have at least one subunit of the DN mutant in the resulting oligomeric-complex is high.

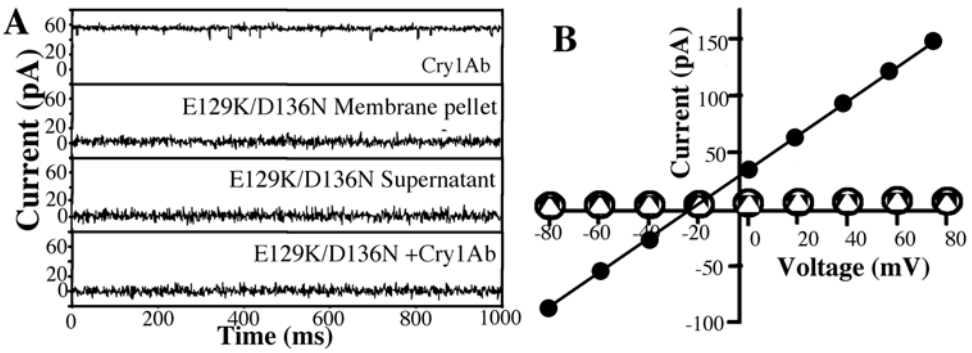

Figure 4. *In vitro* analysis of the Dominant Negative phenotype of E129K/D136N mutant. Pore formation activity of oligomeric structures obtained as described in figure 2C. Panel A, Representative ionic channel records in lipid bilayers of most common transitions induced by oligomer structures of Cry1Ab, E129K/D136N and a 1:1 mixture of Cry1Ab: E129K/D136N. The observed responses with wild type Cry1Ab showed stable channels with high open probability. No ionic channels were observed either for the double mutant E129K/D136N or for the 1:1 mixture of Cry1Ab with the double mutant. Records were obtained in 300:10 mM KCl (*cis:trans*), 10 mM CHES pH 9, at +60 mV. Panel B, Current/voltage (I/V) relationship of macroscopic currents induced by oligomers of Cry1Ab (●) and by oligomers produced from a 1:1 mixture of Cry1Ab and E129K/D136N (△). The activity of the E129K/D136N mutant was also analyzed in the two fractions obtained after activation, the membrane pellet (○), and supernatant fraction (■).
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If one mutant monomer is enough to completely block the wild type toxin activity, then at 1:1 ratio an effective blockage of toxin action is expected. The fact that we found inhibition of wild type toxin activity at 0.25:1 ratio strongly indicates that a single mutant subunit is sufficient to inactivate the oligomer activity and that oligomerization is an important step in toxin action.

The data presented here provides unequivocal evidence that oligomerization is a key step in the mode of action of Cry1Ab and further supports that pore formation is an important event triggering insect cell death. These data support the pore-forming model of the mode of action of Cry toxins and contradict the model of cell death induced by the interaction with cadherin receptor and subsequent induction of signal transduction pathway.

Recent reports raised the concern that the Cry1A toxins may affect non-target organisms [5–9]. Nevertheless, Cry1A toxins used in transgenic plants have been extensively shown to be specific against target insects and safe to non-target organisms [2–4]. In any case the antitoxins of Cry1A described here could be used to inhibit toxicity of Cry toxins in special conditions like, for example, for attenuation of an accidental effect or a release of unregulated Cry toxin, since they offer an efficient alternative to neutralize and counter the Bt toxin action that would help protect potentially endangered organisms in a particular ecosystem.

Materials and Methods

Construction of Cry1Ab mutants

Mutants were produced by site-directed mutagenesis (QuickChange, Stratagene, La Jolla, CA) using the pHT315Ab harboring *cry1Ab* gene. Appropriate oligonucleotides were synthesized for each mutant. Automated DNA sequencing at UNAM's facilities verified the single point mutations. Acrystalliferous Bt strain 407 was transformed with recombinant plasmids and selected in Luria broth at 30 °C supplemented with 10 μg ml^{-1} erythromycin. For construction of double mutants we used pHT315Ab-D136N harboring a point mutation D136N as template to introduce additional point mutations as E129K or T143D.

Cry1Ab toxin purification

Bt transformant strains were grown at 30°C in nutrient broth sporulation medium with erythromycin until complete sporulation. Crystal inclusions were observed under phase contrast microscopy and purified by sucrose gradients [33]. Crystals were solubilized in 50 mM Na_2CO_3, 0.2% β-mercaptoethanol, pH 10.5. The monomeric toxins were obtained by trypsin activation in a mass ratio of 1:20 (1 h, 37°C). Phenylmethylsulfonyl-fluoride (1 mM final concentration) was added to stop proteolysis. The oligomeric Cry1Ab structure was produced as described [17,21] by incubation with svFv73 antibody (1:4 toxin: antibody ratio) purified as described [17] or with CR12 cadherin fragment (1:1 ratio), purified as described [29,34] and 5% midgut juice from *M. sexta* larvae, in 100 μl of solubilization buffer for 1 h at 37°C in the presence of phosphatidylcholine-small unilamellar vesicles (PC-SUV) at 12 μM final concentration. The membrane fraction was separated by ultracentrifugation (30 min at 100,000×*g*) and the pellet was suspended in 20 μl of 10 mM CHES, 150 mM KCl, pH 9.

Western Blot of Cry1Ab toxin

Cry1Ab and mutant proteins incorporated into PC-SUV or that remained in the soluble fraction were boiled 5 min in Laemmli sample loading buffer, separated in SDS-PAGE and electrotransferred onto nitrocellulose membrane. The proteins were detected in Western blots as described [17,21] using polyclonal anti-Cry1Ab.

Bioassays against *Manduca sexta* larvae

Soluble protoxins (from 0.1 to 2000 ng/cm^2) were applied onto the diet surface of 24-well plates as described [17]. Protein was determined by the Bradford assay. Mortality was recorded after seven days and lethal concentration (LC_{50}) estimated by Probit (Polo-PC LeOra Software). For DN assays different ratios of mutant: wild type (0.25:1, 1:1 and 10:1; w: w) were assayed. The concentration of wild type protoxin used in DN-bioassays was 2 ng of toxin per cm^2 of diet.

Preparation of Brush Border Membrane Vesicles (BBMV)

M. sexta eggs were reared on artificial diet. BBMV from fourth instar *M. sexta* larvae were prepared as reported [35].

Toxin binding assay

Binding assays were done with 10 μg BBMV protein and 5 nM biotinylated Cry1Ab toxins as described [21]. We used 500-fold excess of unlabeled toxins to compete binding. Unbound toxin was washed by centrifugation and resulting membrane pellet was boiled in Laemmli sample loading buffer, loaded onto SDS-PAGE, transferred to nitrocellulose membranes and labeled-toxin bound to the vesicles, was visualized by incubating with streptavidin-HRP conjugate and developed with luminol as described [21].

Pore forming activity of Cry1Ab toxins

Black lipid bilayers were made as reported [36] with Dipalmitoyl-*sn*-Glycero-3-Phosphocholine (DPPC) (Avanti Polar Lipids). Buffers 300 mM KCl, 10 mM CHES, pH 9 and 10 mM KCl, 10 mM CHES, pH 9 were added to the *cis* and *trans* compartments, respectively. Once a bilayer was formed, the membrane or soluble fractions containing the activated Cry1Ab toxins (wild type, mutant or mixture of wild type with mutant) were added to the *cis* compartment. Single-channel currents were recorded with a Dagan 3900A patch-clamp amplifier (Dagan Corp., Minneapolis, MN) as described [21]. Currents were filtered at 200 or 500 Hz, digitalized on-line at 1 or 2 kHz, and analyzed using a Digidata 1200 interface and Axotape and pClamp software (Axon Instruments, Foster City, CA).

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Author Contributions

Conceived and designed the experiments: AB. Performed the experiments: CRA LEZ CMG NJJ SP. Analyzed the data: MS. Contributed reagents/materials/analysis tools: LM. Wrote the paper: LM MS AB.

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Review

Employing phage display to study the mode of action of *Bacillus thuringiensis* Cry toxins

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ABSTRACT

Phage display is an *in vitro* method for selecting polypeptides with desired properties from a large collection of variants. The insecticidal Cry toxins produced by *Bacillus thuringiensis* are highly specific to different insects. Various proteins such as cadherin, aminopeptidase-N (APN) and alkaline phosphatase (ALP) have been characterized as potential Cry-receptors. We used phage display to characterize the Cry toxin–receptor interaction(s). By employing phage-libraries that display single-chain antibodies (scFv) from humans or from immunized rabbits with Cry1Ab toxin or random 12-residues peptides, we have identified the epitopes that mediate binding of lepidopteran Cry1Ab toxin with cadherin and APN receptors from *Manduca sexta* and the interaction of dipteran Cry11Aa toxin with the ALP receptor from *Aedes aegypti*. Finally we displayed in phages the Cry1Ac toxin and discuss the potential for selecting Cry variants with improved toxicity or different specificity.

Contents

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1. Introduction

Bacillus thuringiensis (Bt) is an aerobic, spore-forming bacterium from the *Bacillus cereus* group that is distinguished due to its capacity to produce δ-endotoxins that have insecticidal activity. δ-Endotoxins are produced as crystalline inclusions during the sporulation phase [4]. The δ-endotoxins, or Cry proteins, are specifically toxic to the following insect Orders: Lepidoptera, Coleoptera, Hymenoptera and Diptera [4,31]. One feature that distinguishes Cry toxins is their remarkable specificity, and therefore they are harmless to non-target insects or vertebrates. Cry toxins are being used worldwide for the control of vectors of human diseases or insect agricultural pests as insecticidal sprays or in transgenic plants [4].

2. Bt Cry toxins

Cry toxins belong to the group of pore-forming toxins (PFT) and it is widely accepted that their toxic effect is due to the formation of ionic pores in the membrane of insect epithelial midgut cells, which leads to cell swelling and death. To exert its toxic effect, crystals are ingested by susceptible larvae and solubilized by the alkaline pH and reducing conditions of the midgut. Midgut proteases act on the protoxin giving rise to a protease-resistant 55–60 kDa toxin fragment. The toxin fragment binds to specific midgut membrane-associated proteins resulting in the oligomerization and membrane insertion of the toxin [4]. Toxin–receptor interaction is a key step that determines insect specificity [4,31]. Even more, the principal mechanism of resistance to Cry toxins are mutations that affect toxin–receptor interaction [8]. Different proteins such as cadherins, aminopeptidase-N (APN), and alkaline phosphatase (ALP) have been characterized as Cry-receptors in different insect species [17,18,21,33]. Thus, understanding the molecular basis of the interaction of Cry toxins with their receptor molecules would be useful not only for engineering Cry proteins with different specificities or with enhanced insecticidal activity but also for coping with the problem of insect resistance in the field.

The three-dimensional structures of six Cry toxins with different insect specificities have been solved [2,3,9,15,22,26]. These toxins are composed of three domains—domain I, a seven α-helix bundle involved in membrane insertion, oligomer formation and pore formation [4]; domain II, a three anti-parallel β-sheets packed around a hydrophobic core in a ''β-prism'' involved in receptor interaction [4]; and domain III, a β-sandwich of two antiparallel β-sheets also involved in receptor interaction [4].

3. Phage display

For the past few years we characterized Cry toxin–receptor interaction in lepidopteran and dipteran insects to understand the molecular basis of insect specificity of these toxins. For this purpose we have employed a molecular technique known as phage display. Phage display, first developed in 1985 [32], displays recombinant peptides or proteins on the surface of phage particles, that can be screened by enabling the phage to interact with ligands that are immobilized in tubes (panning). This is a very powerful technique since the selected phages maintain a physical link between the displayed protein (phenotype) and the encoding gene (genotype). Filamentous phages, like M13, have been extensively used to develop different types of phage display libraries that display millions of variants of peptides or antibodies. Phage display involves the fusion of foreign DNA sequences to a coat protein gene enabling the fusion protein to be displayed on the surface of the phage. Most commonly, phage display libraries are constructed using vectors called phagemids, which are hybrids of phage and plasmid vectors. These phagemids contain the origins of replication from the M13 phage and *E. coli*; the gene coat protein III or gene coat IV for protein fusion, and an antibiotic resistance gene [1,25]. To display the fusion protein, *E. coli* cells harboring the phagemids are infected with a helper phage that provides all the necessary components for phage assembly.

In this review we will summarize the types of libraries and the panning procedures used to characterize the Cry toxin–receptor interaction. Our experimental approach has been to select, by panning, ligands of the toxin or the receptor that compete the toxin–receptor interaction. This approach has enabled us to map the binding epitopes in the Cry toxins and in the receptors, identify novel receptor molecules, study receptor localization in the insect gut and most importantly, to determine how receptors promote toxicity. Finally, we will discuss the potential of displaying Cry toxins on the surface of phages as a way to select toxins with different specificities or that overcome insect resistance to Cry toxins.

4. A synthetic human scFv library—selection of an scFv molecule that mimics a cadherin receptor

Our first attempt to characterize the amino acid epitopes involved in the interaction of Cry toxins with their receptor molecules was with the lepidopteran insect *Manduca sexta* and the Cry1Ab toxin [10]. At that time, two *M. sexta* proteins that bind Cry1A toxins had been cloned and characterized, a 210 kDa cadherin protein known as Bt-R_1 and a 120 kDa glycosylphosphatidyl-inositol (GPI)-anchored aminopeptidase-N [21,33]. As a way to determine which was the functional receptor and the amino acid epitopes involved in the toxin–receptor interaction, we decided to select by phage display peptides that bind Cry1Ab toxin and identify those that competed toxin interaction with both receptor molecules, then we used these phages as tools to determine the role of both *M. sexta* proteins in toxicity. Also, we expected that some of the peptides that competed the interaction of Cry toxins with their receptor could share sequence similarity with discrete receptor amino acid sequences that were involved in toxin binding as has been shown for several examples of interacting proteins [20]. For the first panning procedure we used the Nissim synthetic phage-antibody library. This library, with a diversity of 1×10^8, contains a diverse repertoire of *in vitro* rearranged human variable heavy (VH) genes containing a random VH-CDR3 of 4–12 amino acids residues in length [27]. Antigen-recognition by antibodies is determined mainly by

CDR regions in the variable light (VL) and VH fragments. To facilitate the display of antibody molecules in phages, the VL and VH fragments are assembled as single-chain molecules with a $(Gly_4Ser)_3$ linker peptide resulting in the construction of scFv genes fused to the pIII gene in phagemids [1,27]. scFv antibodies are likely to retain the binding properties of the whole antibody molecule [1,27]. For the panning procedure, we immobilized Cry1Ab toxin in immunotubes and after several panning rounds of selection we identified three scFv phages that bound specifically to Cry1Ab toxin. Using ligand blots assays and by competing the binding of Cry1Ab toxin with *M. sexta* brush border membrane vesicles (BBMV) blotted proteins, we identified one scFv (scFv73) that competed the binding of Cry1Ab with the 210 kDa Bt-R_1 molecule but not with the 120 kDa APN [10]. Antibody scFv73 inhibited the toxicity of Cry1Ab toxin in bioassays. Sequence analysis of the CDR3 region of scFv73 led to the identification of a eight amino acid region ($_{869}$HITDTNNK$_{876}$) in cadherin repeat 7 of Bt-R_1 [10]. Synthetic peptides corresponding to the Bt-R_1 amino acid region or to scFv73 CDR3 competed the binding of Cry1Ab with Bt-R_1 [10].

Use of the scFv73 antibody was also very important to map the cognate-binding region in the Cry1Ab toxin [11]. Previous work of other groups demonstrated by site directed mutagenesis that domain II loops located in the apex of the β-prism were important for receptor recognition [4,31]. We determined if these loop regions were involved in the interaction of Cry1Ab toxin with scFv73. To do this, we performed binding competition of scFv73 binding with Cry1Ab toxin in Western blots using synthetic peptides corresponding to each of the Cry1Ab loop regions (loop 1–loop 3) [11]. Only loop 2 peptide competed the binding of scFv73 with Cry1Ab toxin [11]. Interestingly a mutant loop 2 synthetic peptide that contained two-amino acid residue changes that in the context of the whole toxin affected binding and toxicity did not compete the binding of scFv73 with Cry1Ab [11]. These results indicate that the Cry1Ab domain II loop 2 region interacts with the Bt-R_1 $_{869}$HITDTNNK$_{876}$ amino acid region [11] (Fig. 1).

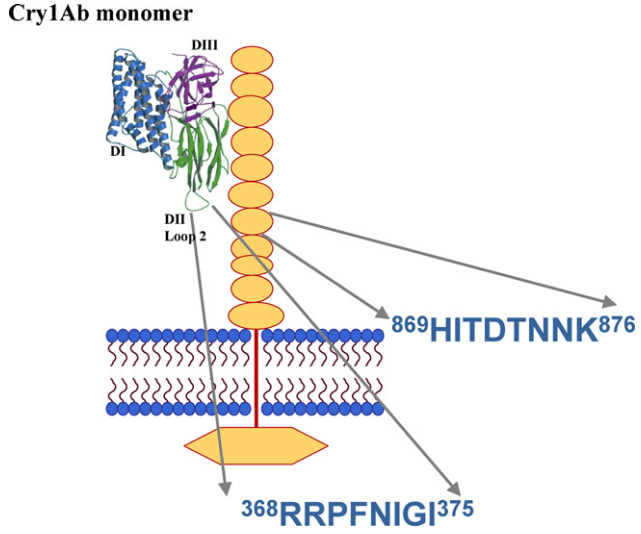

Fig. 1 – Cry1Ab toxin binds *Manduca sexta* cadherin repeat 7 by means of domain II loop 2.

Antibody scFv73 was also very useful to determine the role of the cadherin receptor in promoting toxicity. For several other pore-forming toxins, binding to a receptor facilitates a proteolytic cleavage that finally induces the formation of oligomeric structures that are capable of inserting into membranes [29]. Using scFv73 as a surrogate of Bt-R_1, we demonstrated that Cry1Ab toxin forms a soluble 250 kDa oligomer when activated *in vitro* by *M. sexta* midgut-proteases in the presence of scFv73 [12]. Subsequently, we demonstrated that the 250 kDa oligomer is membrane insertion competent and forms stable pores with high conductances and open probability, in contrast to the 60 kDa monomeric structure that barely inserts into membranes and induces unstable low-conductance pores in black lipid bilayers [30]. Also, we demonstrated that the 250 kDa oligomer is formed in the presence of cadherin fragments containing the toxin binding regions [13]. These results led us to propose that binding to cadherin facilitates the formation of a pre-pore oligomer that is membrane insertion competent (Fig. 2).

5. An immune rabbit scFv library—mapping the binding epitopes of oligomeric toxin and APN receptor

In order to determine the role of other Cry1Ab toxin regions in binding to the receptor molecules, we attempted to isolate other scFv antibodies that recognize other domain II loop regions or domain III, previously shown to be involved in receptor interaction [4,31]. Nevertheless, different attempts of panning using the Nissim library were unsuccessful. Therefore we decided to construct a new scFv phage display library that would be enriched in scFv molecules that recognize Cry1Ab toxin. For this purpose, phage display libraries constructed from a host immunized with the target antigen favors selection of high-affinity, specific antibodies generated during immunization process [14]. A Cry1Ab-immune M13 phage-repertoire was constructed using antibody gene transcripts of bone marrow and spleen from a rabbit immunized with Cry1Ab toxin [14]. These were the first immune-libraries described against Cry toxins.

We also used novel panning protocols to identify antibodies that recognize exposed regions of domain II and III. scFv-antibodies to domain II loop 3 were selected by panning against a domain II loop 3 synthetic peptide. Anti-domain III-scFv molecules were identified by selecting scFv phages that bound Cry1Ab toxin in the presence of soluble Cry1Ac toxin, which has similar domains I and II to Cry1Ab, but a non-related domain III. Then, toxin overlay binding competition assays in the presence of synthetic peptides were used to show that domain II loop 3 of Cry1Ab is an important epitope for interaction with the Bt-R_1 receptor. This interaction between the toxin and the anti-domain II loop 3 scFv antibody also promoted the formation of the pre-pore oligomer as a previously observed with an anti-domain II loop 2 scFv73 antibody [14]. The selected anti-loop 3 antibody (scFvL3-3) molecule lowered the toxicity of Cry1Ab to *M. sexta* larvae in bioassays.

The Cry1Ab epitopes involved in the interaction of the oligomeric structure with APN were also mapped by ELISA and

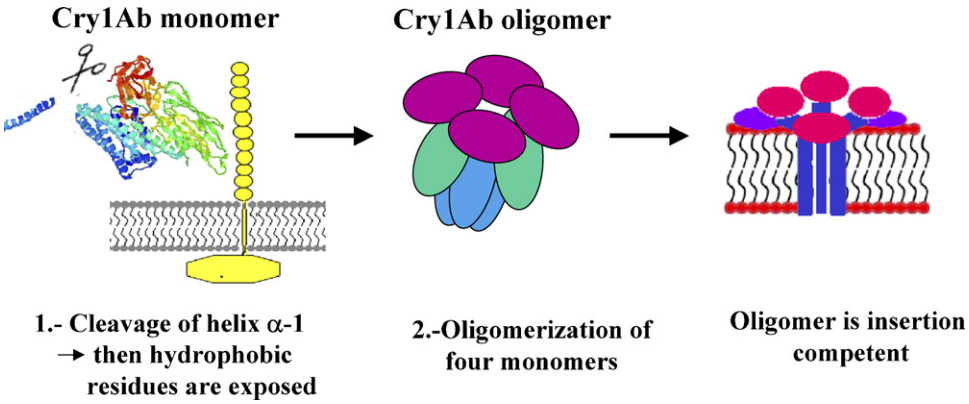

Fig. 2 – Binding of Cry1Ab toxin to the *Manduca sexta* cadherin receptor promotes cleavage of helix α-1 in domain I and the subsequent formation of a pre-pore oligomeric structure that is membrane insertion competent.

toxin overlay binding competition assays. The β16 and β22 from domain III play an important role in this interaction since a scFv-antibody that recognizes these regions (scFvM22) inhibited the interaction of the pre-pore oligomeric structure with APN and lowered the toxicity of Cry1Ab to *M. sexta* larvae in bioassays [14]. scFvM22 did not promote the *in vitro* formation of a pre-pore oligomeric structure. Our results show that interaction with both receptors is important for *in vivo* toxicity.

Finally, we found that scFvL3-3 and scFv73 antibodies preferentially recognized the monomeric toxin rather than the pre-pore structure, suggesting a conformational change in the domain II loop regions is recognized by these antibodies [14]. Overall, these results indicated that the first interaction of Cry1Ab with Bt-R_1 through domain II loop regions, promotes the formation of the pre-pore oligomeric structure with a subtle change in the structure of these exposed domain II loops; then the pre-pore interacts with APN through a different domain III region (β16) (Fig. 3).

6. Random peptide library—identification of an ALP receptor and its interaction with Cry toxins in mosquitoes

In the case of mosquitocidal toxins we decided to work with a Cry11Aa toxin that is particularly toxic to *Aedes aegypti* that is

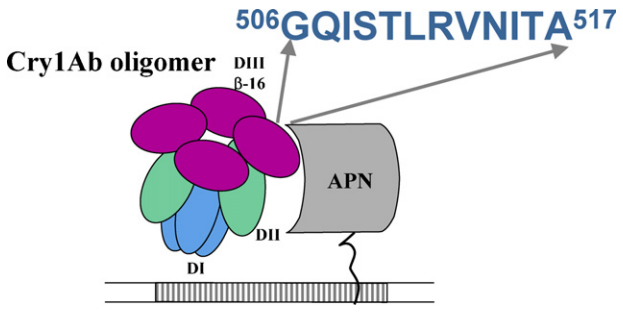

Fig. 3 – The Cry1Ab pre-pore oligomer binds aminopeptidase-N (APN) from *Manduca sexta* by means of β-16 in domain III.

the vector of dengue in humans. *Bacillus thuringiensis* subs *israelensis* (Bti) produces a crystal inclusion composed of six toxins: Cry4Aa, Cry4Ba, Cry10Aa, Cry11Aa, Cyt1Aa and Cyt2Ba that are highly active against dipteran insects, vectors of human diseases [23]. In contrast to *M. sexta* and Cry1Ab, no receptor molecules had been identified in *Ae. aegypti* nor the binding regions in the toxin. We decided to perform panning against Cry11Aa to select molecules that competed for the binding of the toxin with *Ae. aegypti* BBMV and also panning against the BBMV to select molecules that bind the receptor. This is an example that shows that molecules that bind to a receptor molecule can be selected without the need of having a pure preparation of this receptor [7]. We used a commercial phage-peptides library obtained from New England BioLabs Inc. (Ph.D.-12 Phage Display Peptide Library Kit). This library has a complexity of 2.7×10^9 transformants, and is based on a combinatorial library of random 12-mers fused to the coat protein pIII of the M13 phage. We demonstrated that the putative exposed loop α-8 of Cry11Aa toxin, located in domain II, is an important epitope involved in receptor interaction and toxicity. Four exposed loops and three putative exposed regions in domain II of Cry11A were predicted by modeling the three-dimensional structure of the mosquitocidal Cry11Aa toxin [6]. Synthetic peptides corresponding to these regions were used in heterologous binding competition assays of Cry11Aa to *Ae. aegypti* BBMV. We identified three regions of Cry11A toxin, corresponding to loop α-8, β4 and loop 3 that are involved in binding of this toxin to the BBMV [6]. We isolated peptide-displaying phages that bind to Cry11Aa toxin (P5.tox) or to *Ae. aegypti* BBMV (P1.BBMV) [6,7]. These phages interfered with the interaction of the toxin with the BBMV and attenuated the toxicity of Cry11Aa in bioassays [6,7]. The binding of both phages was competed by loop α-8 synthetic peptide, suggesting that phage P5.tox binds the toxin by interacting with loop α-8 while P1.BBMV interacts with the receptor by using an epitope similar to this region [6,7].

A 65 kDa GPI-anchored ALP was identified as a binding Cry11Aa protein in *Ae. aegypti* BBMV. P1.BBMV bound specifically to ALP demonstrating that ALP is a functional receptor of Cry11Aa [7]. On the other hand, immunocytochemical localization of the binding of phage P1.BBMV and Cry11Aa toxin to midgut tissue sections from the mosquito

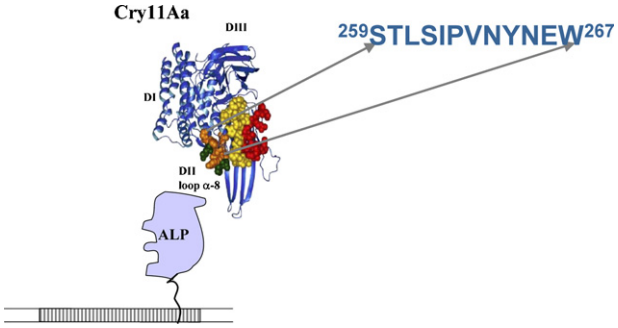

Fig. 4 – The Cry11Aa toxin binds *Aedes aegypti* alkaline phosphatase (ALP) by means of the α-8 loop in domain II.

revealed that Cry11Aa and P1.BBMV strongly recognized the same regions in the midgut tissue. These regions corresponded to the microvilli membrane from the caeca and from the posterior part of the midgut indicating that ALP colocalizes with the binding sites of Cry11Aa [7]. Our results identify domain II loop α-8 as a key determinant for the binding of Cry11Aa toxin to GPI-anchored ALP [6,7] (Fig. 4).

7. Displaying Cry toxins in phages and selection of novel variants

One of the most important features of phage display is that it allows a rapid selection of variants with improved binding characteristics [1,25]. Even more, further mutagenesis and selection by panning allows *in vitro* molecular evolution of proteins [5]. This seems particularly interesting for Cry toxins since for many insect pests there are no Cry toxins available for their control. Also, a major threat for the use of Bt toxins in transgenic plants is the appearance of insect resistance. It has been demonstrated that a single nucleotide change in the *Heliothis virescens* cadherin receptor gene produces an amino acid change that abolishes Cry1A toxin binding [35]. Therefore the need of developing an efficient method that allows genetic evolution of Cry toxins to kill novel targets or to recover toxicity to resistant insects, in the case of the appearance of resistance in the field, will be greatly desirable.

Cry1A toxins have been displayed in three different phages. The first attempts to display Cry1A toxins were using M13 phages [19,24]. In the case of the filamentous M13 experiments, the Cry1Aa toxin was not properly displayed resulting in deletions of the fused protein [24]. A complete Cry1Ac was efficiently displayed in M13 showing toxicity to *M. sexta* larvae, however, the displayed Cry1Ac protein did not bind to functional receptors *in vitro* suggesting structural constraints of the displayed toxin [19]. M13 phage display systems have an intrinsic problem in displaying big proteins since fusion proteins have to be transported into the *E. coli* periplasm where phage assembly occurs.

To circumvent this problem, λ and T7 phages have been used to display Cry1Ac toxin since the assembling of phage particles in both systems occur in the cytoplasm of bacterial cells, thus allowing the display of bigger proteins [28,34]. In the case of λ, the Cry1Ac protein was fused with the capsid protein D and displayed on the surface of phage particles. The displayed Cry1Ac toxin retained toxicity and the capacity to interact with the *M. sexta* APN receptor [34]. More recently we described the use of T7 phage display system to display Cry1Ac toxin [28]. The *cry1Ac* gene was fused to the 3′ end of the T7 10B capsid protein gene and the chimeric protein was displayed on the surface of T7 phage. The T7-Cry1Ac bind receptors recognized by Cry1Ac toxin and retained toxicity against *M. sexta* larvae. Also, we showed that T7-Cry1Ac phage particles bind specifically to *M. sexta* BBMV that contain the native receptor molecules [28]. Nevertheless, a problem with both λ and T7 displaying systems is that for displaying the fusion protein both systems rely on *in vitro* packaging systems that under the best scenario allow for the production of up to 10^7 to 10^8 recombinant phage particles/μg of DNA, making the construction of libraries with large number of variants inefficient. Recently the T7 system was used successfully to select Cry1Aa toxin variants with higher toxicity by selecting phages by panning domain II loop 2 variants that bound the *B. mori* cadherin protein (Bt-R_{175}) with higher affinity [16]. This result shows that it is possible to select novel Cry toxins with desirable binding properties from a pool of variants.

8. Concluding remarks

To exert their toxic effect, Cry toxins interact with several receptor proteins and undergo drastic structural changes allowing a soluble protoxin molecule to insert as oligomers in the membranes of their target cells, thus forming ionic pores. Several receptor molecules in different insect species have been shown to be involved in this complex process. We have exploited the potential of phage display methodology to determine the identity and localization of one receptor molecule in mosquitoes and to determine the sequential participation of two receptor molecules leading to pore formation in lepidopteran insects. We believe that phage display will also be useful for the *in vitro* evolution of Cry toxins for selecting toxins with novel specificities and for selecting toxin mutants that kill resistant insects. Such a system will be of great value to assure long-term use of Bt toxins for insect management; this is highly desirable since the use of these toxins is recognized as an environmentally friendly technology.

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