UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO

PROGRAMA DE DOCTORADO EN CIENCIAS BIOMÉDICAS

INSTITUTO DE FISIOLOGÍA CELULAR

"Características de las vías de transducción de señales que medían la respuesta a feromonas de apareamiento y a estrés osmótico en la levadura *Kluyveromyces lactis*".

T E S I S

QUE PARA OBTENER EL TÍTULO DE:

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

R E S E N T A:

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CIUDAD UNIVERSITARIA, MÉXICO, D.F. 2009

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A mi padre que siempre
ha sido pieza fundamental
para que siga adelante
y cumpla mis objetivos

Este trabajo fue realizado con el apoyo del CONACYT con los proyectos: **44178**, **40406** y con la beca de Doctorado **No. 185969**. Por el PAPIIT, DGAPA, UNAM **No. IN211906**.

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AGRADECIMIENTOS

A mi familia, por haber me apoyado desde el inicio y seguirlo haciendo hasta ahora, a los nuevos miembros que han venido durante estos años a alegrarnos y darnos motivos para hacer las cosas.

Al Dr. Roberto Coria Ortega, por todo el apoyo durante todos estos años en la realización de mi trabajo de investigación, gracias por hacerme parte de este gran grupo de trabajo y hacer lo posible para que me desarrollara de manera independiente cuestionando las ideas o propuestas que tenía para mi trabajo.

A los miembros del jurado, Dra. Alicia González, Dra. Patricia Coello, Dr. Roberto Hernández y Dr. Rafael Camacho, gracias por enriquecer este trabajo con sus opiniones y críticas.

A los miembros de mi comité tutoral, la Dra. Laura Camarena y la Dra. Marina Macías, gracias por todas las críticas a mi trabajo y mi desempeño como estudiante que siempre fueron para animarme a mejorar.

A los miembros del laboratorio, Laura por su incondicional ayuda en todos los aspectos para hacer siempre las cosas mucho más sencillas; Mary gracias por siempre brindarme el apoyo para que nunca faltara nada; Edith, Nancy, Miriam y Rocío gracias por animarme a seguir y no dejar las cosas; Natalia, Eunice y Luis Pablo gracias por recordarme día a día que jamás se debe de perder el ánimo con el que uno se inicia; Abril, por aceptar las ideas propuestas y seguir adelante aunque yo no te pudiera brindar el tiempo necesario.

A las integrantes de la Unidad de Biología Molecular, Dra. Laura Ongay gracias por todas las críticas siempre constructivas que me han ayudado en mi vida académica; Guadalupe Códiz y Minerva Mora gracias por siempre apoyarme de la mejor manera para que mi trabajo se pudiera realizar.

A la Unidad de Cómputo, Gerardo Coello, Ivette Rosas, Juan Barbosa y Sergio Rojas por todo el apoyo brindado y evitar muchos dolores de cabeza.

Al personal que labora en el Doctorado en Ciencias Biomédicas que siempre me apoyó y resolvió cualquier problema de buena manera a pesar de que en años recientes los trámites se han hecho más complicados, engorrosos y no siempre recibí el apoyo necesario de la coordinación.

A mis inseparables amigos compañeros de este camino, que desde hace 10 años que iniciamos me han demostrado que no importa donde nos encontremos siempre podré recibir su apoyo y su cariño, Eda, Cristina, Rosario, Maritrini y Luis.

A Israel y José Ramón por su agradable compañía a lo largo de todos estos años de amistad, gracias por estar aquí.

A Aline, Iliana y Sergio por su amistad y saber que siempre puedo contar con ustedes a pesar del tiempo y la distancia.

A Liz, gracias por todo este tiempo sabes que fuiste de gran apoyo en momentos muy difíciles.

A todos los biomédicos que he conocido durante todos estos años y que omito sus nombres para no dejar de nombrar a ninguno, gracias por ser unos excelentes colegas se que la mayoría llegará muy lejos y cumplirá sus objetivos.

A todos mis ex alumnos de la Facultad de Medicina que se preocupaban y me daban ánimos para continuar y continuar con mis estudios.

RESUMEN

El apareamiento en levadura se inicia por la unión de feromonas a un receptor acoplado a proteína G que se expresa en células haploides. Nosotros analizamos el papel de los receptores KlSte2 y KlSte3 en *Kluyveromyces lactis* de la vía de respuesta a feromona, así como los genes que codifican para cinasas y proteínas cinasas activadas por mitógenos (MAPK). Las cepas mutantes de componentes de la vía de transducción de señales fueron probadas por su capacidad para aparearse y responder a estrés osmótico. La expresión de cada uno de los genes de los receptores es sexo específico. Mutantes de ambos genes son estériles y el defecto de cada gen puede revertir con la expresión mediante un plásmido. La región citoplásmica del extremo carboxilo terminal de KlSte3p interacciona fuertemente con la subunidad (Gα) KlGpa1p que esta involucrada en la transducción estimulada por la feromona para inducir el apareamiento. La MAPK cinasa cinasa KlSte11p (MAPKKK) participa tanto en apareamiento como en respuesta osmótica, mientras que la proteína de andamiaje KlSte5p y la MAPK KlFus3p son específicos de apareamiento. La cinasa activada por p21 Klste20 y la cinasa KlSte50 participan en ambas vías. Experimentos de asociación de proteínas mostraron que KlSte50p y KlSte20p interaccionan con Gα y Gβ, respectivamente. Además KlSte50p y KlSte20p mostraron interacción con KlSte11p. Mutantes de *KlHOG1* y *KlPBS2* que son genes de la respuesta canónica a estrés osmótico, resultaron sensibles a altas concentraciones de sal y altas concentraciones de sorbitol, pero son dispensables en apareamiento. La mutante de la MAPKK KlSte7p tiene un fuerte efecto en apareamiento y también muestra sensibilidad a estrés osmótico. Finalmente, encontramos evidencia de interacción física entre KlSte7p y KlHog1p, además de disminuir los niveles de fosforilación de KlHog1p después de un choque hiperosmótico en células que no expresan KlSte7p. Este estudio nos revela nuevos papeles para componentes de sistemas de transducción de señales en levaduras.

SUMMARY

Mating in yeast is initiated by binding of pheromone to G-protein-coupled receptors expressed in haploid cells. We analysed the role of KlSte2p and KlSte3p receptors in the *Kluyveromyces lactis* mating pathway as well as genes encoding kinases and mitogen-activated protein kinases (MAPKs). The mutated strains were assayed by their capacity to mate and to respond to hyperosmotic stress. The expression of each receptor gene is sex-specific. Disruption mutants of both genes were found to be sterile, and this defect is reversed by plasmidic copies of each gene. The cytoplasmic C-terminus of KlSte3p interacts strongly with the KlGpa1p (Gα) subunit, which is involved in the transduction of the pheromone stimulus to induce mating. The Ste11p (KlSte11p) MAPK kinase kinase (MAPKKK) was found to act in both mating and osmoresponse pathways while the scaffold KlSte5p and the MAPK KlFus3p appeared to be specific for mating. The p21-activated kinase KlSte20p and the kinase KlSte50p participated in both pathways. Protein association experiments showed interaction of KlSte50p and KlSte20p with Gα and Gβ, respectively, the G protein subunits involved in the mating pathway. Both KlSte50p and KlSte20p also showed interaction with KlSte11p. Disruption mutants of the *K. lactis* PBS2 (KlPBS2) and KlHOG1 genes of the canonical osmotic response pathway resulted in mutations sensitive to high salt and high sorbitol but dispensable for mating. Mutations that eliminate the MAPKK KlSte7p activity had a strong effect on mating and also showed sensitivity to osmotic stress. Finally, we found evidence of physical interaction between KlSte7p and KlHog1p, in addition to diminished Hog1p phosphorylation after a hyperosmotic shock in cells lacking KlSte7p. This study reveals novel roles for components of transduction systems in yeast.

ESTRUCTURA DE LA TESIS

Este trabajo de Tesis se organiza de la siguiente manera:

Dentro de la introducción se anexa como antecedentes la revisión “The pheromone response pathway of Kluyveromyces lactis”, donde se explican los antecedentes que teníamos del sistema de respuesta a feromona en *K. lactis* al comenzar este trabajo y como complemento de la introducción anexo el artículo “Kluyveromyces lactis sexual pheromones. Gene structures and cellular responses to alpha-factor”, en donde describimos a la feromona α.

Los Materiales y Métodos se describen en detalle en cada uno de los artículos “The KlSTE2 and KlSTE3 genes encode MATalpha- and MATa-specific G-protein-coupled receptors, respectively, which are required for mating of Kluyveromyces lactis haploid cells” y “Protein kinases involved in mating and osmotic stress in the yeast Kluyveromyces lactis”, por lo que sólo se describen de manera general en este trabajo.

Los Resultados se presentan en los dos artículos mencionados anteriormente.

En la Discusión se condensan ambos artículos y se tratan de una manera más extensa.

INTRODUCCIÓN

Los organismos están expuestos a una gran cantidad de estímulos externos (cambios de temperatura, nutrientes, iones, hormonas, drogas, etc.) que tienen como consecuencia la activación de una respuesta específica. Muchas veces, estos estímulos causan un estrés que es necesario contener de manera inmediata para poder sobrevivir. Es importante conocer la manera como se induce este estímulo y cual es la respuesta de la célula, ya que al ser esencial para la supervivencia, es necesario entender estos procesos para poder evitarlos y actuar una vez que se presentan.

Las levaduras son un modelo atractivo para poder estudiar la respuesta a estrés, ya que al ser un eucarionte unicelular y de vida libre, está expuesta a cambios drásticos en su medio y ha desarrollado una serie de mecanismos para responder a una gran variedad de estímulos. Se sabe que estas respuestas están mediadas generalmente por vías de señalización que dependen de proteínas que se encuentran en la membrana plasmática. Éstas al ser activadas transmiten la señal dentro de la célula por medio de la fosforilación de proteínas cinasas, que a su vez fosforilan a otras proteínas y que tienen como consecuencia la activación o inhibición de la transcripción de grupos específicos de genes (Chen and Thorner, 2007). Esta forma de responder a los diferentes estímulos está conservada en el resto de los eucariontes.

El último elemento de la cascada de fosforilación, que es el responsable de la regulación de genes ya sea directa o indirectamente, se le denominó proteína cinasa activada por mitógenos (MAPK por sus siglas en inglés) por lo que a estas vías de señalización se les conoce como vías de MAP cinasas, compuestas generalmente de tres elementos. Una vez llegado el estímulo, este se traduce en la activación del primer elemento, una MAPK cinasa cinasa (MKKK) mediante otra cinasa o alguna proteína de unión a GTP de la familia Ras o Rho. Estas MAPKKK activadas, fosforilan al segundo elemento, una

MAPK cinasa (MKK) para que ésta a su vez active por medio de fosforilación al tercer elemento, la MAP cinasa (MAPK). Las MAP cinasas son proteínas que generalmente al ser fosforilados se translocan al núcleo para activar la expresión de genes o fosforilar a otras proteínas que intervienen en el control de la activación transcripcional (Widmann et al., 1999).

En la levadura *Saccharomyces cerevisiae* se han descrito varias vías en donde intervienen MAP cinasas responden a distintos estímulos como estrés osmótico, integridad de la pared celular, respuesta a feromona y falta de nutrientes. A pesar de activarse por estímulos distintos, comparten algunos elementos que transducen la señal, aunque las respuestas son distintas; es por eso que es necesario estudiar más la regulación de estos sistemas para entender qué delimita o da la especificidad.

Sistema de Respuesta a Feromona en S. cerevisiae.

Durante el ciclo de vida de hongos unicelulares, como *S. cerevisiae*, las células son estables tanto en su forma haploide como en su forma diploide. Existen dos tipos de células haploides, las células de sexo ***a*** y las células de sexo α. Las células de los dos sexos son morfológicamente idénticas, y sólo se diferencian por la expresión de genes específicos según el sexo, regulados por los alelos del locus *MAT*; y se denominan *MAT**a*** para las células ***a*** y *MAT*α para las células α. Tanto las células haploides como las diploides tienen un crecimiento vegetativo en el cual se reproducen por gemación. Sólo cuando se encuentran en el medio dos células del sexo contrario se reproducen sexualmente (Dickinson and Schweizer, 1999).

Cada tipo celular haploide secreta al medio una feromona que se conoce como factor sexual (Betz and Duntze, 1979; Betz et al., 1977; Duntze et al., 1970). Éstos se unen a receptores de siete dominios transmembranales. Las dos feromonas son sintetizadas como precursores que sufren cortes proteolíticos

para activarse (Brake et al., 1983; Julius et al., 1983; Michaelis and Herskowitz, 1988). El factor-α, que producen las células *MATα*, es un péptido de 13 residuos (Brake et al., 1983; Julius et al., 1983), mientras que el factor-**a** consta de 12 aminoácidos y una modificación postraduccional en el extremo carboxilo terminal, que consiste en la adición de una grupo isoprenoide de 15 carbonos llamado farnesilo (Anderegg et al., 1988; Becker et al., 1987; Betz et al., 1987; Michaelis and Herskowitz, 1988).

Al unirse a su receptor, estos factores inducen una serie de cambios dentro de las células que culminan en la interrupción del ciclo celular durante la fase G_1; el reordenamiento de los filamentos de actina que favorece el crecimiento polarizado de las células (“shmoo”); cambios en la composición de la pared celular y la membrana plasmática para favorecer la fusión celular y la modificación de la envoltura nuclear para poder llevar a cabo la fusión de núcleos (Marsh L. and M.D., 1997; Sprague G.F. Jr and J.W., 1993).

Para poder llevar a cabo esta serie de cambios en las levaduras, el estímulo es transmitido por una cascada de señalización. Los primeros elementos de la vía son los receptores de la feromona. Estos receptores, pertenecen a la familia de receptores de siete dominios transmembranales acoplados a proteínas G; Ste2p es el receptor del factor-α y se expresa en las células **a**, mientras que Ste3p es el receptor del factor-**a** y se expresa en el otro tipo celular. La proteína G heterotrimérica a la que están acoplados estos receptores, se compone de Gpa1p que es la subunidad α y de Ste4p-Ste18p que son el dímero βγ (**Figura 1A**).

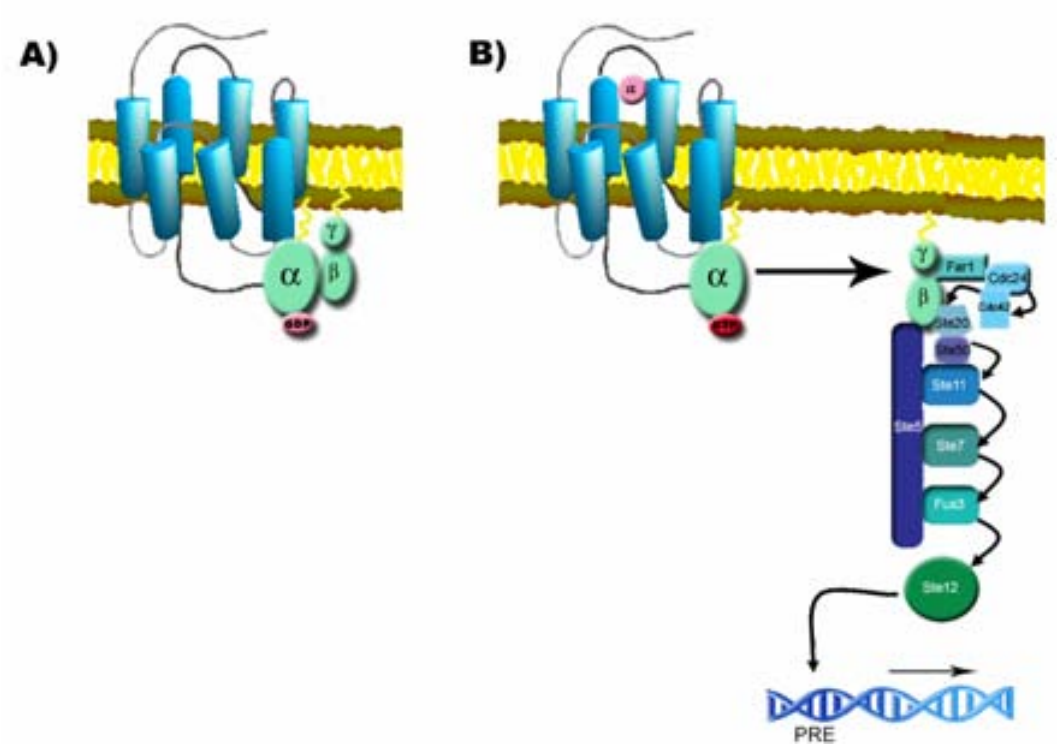

Figura 1. Vía de respuesta a feromona sexual en *S. cerevisiae*. **A)** Cuando el sistema no esta activo, las tres subunidades que componen la proteína G heterotrimérica se encuentran unidas. **B)** Una vez que la feromona activa al receptor, los elementos que componen la proteína G heterotrimérica se separan, debido al intercambio de GDP por GTP en la subunidad α y es el dímero $\beta\gamma$ el responsable de transducir la señal mediante una cascada de fosforilaciones (flechas negras), que tiene como consecuencia la activación de la transcripción de genes que contienen elementos de respuesta a feromona (PRE) en su región promotora, por medio del factor transcripcional Ste12p.

Una vez que la feromona se une al receptor, éste tiene un cambio conformacional que activa a la proteína G promoviendo el intercambio de GDP por GTP en la subunidad α, al estar unida a GTP α pierde su afinidad por el dímero $\beta\gamma$ y así el dímero activa a sus moléculas efectoras induciendo cambios conformacionales al interactuar con sus proteínas blanco: Far1p, Ste20p y Ste5p. Far1p es una proteína adaptadora del factor de intercambio de nucleótidos de guanina (GEF por sus signas en inglés) Cdc24p, que a su vez activa a la GTPasa monomérica semejante a Ras, Cdc42p; así, al momento en que el dímero $\beta\gamma$ es liberado, Far1p recluta a Cdc24p y éste es capaz de activar a Cdc42p, que a su vez activa a Ste20p. La activación de estas proteínas es

necesaria para reorganizar los filamentos de actina y para crecimiento polarizado, eventos necesarios para la fusión celular (Butty et al., 1998; Nern and Arkowitz, 1999; Peter et al., 1996; Wiget et al., 2004; Zhao et al., 1995).

La otra molécula que se une al dímero βγ es Ste20p, que pertenece a la familia de proteínas cinasas activadas por p21 (PAK) y es una cinasa de serinas y treoninas que cumple el papel de una MAPKKK cinasa al activar a Ste11p (MAPKK cinasa) (Drogen et al., 2000; Leberer et al., 1992). Ste11p utiliza a su proteína accesoria Ste50p para realizar esta interacción, ya que Ste50p cuenta con dominios de unión a Ste20p y además a Cdc42p (Ramezani-Rad, 2003; Xu et al., 1996).

Por último Ste5p, tercer efector de βγ, es una proteína de andamiaje que se une al resto de los componentes de la vía: Ste11p, Ste7p (MAPK cinasa) y Fus3p (MAP cinasa), de esta manera se recluta el complejo de MAP cinasas necesarias para poder transducir la señal (Choi et al., 1994; Marcus et al., 1994; Printen and Sprague, 1994). Finalmente, una vez que Fus3p está activada, fosforila al factor transcripcional Ste12p que se une a los elementos de respuesta a feromona (PRE por sus siglas en inlgés) (**Figura 1B**), lo cual promueve la transcripción de los genes necesarios para la conjugación de células (Elion et al., 1993; Errede and Ammerer, 1989; Hagen et al., 1991). De esta forma, al unirse la feromona a su receptor activa el sistema y por medio de los tres efectores del dímero βγ, se asegura que se recluten los complejos necesarios, se propague la señal y se transcriban los genes de respuesta a feromona.

Receptores de feromonas en S. cerevisiae.

El receptor de feromonas es el primer elemento de la vía y es el responsable de transmitir la señal. Deben de tener la especificidad para

activarse sólo por la unión de su ligando y no por otras feromonas de especies distintas que podrían estar en el medio.

Estos receptores pertenecen a la gran familia de receptores de siete dominios transmembranales acoplados a proteínas G. A pesar de que la secuencias de aminoácidos de Ste2p y Ste3p no son similares, estructuralmente son semejantes, tienen siete dominios transmembranales, tres asas extracelulares y tres intracelulares, con el extremo amino extracelular y el extremo carboxilo intracelular (Burkholder and Hartwell, 1985; Hagen et al., 1986).

El receptor más estudiado es Ste2p, debido a que su ligando el factor-α no requiere de la adición de lípidos, como en el caso del factor-**a**, motivo por el cual se ha utilizado desde hace mucho años como un modelo de estudio de receptor de hormonas. Se ha propuesto que para la unión del factor-α a Ste2p, las asas extracelulares forman un hueco donde se une el ligando, siendo esenciales la serina 47 y treonina 48 que corresponden a la interfase entre el primer dominio transmembranal y la parte extracelular y la fenilalanina 204 y tirosina 266 que se encuentran también en la interfase de los dominios transmembranales 5 y 6 respectivamente (Lee et al., 2001; Lee et al., 2002; Lin et al., 2003; Naider and Becker, 2004). Por medio de la primera y tercera asas intracelulares y el extremo carboxilo terminal, el receptor, es capaz de interactuar directamente con las subunidades α y β de la proteína G heterotrimérica y esta interacción es necesaria para que se pueda transmitir la señal (Blumer and Thorner, 1990; Coria et al., 1996; Chinault et al., 2004; Duran-Avelar et al., 2001; Ongay-Larios et al., 2000). Para que el receptor pueda funcionar de una manera adecuada, es necesaria la formación de oligómeros que se ensamblan desde el retículo endoplásmico por medio del motivo GXXXG del primer cruce transmembranal, siendo también importantes el segundo cruce transmembranal y el extremo amino, aunque recientemente se ha propuesto que la unión de su ligando puede favorecer la formación de oligómeros (Overton and

Blumer, 2000; Overton and Blumer, 2002; Overton et al., 2003; Shi et al., 2009). El receptor no solo es el encargado de detectar la señal del medio, sino que también juega un papel importante durante la fusión celular, ya que mutantes que carecen del extremo amino terminal, tanto en Ste2p como en Ste3p, tienen defectos en la fusión de membranas (Shi et al., 2007).

Respuesta a estrés osmótico en S. cerevisiae.

Las levaduras, por las características de su vida libre, están expuestas a cambios constantes en su medio, por este motivo necesitan la capacidad de responder a estos estímulos adaptándose al medio para poder sobrevivir. Esta respuesta se va a traducir en el cambio de expresión de genes, en la vida media de proteínas y en la síntesis de moléculas que le ayuden a sobrevivir. Uno de los cambios más frecuentes en el medio es la concentración de solutos, cuando ésta sobrepasa la osmolaridad interna de la célula, la presión de turgencia disminuye. Para restablecer el balance osmótico *S. cerevisiae* incrementa la síntesis de glicerol, un soluto intracelular inerte que es soluble en agua. La vía de transducción de señales que es activada después de un choque osmótico se le conoce como la cascada de glicerol por alta osmolaridad (HOG por sus siglas en ingles).

La vía de HOG es una cascada de MAP cinasas que tiene como consecuencia la fosforilación y activación de Hog1p. Ésta proteína es el homólogo en levaduras de p38, una MAP cinasa de mamíferos activada por estrés, conocidas como SAPK por sus siglas en inglés (Han et al., 1994).

Vía de HOG en S. cerevisiae.

La vía de HOG es activada a través de dos diferentes ramas, SHO1 y SLN1; la primera es mediada por las proteínas transmembranales semejantes a mucina Hkr1p y Msb2p (**Figura 2**), posibles osmosensores de esta rama (Tatebayashi et al., 2007). Estas dos proteínas activan a Sho1p, que a su vez junto con Msb2p activan a la GTPasa Cdc42p, que estimula a Ste20p (Raitt et al., 2000). Una vez fosforilada, Ste20p activa a Ste11p (como ocurre en respuesta a feromona) que a su vez activa por fosforilación a la MAPK cinasa Pbs2p. Estos eventos ocurren en la membrana plasmática, donde Sho1p, Hkr1 y Msb2 tienen interacción física (Tatebayashi et al., 2007). Además, Sho1p usa su dominio SH3 citosólico para formar un complejo que se compone de Ste11p, su proteína adaptadora Ste50p y Pbs2p. Por otra parte, Cdc42p activado se une al dominio citosólico de Msb2p y a Ste50p (Cullen et al., 2004; Maeda et al., 1995; Tatebayashi et al., 2006). De esta manera, se forman complejos que favorecen la interacción y promueven la especificidad de las proteínas que conforman las ramas, a pesar de que se compartan con otras vías de diferentes respuestas. Al estar ensamblados los complejos antes de que se produzca el estímulo se favorece que la señal se pueda transmitir más rápido.

La rama SLN1 es activada por la cinasa de histidinas transmembranal Sln1p, que además funciona como osmosensor y es parte del sistema de fosforrelevo que incluye a las proteínas Sln1p-Ypd1p-Ssk1p, homólogo a los sistemas de dos componentes descritos en procariontes (Maeda et al., 1994; Posas et al., 1996). Cuando las células están en condiciones iso-osmóticas, Sln1p se auto-fosforila y transfiere un fosfato a Ypd1p, que subsecuentemente transfiere el fosfato a la proteína reguladora Ssk1p (**Figura 2**). Cuando Ssk1p está fosforilada se evita su interacción con las MAPKK cinasas Ssk2p y Ssk22p. Cuando la presión de turgencia cambia, la auto-fosforilación de Sln1p se inhibe, teniendo como consecuencia que Ssk1p se desfosforile y así puede unirse a Ssk2p y Ssk22p para activarlas (Posas and Saito, 1998).

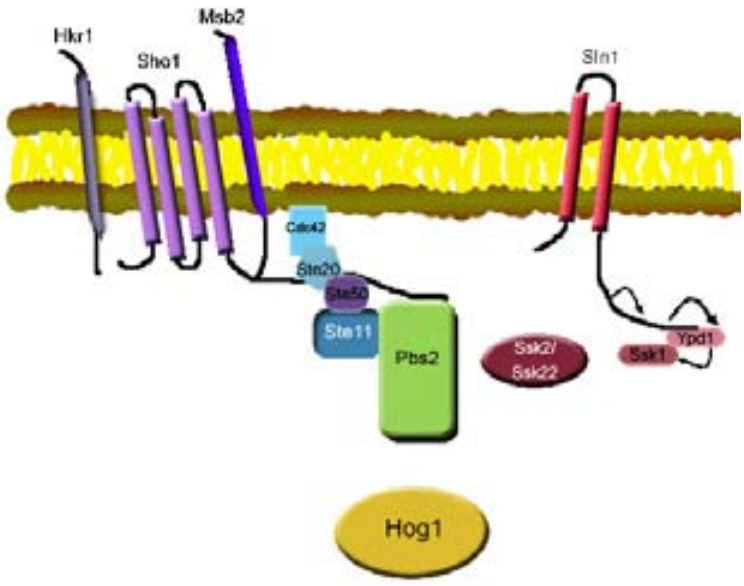

Figura 2. Vía de respuesta estrés osmótico (HOG) en *S. cerevisiae*. Se compone de las ramas SHO y SLN. Cuando el sistema no esta activo la rama de SHO se encuentra como un complejo en la membrana plasmática sin que haya fosforilación de sus elementos. La rama de SLN cuando no esta activa Sln1p se autofosforila y ésta transfiere el fosfato a Ypd1 que a su vez fosforila a Ssk1, de ésta manera se impide que Ssk1 se una a Ssk2 o Ssk22 manteniendo el sistema apagado.

Las dos ramas, SHO1 y SLN1, convergen cuando las MAPKK cinasas Ste11p, Ssk2p y Ssk22p activan y fosforilan a Pbs2p, que a su vez activa a Hog1p (**Figura 3**) por medio de la fosforilación en la Treonina 174 y la Tirosina 176 (Maeda et al., 1995). Una vez fosforilado Hog1, se transloca al núcleo y afecta la expresión de un gran número de genes (O'Rourke and Herskowitz, 2004; Posas et al., 2000; Rep et al., 2000), gracias a su interacción con activadores transcripcionales como Hot1p, Msn1p, Msn2p/Msn4p y Smp1p, así como con el represor transcripcional Sko1. Adicionalmente, interactua con la maquinaria remodeladota de la cromatina y la RNA Polimerasa II (de Nadal et al., 2003; De Nadal et al., 2004; Martinez-Pastor et al., 1996; Proft and Serrano, 1999; Rep et al., 2000; Rep et al., 2001; Rep et al., 1999).

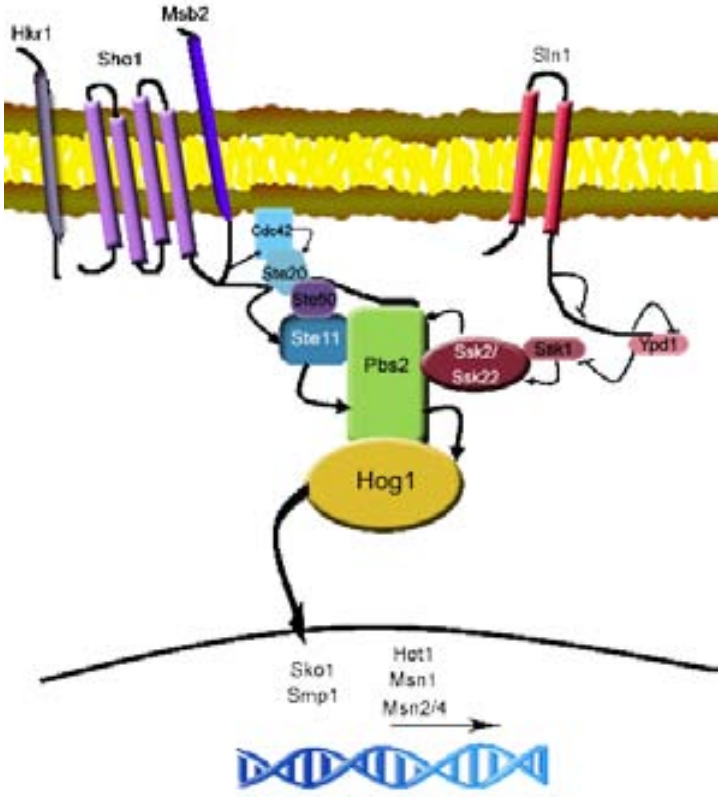

Figura 3. Vía de respuesta estrés osmótico (HOG) activada en *S. cerevisiae*. Una vez que se altera la presión osmótica de la célula, la rama de SHO induce un cambio conformacional en Cdc42p activando su dominio de cinasa que tiene como consecuencia la transducción de la señal por medio de fosforilación de proteínas (indicadas con flechas). La rama de SLN al ser activada se inhibe la fosforilación en el sistema de fosforrelevo que al no fosforilar a Ssk1p, ésta pierde afinidad por Ypd1p, activa su dominio de cinasa y se vuelve afín a Ssk2p y Ssk22p fosforilandolas para activarlas. Ambas vías convergen con la activación de Pbs2p y la fosforilación de Hog1p que se importa al núcleo y es capas de alterar la expresión de varios genes.

Aunque las dos vías convergen en el mismo punto, la rama de SLN1 responde a cambios en la presión de turgencia, además de tener un bajo umbral de activación por KCl (.0625 mM a .25 mM), mientras que la rama de SHO1 es activada en condiciones de hiperosmolaridad (por ejemplo, NaCl 0.5 a 1 M) y no responde a cambios en la presión de turgencia (Maeda et al., 1995; O'Rourke and Herskowitz, 2004; Reiser et al., 2003; Van Wuytswinkel et al., 2000). Esta activación diferencial es esencial para la supervivencia de las levaduras en su cambiante entorno. Si una de las ramas es inactivada, la otra puede rescatar a la

célula en alta osmolaridad, pero si se eliminan las dos ramas las células se hacen sensibles a estos cambios y mueren.

Kluyveromyces lactis como modelo de transducción de señales.

K. lactis es una levadura petite negativa, es incapaz de crecer en ausencia de oxígeno con sustratos fermentables; además de ser negativa para el efecto Crabtree, es decir, en condiciones de aerobiosis y grandes concentraciones de glucosa, produce biomasa a diferencia de *S. cerevisiae* que produce etanol, sin embargo puede utilizar una amplia gama de fuentes de carbono como lactosa (Gonzalez-Siso et al., 2000). A pesar de ser un ascomiceto cercano a *S. cerevisiae*, se estima que estas dos especies divergieron hace aproximadamente 80 millones de años, poco antes de que el ancestro de *S. cerevisiae* duplicara su genoma (Thomson et al., 2005). Todas estas características hacen a *K. lactis* un modelo atractivo por sus diferencias metabólicas y fisiológicas.

El proceso de conjugación sexual de estas dos levaduras se lleva a cabo de manera semejante. Como *S. cerevisiae*, *K. lactis* también presenta dos tipos celulares denominados **a** y α.

En nuestro laboratorio nos hemos interesado en estudiar los sistemas de transducción de señales en *K. lactis* encontrando diferencias importantes con las descritas en *S. cerevisiae* (Coria et al., 2006).

Sistema de respuesta a feromona en K. lactis.

El primer sistema en el que nos interesamos fue el de respuesta a feromonas. En este sistema describimos que la subunidad α de la proteína G

heterotrimérica (KlGpa1p), actúa como una proteína activadora de la señal, ya que la cepa *ΔKlgpa1* es viable y cuando hacemos ensayos de apareamiento, es incapaz de formar células diploides con la misma eficiencia que la cepa silvestre. Estos resultados son distintos a lo que ocurre en *S. cerevisiae,* donde Gpa1p juega un papel negativo uniéndose al dímero βγ para evitar que active a sus efectores y la mutación de *GPA1* es letal, ya que las células al tener activada la respuesta de manera constitutiva se quedan en fase G_1 indefinidamente (Jahng et al., 1988; Savinon-Tejeda et al., 2001). También tenemos indicios de que *KlGPA1* regula la expresión de genes blanco de la vía como *KlFUS1*, ya que utilizando un reportero con el promotor de éste gen fusionado al gen *LacZ* de *E. coli* tenemos actividad de la enzima β-galactosidasa cuando expresamos una KlGpa1p constitutivamente activa, incluso mayor que la obtenida induciendo sólo con feromona y la activación es dependiente del factor de transcripción de la vía KlSte12p (Lloret et al., 2003). La subunidad β de la proteína G heterotrimérica está codificada por el gen *KlSTE4*, cuya mutante es completamente estéril y la activación de la vía también depende de KlSte12p (Kawasaki et al., 2005). **Figura 4**.

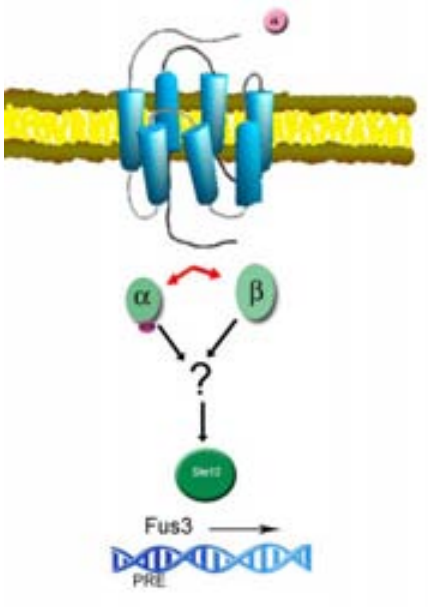

Figura 4. Vía de respuesta a feromona en *K. lactis*. Tanto la subunidad α (KlGpa1p) como la subunidad β (KlSte4p) tienen un papel positivo en la transducción de la señal y activan mediante el fector transcripcional KlSte12p al reportero de respuesta a feromona *KlFus3*.

Hasta el momento no se han descrito los receptores para K. lactis, ni se sabe con que proteínas podrían interactuar para llevar a cabo la activación de la vía. El conocimiento que tenemos de la vía de respuesta a feromona sexual está descrito en la siguiente revisión, donde ya habíamos comenzado a trabajar con algunas de las mutantes de este trabajo. También anexo un trabajo sobre las feromonas sexuales en *K. lactis* que recientemente publicamos.

“The pheromone response pathway of Kluyveromyces lactis

MINIREVIEW

The pheromone response pathway of *Kluyveromyces lactis*

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Received 9 May 2005; revised 29 August 2005; accepted 19 September 2005.
First published online 19 December 2005.

doi:10.1111/j.1567-1364.2005.00022.x

Editor: Lex Scheffers

Keywords
pheromone response; *Kluyveromyces lactis*; signal transduction; G-protein; yeast.

Abstract

The mating pheromone response pathway in *Saccharomyces cerevisiae* is one of the best understood signalling pathways in eukaryotes. Comparison of this system with pathways in other fungal species has generated surprises and insights. Cloning and targetted disruption of genes encoding components of the pheromone response pathway has allowed the attribution of specific functions to these signal transduction components. In this review we describe current knowledge of the *Kluyveromyces lactis* mating system, and compare it with the well-understood *S. cerevisiae* pathway, emphasizing the similarities and differences in the heterotrimeric G protein activity. This mating pathway is controlled positively by both the Gα and the Gβ subunits of the heterotrimeric G protein.

Introduction

Signal transduction pathways allow cells to respond to environmental stimuli and to adapt their gene expression program. Fungal cells can sense many different chemical and physical signals, such as nutrients, osmolarity, pH, light, etc. One common mechanism for responding to extracellular signals uses cell-surface receptors coupled to intracellular heterotrimeric guanine nucleotide-binding proteins (G proteins). Regulatory networks identified in microorganisms often serve as paradigms for those of vertebrates. One of the most studied examples is the pheromone response pathway in the yeast *Saccharomyces cerevisiae*. In this system, yeast haploid cells sense the presence of potential mating partners by recognizing sex-specific pheromones secreted by cells of the opposite mating type. The standard model of G-protein-coupled receptor signalling, which regulates the mating response, postulates that a haploid cell starts the mating process when a sexual pheromone, produced by the mating partner, binds to its specific membrane-spanning receptor. The pheromone stimulus is transmitted via a heterotrimeric G protein, composed of Gα(Gpa1p), Gβ(Ste4p) and Gγ(Ste18p) subunits, coupled to the pheromone receptor (Dietzel & Kurjan, 1987; Whiteway *et al.*, 1989). The Gpa1p subunit negatively regulates the response in the absence of pheromone, and the Ste4p/Ste18p dimer acts positively to trigger the response after binding of pheromone, via a phosphorylation cascade that involves at least Ste20p, the MAP kinase module and transcription activators that finally lead to growth arrest, shmoo formation, induction of genes required for membrane fusion, including *FUS1*, and diploid formation (Herskowitz, 1995).

Pheromone response components have been identified and characterized in a number of fungal species. For many of them, targetted gene disruptions have revealed interesting clues about their functions. In this review we summarize findings concerning the *Kluyveromyces lactis* pheromone response system, and compare them with current knowledge of other yeast species, particularly of *S. cerevisiae*. Most of the gene products described here have been obtained by standard cloning techniques; however, some have been identified by sequence comparison with their *S. cerevisiae* counterparts by blast-searching the *K. lactis* database (http://

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Table 1. Putative and proven components of the *Kluyveromyces lactis* pheromone response pathway

				Saccharomyces cerevisiae homologue		
Gene name	Mutant phenotype	Function	Identifier in the *K. lactis* database	Identity (%)	E value	Reference
KlSte2	unknown	ND	KLLA0F25102g	48	5e-92	
KlSte3	sterile	Pheromone receptor	KLLA0A06534g	51	1e-131	Torres-Quiroz *et al.* (unpublished)
KlGpa1	sterile	Acts as positive element in the mating pathway	KLLA0F25916g	62	8e-154	Savinón-Tejeda *et al.* (2001)
KlSte4	sterile	Acts as positive element in the mating pathway	KLLA0D06787g	52	1e-119	Kawasaki *et al.* (2005)
KlSte18	fertile	Unknown	KLLA0E06138g	53	3e-24	
KlSte20	unknown	Unknown	KLLA0B13607g	50	0	
KlSte5	sterile	Unknown	KLLA0F12023g	27	3e-56	
KlSte11	unknown	Unknown	KLLA0B13112g	58	1e-60	
KlSte7	unknown	Unknown	KLLA0C16577g	52	1e-130	
KlFus3	unknown	Unknown	KLLA0E10527g	69	1e-150	
KlSte12	sterile	Transcription activation of mating genes	KLLA0E17193g	35	1e-107	Yuan *et al.* (1993) Lloret *et al.* (2003)
KlFus1	sterile	Cell fusion	KLLA0C01540g	33	1e-17	Lloret *et al.* (2003)

cbi.labri.fr/Genolevures/elt/KLLA). Table 1 summarizes our current knowledge of some of the components that participate in the *K. lactis* mating system, as well as putative gene products that, by analogy to *S. cerevisiae*, might have a role in the mating process. In Table 2 we include putative gene products belonging to three more hemiascomycetes species that have been studied in the 'Genolevures' project (http://cbi.labri.fr/Genolevures/) and that showed significant homology to those of *K. lactis*. The average sequence identity between the *K. lactis* proteins with their orthologues from other species are: 49% for *S. cerevisiae*, 42% for *Candida glabrata*, 34% for *Debaryomyces hansenii*, and 30% for *Yarrowia lipolytica*. These data are consistent with the findings obtained by comparative genomic studies of these yeast species (Dujon *et al.*, 2004).

Pheromone receptors

Mating is initiated by the binding of sexual pheromone to a seven-transmembrane G-protein-coupled receptor (known as a serpentine receptor or GPCR). Activated receptors catalyze the exchange of GDP for GTP at the high-affinity binding site for guanine nucleotides, located in the α subunit, thus initiating a well-regulated dissociation–reassociation cycle. Although the primary sequences of receptors do not show similarity, GPCRs share parallelism in their topological arrangement in the plasma membrane, consisting of seven hydrophobic and potentially α-helical segments that span the lipid bilayer (Dohlman & Thorner, 2001). The N terminus of these receptors is located extracellularly and contains essential residues for pheromone binding. The long C terminus extends into the cytoplasm.

Carboxyl-terminal tails of serpentine receptors vary considerably in their amino acid sequence. In the case of the *S. cerevisiae* α-pheromone receptor, ScSte2p, the cytoplasmic carboxyl-terminal tail extends at least 130 amino acid residues out from the membrane. Although the C terminus of the receptor interacts physically with its associated heterotrimeric G protein (Bourne, 1997; Dosil *et al.*, 2000), mutagenesis studies have shown that it is not required for ligand binding, but is involved in desensitization after pheromone treatment (Konopka *et al.*, 1988; Reneke *et al.*, 1988), and in the formation of a pre-activation complex with its associated G protein (Dosil *et al.*, 2000).

The *K. lactis* genome encodes two putative pheromone receptors, KlSte2p and KlSte3p, which show an overall 50% identity with their *S. cerevisiae* homologues. Conserved amino acids are mostly located in transmembrane regions and at the second and third intracellular loops. Remarkably, the amino acid sequence of the third intracellular loop is the most highly conserved (around 90% between species). This loop plays a key role in maintaining a productive G-protein coupling (Ćelić *et al.*, 2003). Several other studies with a variety of G-protein-coupled receptors have suggested that not only the third but also the second intracellular loop and the carboxyl-terminal tail are important sites of interaction with the G-protein α subunit (Bourne, 1997; Wess, 1997; Durán-Avelar *et al.*, 2001; Meng & Bourne, 2001). Nonetheless, the second loop can be removed from the *S. cerevisiae* Ste2p receptor with minimal effects on mating (Martin *et al.*, 1999).

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Table 2. Comparison of the *Kluyveromyces lactis* pheromone response pathway components with putative orthologous proteins from different yeast species

Gene product	ORF*	Identity (%)	Similarity (%)	E value
KlSte2p	CAGL0K12430g	35	52	1e-57
	DEHA0A11110g	30	51	8e-43
	YALI0F03905g	26	37	2e-28
KlSte3p	CAGL0M08184g	44	61	1e-117
	DEHA0D04708g	34	52	4e-69
	YALI0F11913g	26	47	4e-48
KlGpa1p	CAGL0F06677g	61	76	1e-149
	DEHA0D14894g	57	71	1e-136
	YALI0E11627g	41	58	3e-87
KlSte4p	CAGL0L02761g	44	60	1e-102
	DEHA0C17600g	38	55	5e-83
	YALI0E01364g	27	42	8e-44
KlSte18p	CAGL0M09207g	47	65	2e-21
	DEHA0G25388g	16	26	0.015
	YALI0A06699g	27	41	3e-10
KlSte20p	CAGL0K02673g	43	54	1e-178
	DEHA0E23529g	39	52	1e-122
	YALI0F00572g	33	43	1e-118
KlSte5 p	CAGL0L06336g	22	36	1e-23
	DEHA0G13684g	18	30	9e-08
	YALI0F20680g	17	31	3e-04
KlSte11p	CAGL0B02739g	55	71	0.0
	DEHA0B04895g	40	54	1e-137
	YALI0F13629g	31	41	1e-112
KlSte7 p	CAGL0I03498g	42	59	e-102
	DEHA0F15719g	34	48	2e-67
	YALI0F21065g	33	46	2e-67
KlFus3p	CAGL0J04290g	67	79	e-146
	DEHA0E21219g	55	70	1e-117
	YALI0E23496g	57	70	1e-124
KlSte12p	CAGL0M01254g	31	46	1e-81
	DEHA0F27445g	31	43	5e-80
	YALI0E16236g	27	36	2e-63
KlFus1p	CAGL0B00968g	23	32	3e-17
	DEHA0E14377g	24	38	1e-09
	YALI0E29755g	16	26	5e-05

*ORFs from *Candida glabrata* (CAGL), *Debaryomyces hansenii* (DEHA) and *Yarrowia lipolytica* (YALI).

We have analysed the role of KlSte3p in the mating pathway (unpublished data). From sequence analysis, KlSte3p is the homologue of the *S. cerevisiae* a-pheromone receptor. From expression experiments, however, the *KlSTE3* gene is expressed in *K. lactis* cells typified as *MATa*, and therefore is the receptor for the *K. lactis* α pheromone. This gene is silent in *MAT*α cells, whereas it is highly expressed in *MATa* cells. As expected, disruption mutants appeared sterile. KlSte3p interacts strongly with the Klgpa1p (Gα) subunit, which is involved in transducing the pheromone stimulus to induce mating (see below). An in-frame GFP–KlSte3p fusion protein, which retains its functional role in mating, was located mainly in the plasma membrane of haploid cells.

The G protein

The heterotrimeric G proteins are intracellular switches that alternate between a GDP-bound off-state and a GTP-bound on-state. Membrane-bound heterotrimers composed of Gα, Gβ and Gγ subunits are closely associated with the intracellular faces of GPCRs. The GDP-Gα subunit binds to the heterodimer Gβγ, and this association allows functional coupling to GPCRs (Chen & Manning, 2001). Agonist binding to GPCRs induces the release of bound GDP by Gα, and then Gα binds GTP, resulting in conformational changes within the flexible regions of Gα (Wall *et al.*, 1998) and dissociation of Gβγ. Both GTP-Gα and Gβγ are capable of signal transduction by activating downstream effectors (McCudden *et al.*, 2005).

In *S. cerevisiae*, the heterotrimer Gpa1p(Gα)/Ste4p(Gβ)/Ste18p(Gγ) is required for response to mating pheromones. This G protein is the same in *MATa* and *MAT*α cells. Upon interaction of pheromones with their respective receptor, the G protein dissociates into Gpa1p(GTP) and the Ste4p/Ste18p dimer, which in turn initiates a cascade of events that results in transcriptional activation of genes required for mating (Bardwell, 2004). Mating between haploid cells of the opposite mating types leads to the formation of a diploid *MAT*α/*MATa* cell.

Two genes encoding G protein α subunits have been cloned and studied in *K. lactis*: *KlGPA1* (Saviñón-Tejeda *et al.*, 2001) and *KlGPA2* (Saviñón-Tejeda *et al.*, 1996). The full sequence of the G protein designated KlGpa1p spans 447 amino acid residues with a deduced molecular mass of 50 739 Da. Analysis of the primary structure showed a high degree of identity (62%) and similarity (72%) with the Gpa1p from *S. cerevisiae*. KlGpa1p contains the characteristic structural domains conserved in Gα(s) from different organisms (Bölker, 1998). It contains the typical G1 and G2 regions involved in the binding and hydrolysis of the GTP. It also presents the consensus amino terminus end (MG-XXXSXX) that has been identified as the *N*-myristoylation target in members of the mammalian Gαi subunits that inhibit adenylyl cyclase (Johnson *et al.*, 1994). However, unlike members of this family, KlGpa1p does not contain the cysteine residue at its carboxyl end that is ADP-ribosylated by pertussis toxin (Johnson *et al.*, 1978). Just as Gpa1p from *S. cerevisiae*, KlGpa1p contains an extra internal fragment of 75 amino acid residues between the conserved G1 region and the first module of the G2 region.

In contrast to the case for *S. cerevisiae*, where the G protein α subunit negatively regulates the mating response in the absence of pheromone, it has been observed that in *K. lactis* the Gα protein is required for mating, and mutants lacking the Gα subunit are partially sterile (Saviñón-Tejeda *et al.*, 2001). Disruption of *KlGPA1* rendered viable cells with a reduced capacity to mate, indicating that KlGpa1p

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plays a positive role in the mating pathway. Mutations on the critical glutamine (Q^{298}) rendered GTPase-deficient Gα subunits and thus a constitutively active subunit. This mutation has no effect on the mating of wild-type cells, but complements and restores mating in the deficient Δ*Klgpa1* strain (Lloret *et al.*, 2003). The same effect is observed in both *MAT***a** and *MAT*α strains. The equivalent mutation on ScGpa1p induces slow growth of normal haploid *S. cerevisiae* cells (Dohlman *et al.*, 1996), indicating constitutive activation of the pheromone response pathway as a result of inefficient association with the Gβγ dimer. Because some studies have indicated direct interaction of ScGpa1p with the mating-specific MAPK Ste50p (Metodiev *et al.*, 2002) and direct coupling of the activated Gα subunit with Scp160 (an RNA-binding protein) (Guo *et al.*, 2003), a positive role of ScGpa1p in mating cannot be discarded. Gα subunits playing positive roles in mating have been described in other fungal species, for example Gpa1p (homologous to ScGpa2p, which is involved in cAMP regulation) in the basidiomycete *Cryptococcus neoformans* (Tolkacheva *et al.*, 1994; Alspaugh *et al.*, 1997), Gpa3p in *Ustilago maydis* (Regenfelder *et al.*, 1996), and Gpa1p in the fission yeast *Schizosaccharomyces pombe* (Obara *et al.*, 1991).

In addition to the pheromone response pathway, in *K. lactis* a cAMP-nutrient-sensing signalling pathway regulated by a Gα2 (KlGpa2p) subunit has been identified (Saviñón-Tejeda *et al.*, 1996), which is unrelated to the mating pathway. Cells lacking KlGpa2p are viable (and fertile) but fail to respond to a transient stimulus of glucose and the cAMP level drops significantly, indicating that the G protein containing KlGpa2p is involved in regulating the activity of adenylyl cyclase and participates in a pathway related to the monitoring of the nutrient status of the cell.

The Gβγ subunit is a functional heterodimer that forms a stable structural unit. All Gβ subunits contain seven WD-40 repeats forming small antiparallel β strands (van der Voorn & Ploegh, 1992). Crystal structures of mammalian Gαβγ (Wall *et al.*, 1995) and free Gβγ (Sondek *et al.*, 1996) reveal that the Gβ subunit folds in a highly symmetric β propeller containing seven β sheets that form the blades of the propeller. Each blade consists of four antiparallel strands, radiating outwards from a central core. The N-terminal region of the Gβ subunit is involved in the interhelical dimerization with the N-terminus of the Gγ subunit by forming an α-helical coiled-coil structure.

The *STE4* gene encodes the Gβ subunit (Whiteway *et al.*, 1989), which associates with the *GPA1* (Gα) (Dietzel & Kurjan, 1987; Nakafuku *et al.*, 1987) and *STE18* (Gγ) (Whiteway *et al.*, 1989) gene products, forming the heterotrimeric G protein involved in the pheromone response pathway in the yeast *S. cerevisiae*. The mating response is mediated by free Ste4p/Ste18p dimer, which can be generated by pheromone induction, Gpa1p inactivation or Ste4p overproduction (Nakayama *et al.*, 1988; Nomoto *et al.*, 1990). These events lead to transcriptional activation of genes, changes in morphology (*shmoo* formation), and growth arrest. Lack of Ste4p gives rise to the inability to respond to sex pheromone and therefore to sterility (Whiteway *et al.*, 1989).

The gene encoding the heterotrimeric G-protein β subunit *KlSTE4* from *K. lactis* was cloned, and its role in mating was analysed (Kawasaki *et al.*, 2005). In order to analyse the function of KlSte4p, a disruption mutant was created by homologous recombination. Mutant strains of both *MAT***a** and *MAT*α cells were sterile, but had no defect in vegetative growth. The full ORF is 1310 bp long, including the stop codon, and encodes a putative protein of 436 amino acid residues whose predicted molecular weight is 48.6 kDa. The primary structure of the protein shows identity with reported Gβ subunits from different sources and shows the WD motif arrangement characteristic of this family. The predicted KlSte4 protein is 63% similar and 52% identical to Ste4p from *S. cerevisiae*.

KlSte4p shows amino acid residues that in *S. cerevisiae* have been involved in the association with the Gpa1p subunit, as well as those involved in the interaction with the Gγ (Ste18p) subunit (Leberer *et al.*, 1992; Whiteway *et al.*, 1994). Moreover, the KlSte4 protein contains a coil-coiled N terminus with a similar length to that of ScSte4p, which has been implicated in functional interaction with the Gγ subunit (Coria *et al.*, 1996; Lambright *et al.*, 1996). KlSte4p also contains residues that in ScSte4 have been involved in association with the protein kinase Ste20p, which is required to link the G-protein to downstream signalling components (see below) (Leberer *et al.*, 1992). Interestingly, KlSte4p also conserves the residues H^{403}, R^{406} and W^{424}, which have been implicated in the conformational change of mammalian Gβ for effector interaction (Myung & Garrison, 2000).

The ScSte18p subunit is an essential component in the mating pheromone response pathway of *S. cerevisiae*. A *ste18* mutant strain is unable to respond to pheromone (Whiteway *et al.*, 1989). ScSte18p is post-translationally modified by a reaction that farnesylates the cysteine residue of the CAAX motif located at its C terminus, trimming the three C-terminal amino acids, and carboxymethylating the now C-terminally located polyisoprenyl-cysteine (Sanford *et al.*, 1991). KlSte18p shows 53% identity with ScSte18p and contains the CAAX polyisoprenylation signal at its C terminus. Unpublished observations have suggested that KlSte18p may interact with the KlSte4p subunit in order to form the heterodimer. However, inactivation experiments point to the hypothesis that KlSte18p is not essential for the mating response, suggesting that either the KlSte4p subunit is directly modified for membrane targetting, or KlSte4p is not required at the plasma membrane to trigger the

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pheromone response. These hypotheses are expected to be tested by analysing the structure of the active Gβ subunit.

The downstream effects of the G protein

In the yeast *S. cerevisiae*, following G protein activation by the sexual pheromone, the membrane-bound Gβγ dimer transmits the signal to at least three different effectors: the Ste20 protein kinase, a Ste5p/Ste11p complex, and a Far1/Cdc24 complex. Whereas the farnesylated and palmitoylated Ste18p anchors the Gβγ dimer to the membrane, the Ste4p binds to each of the effectors. The first effector is the Ste20p kinase, a member of the p21-activated protein kinase (PAK) family (Leberer *et al.*, 2000). Binding of Gβγ to a short conserved motif in the carboxyl terminus of Ste20 is required to initiate the activation cycle of the kinase. This occurs when the CRIB domain of Ste20p binds to Cdc42 [a small Rho-like G protein (Ash *et al.*, 2003), which is attached to the membrane], uncovering its activation loop, and thereby permitting autophosphorylation (Menser *et al.*, 1994). We have observed that *K. lactis* expresses the KlSte20p kinase, which is 50% identical to ScSte20p. It shows a fully conserved Gβ binding motif at its carboxyl terminus and high conservation of the CRIB and kinase domains located in the N-terminal and C-terminal regions, respectively (Lamson *et al.*, 2002). Studies on the participation of this KlSte20p kinase in the mating process of *K. lactis* are currently in progress.

The second effector of Gβγ in *S. cerevisiae* is Ste5p. This protein functions as a scaffold, forming a complex with a MAP kinase module (mitogen activated protein kinases) composed of Ste11p (MAPKKK), Ste7p (MAPKK), and Fus3p (MAPK) (Choi *et al.*, 1994; Kranz *et al.*, 1994). Ste5p has no catalytic activity but acts in the first instance as an adapter, binding to both Gβ and to Ste11 protein kinase. As a result, Ste11p is located near the membrane and is phosphorylated and activated by the Ste20p kinase (Pryciak & Huntress, 1998). We have characterized a *K. lactis* mutant lacking the *KlSTE5* gene. As expected, it fails to respond to pheromone and therefore is unable to mate (unpublished results).

Genes coding for the conserved MAP kinase components have been found after a blast search of the *K. lactis* genome data base. We identified the putative homologues KlSte11p, KlSte7p and KlFus3p, which are 58, 52 and 69% identical to the corresponding *S. cerevisiae* proteins (Table 1).

In the pheromone response pathway of *S. cerevisiae*, a key substrate of Fus3p (MAPK) is the Ste12p/Dig1p and Dig2p transcription factor complex, which is required to selectively induce transcription of genes required for mating. Strains lacking the Ste12 transcription factor are defective in mating (Kirkman-Correja *et al.*, 1993). Ste12p binds to a DNA motif (A/TGAAACA) in the promoter regions of genes required for mating (Harrison & DeLisi, 2002). Dig1p and Dig2p seem to regulate Ste12p activity negatively (Tedford *et al.*, 1997).

So far, studies on the transcription activation complex in *K. lactis* were ascribed to KlSte12p. This gene was cloned and expressed in sterile *S. cerevisiae* cells lacking their native Ste12 protein, and shown to restore diploid formation in a mating type-dependent manner (Yuan *et al.*, 1993). Later, a *Klste12* mutant was constructed that showed a strong effect on the mating efficiency of haploid cells of the two mating types, indicating that this product is essential for proper mating (Lloret *et al.*, 2003). Wild-type cells overexpressing KlSte12p showed increased mating efficiency, and increased expression of the *KlFUS1* gene (required for haploid cell fusion, see below). Based on these studies it was suggested that KlSte12p is an essential element to elicit the pheromone mating response.

Both Gα and Gβ signal through KlSte12

The data obtained so far indicate that in *K. lactis* both KlSte4p and KlGpa1p subunits are required to induce mating by activating the transcription factor KlSte12p. As neither Gα nor Gβ can bypass the lack of KlSte12p, it appears that this transcription factor is a common element in the pathways regulated by both subunits (Saviñón-Tejeda *et al.*, 2001; Lloret *et al.*, 2003; unpublished data). This has been confirmed by epistasis experiments and by determination of the mating phenotype of Δ*Klgpa1*, Δ*Klste4*, Δ*Klste12* mutants and the combination of double mutants. For example, the Δ*Klste4* mutant is not rescued by the constitutively active form of KlGpa1p, and, conversely, the overexpression of KlSte4p does not rescue the Δ*Klgpa1*. By contrast, the transcription of the *KlSTE4* gene is stimulated by the active form of the KlGpa1p subunit, and this stimulation is not observed in a *Klste12* background, indicating that the transcription factor is required for the enhanced transcription of Gβ during the pheromone response process. These studies indicate that at least a partial role of KlGpa1p is to overinduce transcription of *KlSTE4*, via the KlSte12p transcription factor.

In conclusion, the *K. lactis* pheromone response pathway may be summarized as follows: the binding of pheromone to its receptor induces the activation of the Gα and Gβ subunits, triggering two different signal transduction cascades, which converge in the KlSte12p transcription factor that finally induces the expression of genes required for the mating of haploid *K. lactis* cells. For the Gβ subunit this activation is insufficient to trigger the response, and it requires increased transcription to reach a critical concentration. This occurs by stimulation of the KlSte12p transcription factor mediated by the active form of the KlGpa1p subunit. Future studies are needed to elucidate whether both

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the Gα and Gβ subunits activate the KlSte12p factor through the MAP kinase module, or through different intermediate proteins.

KlFus1p is a pheromone response element

Fus1p is a member of the set of genes induced at the transcriptional level by sexual pheromone (Hagen *et al.*, 1991). As a result of stimulation by sexual pheromones, activation of the Ste12 transcription complex triggers directional cell growth in cells of both mating types, resulting in the development of a mating projection directed towards the mating partner (Jackson & Hartwell, 1990). After contact of mating partners, there follows remodelling of the cell wall and fusion of the plasma membranes to form a single zygote. A number of gene products, including Fus1p, are required for cell fusion during yeast mating. Consistent with its role, inactivation of ScFus1p results in defective haploid mating and diploid formation. Fus1p-deficient mutants show only moderate mating impairment when the two mating partners harbor the mutated allele, and this defect is practically imperceptible in crosses between a wild-type strain and a *fus1* partner.

Inactivation of the *Klfus1* gene significantly affects mating, not only between mating parents that both harbor mutated alleles, but also between wild-type and *Klfus1* parents. Still, in these last crosses diploid formation occurs, suggesting that some other gene products might be participating in the cell fusion process in *K. lactis*.

Expression of the KlFus1 gene is dependent on the sexual pheromone stimulus. Moreover, it requires a functional KlSte12 transcription factor and the active forms of both KlGpa1p and KlSte4p subunits (Saviñón-Tejeda *et al.*, 2001; Lloret *et al.*, 2003; unpublished data).

The mating defect of the *S. cerevisiae fus1* mutant is significantly reversed by expression of *KlFUS1*, suggesting that this last protein might have a role in the cell conjugation process of *K. lactis* equivalent to that of ScFus1p in *S. cerevisiae*.

The primary structure of KlFus1p predicts a membrane protein with a single plasma membrane-spanning segment, leading to an amino terminus that faces the periplasma and a long C terminus pointing to the cytoplasmic side. Although no significant homology was found in the amino terminus of the KlFus1p and ScFus1p proteins, the presence of a number of serine and threonine residues in the amino terminus of KlFus1p suggests that this protein may also be glycosylated and projected to the periplasmic space as ScFus1p (Trueheart & Fink, 1989). The highest identity between the two proteins is found in three regions that lie in the cytoplasmic portions. One is located within the KlFus1p residues 204-227, the second within residues 270-286, and the third at the very last 60 residues of the carboxyl terminus. This last region appears to contain an Src homology 3 (SH3) domain (Rodaway *et al.*, 1989), characteristic of motifs involved in protein–protein interactions of molecules that participate in the control of cytoskeletal organization (Mayer & Baltimore, 1993). Fluorescence determination of KlFus1–GFP fusions suggests that the native protein localizes in the plasma membrane in mating-activated cells and moves to the cell projection in elongated cells (Lloret *et al.*, 2003).

The putative α pheromone gene

Mating in *S. cerevisiae* is initiated by the secretion of diffusible peptide pheromones, known as α- and a-factors, by haploid cells of *MATα* and *MATa* types, respectively, which are recognized by GPCR receptors. The mating pheromones are absolutely required to trigger the mating response (Stotzler & Duntze, 1976; Kurjan & Herskowitz, 1982; Anderegg *et al.*, 1988).

A blast search of the *K. lactis* genome database, using the *S. cerevisiae* mature pheromone sequence, allowed the identification of a putative gene that encodes the α-pheromone. The homologous sequence is found within the predicted ORF KLLA0E19173g, as a tandem repeat of 27-27-29-13 amino acid residues. This tandem repeat is located in the second half of a predicted ORF of 187 amino acid residues, suggesting that α-pheromone is synthesized as a precursor and cleaved to produce the mature peptide. By sequence homology, the putative *K. lactis* pheromone is also a peptide of 13 amino acids with seven residues identical to those of the *S. cerevisiae* α-factor. This observation indicates that the α-factors from both yeast species have sequence similarities, suggesting evolutionary relatedness:

Sc α-pher : WHWLQLKPGQPMY,
*Kl*α-pher : W S W I TLRPGQP I F.

The receptors KlSte2p and ScSte2p are 48% identical. Studies using chimeric receptors and pheromones from *S. cerevisiae* and *S. kluyveri* indicated that important elements for pheromone recognition are amino acids 47, 48 and 49 of the respective GPCRs (reviewed in Naider & Becker, 2004). These residues in *S. cerevisiae* are STV, whereas in the putative KlSte2p receptor they are ENI. These residues may be important for interaction with one of the non-conserved residues of the *K.lactis* α-factor.

Outlook

The signal transduction pathways in *K. lactis* share conserved elements found in signalling pathways in other fungal species, especially in *S. cerevisiae*. However, *K. lactis* has shown characteristics special and unique among fungal species. One of the most striking characteristics is the role

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of the KlGpa1 subunit in the mating pathway. This was inferred from genetic studies of a *Klgpa1*-disrupted strain, where disruption of the *KlGpa1* allele resulted in haploid viable cells with a deficiency in mating. By contrast, Gpa1p from *S. cerevisiae* has a negative role in the pheromone response pathway; that is, disruption of the gene causes arrest in the G1 phase, with a transient ability to mate, whereas its overexpression reduces pheromone response and mating.

The pheromone response pathway involves different types of G protein signalling. In *S. cerevisiae* the pheromone signal during mating is transduced by the free βγ dimer, in *Schizosaccharomyces pombe* by a Gα subunit in concert with a Ras protein, in *U. maydis* by a stimulatory Gα subunit that activates cAMP production, and in *K. lactis* by the Gα and the Gβ subunits, most probably acting in parallel.

Finally, the determination of the complete nucleotide sequence of the *K. lactis* genome provided opportunities for the study of the components of the pheromone response pathway.

Acknowledgements

This work was partially funded by grants 44178 from CONACyT, Mexico, and PAPIIT IN209602 from DGAPA, UNAM.

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“Kluyveromyces lactis sexual pheromones. Gene structures and cellular responses to alpha-factor”

Kluyveromyces lactis sexual pheromones. Gene structures and cellular responses to α-factor

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Received 21 December 2006; revised 19 February 2007; accepted 15 March 2007. First published online 16 May 2007.

DOI:10.1111/j.1567-1364.2007.00249.x

Editor: Hiroshi Fukuhara

Keywords
α-pheromone; **a**-pheromone; *Kluyveromyces lactis*; mating; signal transduction.

Abstract

The *Kluyveromyces lactis* genes for sexual pheromones have been analyzed. The α-factor gene encodes a predicted polypeptide of 187 amino acid residues containing four tridecapeptide repeats (WSWITLRPGQPIF). A nucleotide BLAST search of the entire *K. lactis* genome sequence allowed the identification of the nonannotated putative **a**-pheromone gene that encodes a predicted protein of 33 residues containing one copy of the dodecapeptide **a**-factor (WIIPGFVWVPQC). The role of the *K. lactis* structural genes *KlMFα1* and *KlMFA1* in mating has been investigated by the construction of disruption mutations that totally eliminate gene functions. Mutants of both alleles showed sex-dependent sterility, indicating that these are single-copy genes and essential for mating. *MATα*, *Klsst2* mutants, which, by analogy to *Saccharomyces cerevisiae*, are defective in Gα-GTPase activity, showed increased sensitivity to synthetic α-factor and increased capacity to mate. Additionally, *Klbar1* mutants (putatively defective in α-pheromone proteolysis) showed delay in mating but sensitivity to α-pheromone. From these results, it can be deduced that the *K. lactis MAT**a*** cell produces the homolog of the *S. cerevisiae* α-pheromone, whereas the *MATα* cell produces the **a**-pheromone.

Introduction

Mating of haploid cells of *Saccharomyces cerevisiae* is initiated by the secretion of diffusible peptide pheromones that are recognized by receptors on the opposite cell type. The mating pheromones (known as α-factor and **a**-factor) are absolutely required to trigger the mating process; cells that cannot produce these molecules or lack their specific receptors (Ste2p for α-factor or Ste3p for **a**-factor) are sterile (Sprague, 1991). The binding of pheromones to their cognate receptors stimulates several responses, including changes in transcription, growth arrest, and polarized morphogenesis. A receptor G-protein-coupled mitogen-activated protein kinase cascade mediates all the responses to pheromones (Herskowitz, 1995).

The best characterized mating pheromones are those of *Sa. cerevisiae*. The α-factor is encoded by genes *MFα1* and *MFα2* (Kurjan & Herskowitz, 1982; Singh *et al*., 1983), whereas the **a**-factor is encoded by genes *MFA1* and *MFA2* (Michaelis & Herskowitz, 1988). Both pheromones are synthesized as precursors that undergo an ordered set of maturation events (Sprague & Thorner, 1992). In the case of the α-factor, the maturation process includes signal sequence cleavage, glycosylation, and proteolytic processing by three peptidases (Kex2p, Kex1p, and Ste13p) during the transit of the α-factor through the secretory pathway (Fuller *et al*., 1988). During **a**-factor biogenesis, the **a**-factor precursor undergoes prenylation, proteolytic cleavage of the universal motif AAX, carboxylmethylation in the C-terminus, and two proteolytic processing events at the N-terminus (Chen *et al*., 1997).

The mature pheromones are small peptides that interact with a binding pocket formed by the extracellular loops and the extracellular side of some transmembrane regions of the receptor (Naider & Becker, 2004). The mature α-pheromone is a 13-residue peptide composed of two variants; the most abundant is encoded by the *MFα1* gene (WHWLQLKPGQPMY), and its sequence variant (N5R7) is encoded by the *MFα2* gene. Both genes are expressed only in α-cells (Jarvis *et al*., 1988), and their products seem to have the same biological activity (Raths *et al*., 1986).

Two forms of the mature dodecapeptide **a**-factor are also present in *Sa. cerevisiae*, differing in one residue:

YIIKGLFWDPAC, encoded by *MFA1*, and its variant V6, encoded by *MFA2*. These genes are expressed only in *MAT**a*** cells (Fields *et al.*, 1988).

There are significant structural homologies in genes encoding sexual pheromones in a variety of fungal species, e.g. *Candida albicans* (Bennett *et al.*, 2003; Panwar *et al.*, 2003), *Schizosaccharomyces pombe* (Davey, 1998), *Magnaporthe grisea* (Shen *et al.*, 1999), and *Cryptococcus neoformans* (Davidson *et al.*, 2000). In general, one pheromone tends to be farnesylated at its C-terminus, and the other is synthesized as a precursor that has to be cleaved by specific peptidases.

The budding yeast *Kluyveromyces lactis* is a unicellular, haploid organism with a conventionally organized cell cycle, and it is easily subjected to genetic analysis (Wésolowski-Louvel *et al.*, 1996). *MAT**a*** and *MAT*α cells undergo mating when they are mixed together in the same medium. The Gα (Saviñón-Tejeda *et al.*, 2001) and the Gβ (Kawasaki *et al.*, 2005) subunits of the heterotrimeric G-protein trigger the pheromone signal to downstream effectors. Sex-specific G-Protein-coupled (GPC) receptors located at the plasma membrane are essential for this process (Torres-Quiroz *et al.*, 2007). Both G-protein subunits function in the mating pathway via the KlSte12p transcription factor. This factor is thought to bind to pheromone response elements located in promoter regions of genes required for mating.

The induction of growth arrest and polarized morphogenesis by the activation of the pheromone response in *K. lactis* is not evident, neither by mixing *MAT**a*** and *MAT*α haploid cells nor by overexpression of the Gβ subunit (Kawasaki *et al.*, 2005). However, expression of a constitutively active form of the Gα subunit in a Δ*G*α background is able to induce weak growth arrest (Saviñón-Tejeda *et al.*, 2001).

To further characterize the mating response pathway in *K. lactis*, in this work we analyzed the genes encoding the sexual pheromones, constructed disruption mutants, and determined the physiologic responses to synthetic α-factor.

Materials and methods

Strains and media

The yeast strains used in this work were *K. lactis* 155 (*MAT*α, *ade2*, *his3*, *ura3*) and 12/8 (*MAT**a***, *lysA*, *argA*, *ura3*). *Escherichia coli* strains DH5α and Gm33 (for preparation of nonmethylated DNA) were used to propagate plasmids. YPD medium consisted of 1% yeast extract, 2% Bacto peptone and 2% glucose. SD minimal medium consisted of 0.67% yeast nitrogen base without amino acids (Difco) and 2% glucose. SD minimal media were supplemented with the required amino acids and nitrogen bases (50 μg mL^{-1}). Luria–Bertani broth plus ampicillin (100 μg mL^{-1}) was used to propagate recombinant plasmids in bacteria.

Gene disruptions

Putative *KlMF*α*1*, *KlMFA1*, *KlSST2* and *KlBAR1* gene disruptions were achieved by homologous recombination introducing the *URA3* marker. For *KlMF*α*1*, a 1087-bp fragment containing the full ORF, plus 500 bp of the 5′-untranslated region (UTR) and 30 bp of the 3′-UTR, was obtained by PCR amplification and cloned into the pGEM-T-Easy vector (Promega). The EcoRI fragment was then introduced into the YIp352 vector, and PCR amplification was performed, employing divergent primers designed at positions −87 to −103 (reverse complement) and +170 to +187 (coding strand). This generated a linear product that contained the full YIp352 plasmid flanked by 430-bp and 429-bp recombinant ends. For *KlMFA1*, a 510-bp fragment containing the putative full ORF, 188 bp of the 5′-UTR and 221 bp of the 3′-UTR was PCR-amplified using primers that introduce EcoRI sites at positions −174 and +312. The product was ligated into the pGEM-T-Easy vector and subcloned as an EcoRI fragment into YIp352, in which the HindIII site had been previously eliminated. This last plasmid was digested at the naturally occurring HindIII (position +18) and BclI (position +157) sites, giving a linear molecule containing the YIp352 plasmid flanked by 190 bp and 150 bp of recombinant ends. For *KlBAR1*, a 950-bp fragment was amplified using deoxyoligonucleotides designed from positions +230 (forward) to +1183 (reverse). This fragment was ligated into the pGEM-T-Easy vector. An 823-bp EcoRI–BamHI fragment was then ligated into the YIp352 vector prepared with the same enzymes. The YIp352–*KlBAR1* plasmid was then digested with BglII and SacI, giving a linear molecule that contained the full YIp352 flanked by *KlBAR1* recombinant ends of 185 bp and 220 bp. *KlSST2* was amplified using primers designed at positions −20 (forward) and +2188 (reverse). The 2208-bp fragment was ligated into the pGEM-T-Easy vector. *KlSST2* was then ligated as a NotI–PstI (filled in at NotI) fragment into YIp352 digested with SmaI and PstI. The resulting construct was opened at the naturally occurring BclI and SpeI sites, leaving 290 bp and 418 bp as recombinant ends.

Complementation tests

An 1087-bp EcoRI fragment containing the full *KlMF*α*1* ORF flanked by 500 bp of the 5′-UTR and 30 bp of the 3′-UTR was obtained from the pGEM-T-Easy plasmid and was subcloned into YEpKDHis (described in Kawasaki *et al.*, 2005) digested with the same enzyme. An EcoRI fragment containing the full *KlMFA1* ORF, flanked by upstream and downstream sequences of 188 and 221 bp, respectively, was obtained from the pGEM-T-Easy plasmid and was subcloned into the YEpKDHis vector. These constructs were introduced into disruptant yeast strains for mating assays.

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Table 1. Proven and putative genes of the *Kluyveromyces lactis* pheromone response pathway

Gene name	Product	Mutant phenotype*	Relevant mating type affected	Identity (%)	*E*-value	ORF in the *K. lactis* database
KlMFα1	α-Pheromone	Sterile	*MATa*	46†	1e-42	KLLA0E19173g
KlMFA1	*a*-Pheromone	Sterile	*MATα*	45†	2e-05	Nonannotated
KlKEX2	KR endopeptidase	ND	ND	57	0	KLLA0D19811g
KlKEX1	Carboxypeptidase	ND	ND	44	1e-162	KLLA0F09999g
KlSTE13	Dipeptidyl aminopeptidase	ND	ND	39	0	KLLA0D06919g
KlRAM1	Farnesyl-transferase β subunit	ND	ND	50	1e-119	KLLA0F07161g
KlRAM2	Farnesyl-transferase α subunit	ND	ND	50	2e-87	KLLA0E18051
KlSTE14	Farnesyl cysteine carboxyl-methyltransferase	ND	ND	53	8e-75	KLLA0A02167g
KlSTE24	Zinc metalloprotease	ND	ND	69	0	KLLA0D10846g
KlAXL1	Protease	ND	ND	39	0	KLLA0D15631g
KlSTE6	ATP-binding transporter	ND	ND	47	0	KLLA0B14256g
KlBAR1	α-Pheromone endopeptidase	Sensitivity to α-pheromone	*MATα*	35	4e-90	KLLA0D15917g
KlSST2	G-protein regulator	Sensitivity to α-pheromone	*MATα* and *MATa*	41	1e-152	KLLA0D10549g

*Phenotypes relevant to this work, particularly concerning response to α-factor.
†Calculated identity of the precursor protein.

Mating assays

A patch of cells of the strain to be tested was grown on a plate of selective medium for 24 h. The tester strain was grown as a lawn on a YPD plate for 24 h. Both strains were replica-plated onto YPD plates and incubated for different times at 30 °C, allowing cells to mate. Diploids were selected on SD medium by replica plating.

α-Pheromone assays

Synthetic α-pheromone was obtained from GenScrip Corp at 95.5% purity, and suspended in water. For these assays, cells were suspended in YPD medium at a density of 10^6 cells/100 μL, and pheromone was added to a final concentration of 100 μg mL^{-1}. Cells were examined under phase-contrast microscopy and photographed at different times.

Other methods

All PCR products were sequenced in full at the Molecular Biology Facility, IFC, UNAM. Disruption mutants were confirmed by Southern blot. Probes for Southern analysis were labeled with [α-^{32}P]dCTP with the Random Prime Labeling System (Rediprime II, Amersham Biosciences). Standard Southern blot analysis, recombinant DNA technology and yeast genetics procedures were also performed.

Results and Discussion

Mating in *K. lactis* is relatively unknown compared to that in *Sa. cerevisiae*, where the mating pathway has been studied in detail. The first step that triggers mating is sensing of the diffusible pheromones secreted by cells of the opposite mating type. Cells that cannot produce these molecules are sterile (Sprague, 1991). In the current study, we characterized the mating pheromone genes of *K. lactis*, investigated the phenotypic effects of mutations in the structural genes, and constructed mutations that were sensitive to synthetic α-factor. The genes described in this article are listed in Table 1, including those that, by sequence homology, can be predicted to participate in the pheromone maturation process. All genes were identified by BLAST analyses of the *K. lactis* genome sequence database (http://cbi.labri.fr/Genolevures/elt/KLLA).

The gene encoding the α-factor

A BLAST search of the *K. lactis* genome database using the *Sa. cerevisiae* mature pheromone sequence allowed the identification of a single-copy gene in *K. lactis* with structural similarities to the α-mating pheromone from *Sa. cerevisiae*. The homologous sequence was designated *KlMFα1*. Analysis of the sequence revealed a 564-bp ORF that codes for four identical tridecapeptide repeats separated by spacer sequences of eight, 14, 14 and 16 amino acid residues (Fig. 1a). These tandem repeats are located in the second half of a predicted 187 amino acid polypeptide, suggesting that α-pheromone is synthesized as a precursor and cleaved to produce the mature peptide. In agreement with what is known in *Sa. cerevisiae* (Sprague & Thorner, 1992), the *K. lactis* precursor has an N-terminal hydrophobic sequence that can act as signal sequence, two N-X-T motifs for attachment of asparagine-linked carbohydrate, and a segment containing the four α-factor repeats, separated from each other by spacer sequences (Fig. 1a). Each spacer begins

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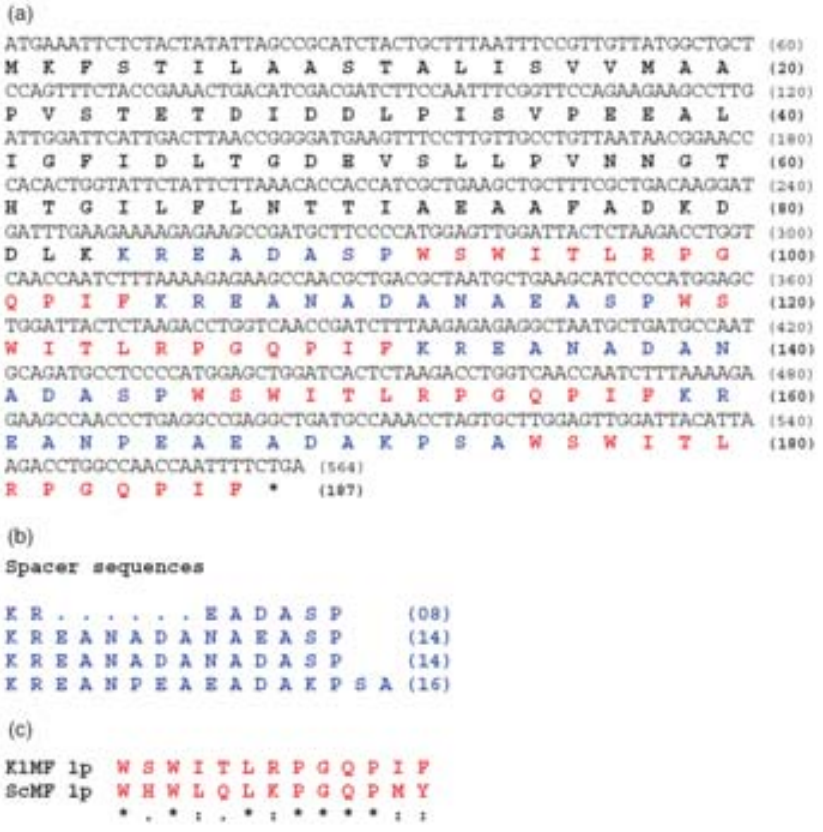

Fig. 1. (a) Nucleotide sequence of the *MFα1* gene and its deduced amino acid sequence. (b) Alignment of putative spacer sequences of α-pheromone. (c) Alignment of mature α-pheromones from *Kluyveromyces lactis* and *Saccharomyces cerevisiae*. Spacer sequences are marked in blue, and mature pheromones are marked in red. *identical amino acids; :, amino acids with similar polarity; ., amino acids with different polarity.

with the basic KR residues, which are followed by two, four or five X-A dipeptides (Fig. 1b). Although the *K. lactis* precursor differs in amino acid sequence from that of *Sa. cerevisiae*, their overall structures are well conserved. The same is true for fungal species as distant as *Ca. albicans* (Bennett *et al.*, 2003; Panwar *et al.*, 2003) and *Sc. pombe* (Davey, 1998). This suggests that the maturation pathway of the α-factor is also well conserved between species. In the case of the *Sa. cerevisiae* pheromone, this pathway includes three proteolytic events: (1) excision of the pheromone repeats by the KR endopeptidase Kex2p (Julius *et al.*, 1984); (2) trimming of the generated C-termini by carboxypeptidase Kex1p (Wagner & Wolf, 1987); and (3) trimming of the N-termini by the action of the dipeptidyl aminopeptidase A, Ste13p (Julius *et al.*, 1983). All of these processing steps can be predicted for the maturation of the *K. lactis* α-pheromone on the basis of the existence of homologous genes for the three peptidases (Table 1).

The putative *K. lactis* α-pheromone is also a tridecapeptide with seven residues identical to those of the *Sa. cerevisiae* α-factor encoded by the *MFα1* gene (Fig. 1c). Studies involving the replacement of each residue of the mature *Sa. cerevisiae* α-factor by L-alanine or D-alanine residues (Naider & Becker, 2004), along with photoaffinity labeling and site-directed mutagenesis studies, have led to the construction of a model for the structure of the peptide bound to its receptor (Ste2p). Three domains are seen in this model: the signaling domain, composed of residues 1–4; the loop domain, composed of residues 7–10; and the binding domain, composed of residues 10–13. According to this model, the nature of the putative interactions involved in receptor and pheromone recognition are conserved in *K. lactis*. First, the W1 and W3 present in both pheromones may contact the conserved aromatic group of the Y residue present in the interface of the sixth transmembrane region (TM6) of both receptors (Torres-Quiroz *et al.*, 2007). Second, Q10 of both factors may contact the receptor's polar residues present on the outside surface of the first transmembrane segment (S and T in *Sa. cerevisiae*; E and N in *K. lactis*). Finally, the aromatic group of Y13 of the *Sa. cerevisiae* α-factor might contact the aromatic F and Y residues located at the extracellular side of the fifth and sixth ScSte2p transmembrane regions (TM5 and TM6), respectively (Naider & Becker, 2004). Interestingly, position 13 of the *K. lactis* pheromone is F, and its putative receptor-interacting residues are Y in TM5 and Y in TM6 (Torres-Quiroz *et al.*, 2007). Regardless of the moderate conservation of the primary sequence of α-factors (50%),

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some of the nonconserved residues could play important roles in species-specific interactions with cognate receptors, and should contribute to the sterility barrier between these two yeasts.

The gene encoding the a-factor

A TBLASTX search of the entire *K. lactis* genome sequence, using the nucleotide sequence of the *ScMFA1* gene as query, allowed us to identify the putative *KlMFA1* gene encoding the **a**-factor (Table 1). *KlMFA1* is a single-copy gene located in chromosome E (reverse complement, frame 1), coordinates 108 4444–108 4545. The *K. lactis* active **a**-pheromone is thought to be generated from a 33 amino acid precursor, containing one copy of the **a**-factor located in its C-terminal end (Fig. 2a). The structure of the gene shows conservation with that of *Sa. cerevisiae*, and even with those of distant fungal species such as *Sc. pombe* (Davey, 1998), *Cr. neoformans* (Davidson *et al.*, 2000), and *Ustilago maydis* (Spellig *et al.*, 1994). The C-terminus of the **a**-precursor contains the universal CAAX motif (CVVA) that is characteristic of these peptides. The maturation pathway of the *Sa. cerevisiae* **a**-factor is very well known. Processing of the CAAX motif involves farnesylation via a thioether linkage of the cysteine residue (Anderegg *et al.*, 1988), proteolysis of the C-terminal tripeptide (AAX), and methyl esterification of the exposed carboxyl group (Sprague & Thorner, 1992). Finally, proteolytic cleavage of the N-terminus, carried out in a two-step process, produces the mature pheromone, which is exported from the cell. All genes encoding the enzymes that participate in *Sa. cerevisiae* **a**-factor processing are present in the *K. lactis* genome: the KlRam1p and KlRam2p farnesyl-transferase β and α subunits respectively; the KlSte14 farnesyl cysteine carboxyl-methyltransferase; the KlSte24 zinc metalloprotease; the KlAxd1 protease; and the KlSte6 ATP-binding transporter (Table 1). The putative mature **a**-pheromone is a dodecapeptide with 50% identity and 56% similarity to the **a**-pheromone from *Sa. cerevisiae* (Fig. 2b).

(a)

```
ATGCAACCAACTCAACAAGCTTCCCAAAATGAATCCGCTGAAAACAAGGAAAAC
M  Q  P  T  Q  Q  A  S  Q  N  E  S  A  E  N  K  E  N
TGGATTATCCCAGGTTTTGTCTGGGTCCCACAATGTGTCGTTGCTTGA
W  I  I  P  G  F  V  W  V  P  Q  C  V  V  A  *
```

(b)

```
KlMfalp     MQP-----TQQASQNESAENKENWIIPGFVWVPQCVVA
ScMfalp     MQPITTASTQATQKDKSSEKKDNYIIKGLFWDPACVIA
            ***      **.:.:::*:*.*:*:**.*..*.*.*.**:*
```

Fig. 2. (a) Nucleotide sequence of the *KlMFA1* gene and its deduced amino acid sequence. (b) Alignment of **a**-pheromone precursors from *Kluyveromyces lactis* and *Saccharomyces cerevisiae*. Mature pheromones are marked in red. *identical amino acids; :, amino acids with similar polarity; ., amino acids with different polarity.

KlMFα1 and *KlMFA1* gene disruptions

In order to ascertain the role of *KlMFα1* and *KlMFA1* in mating, we constructed disruption mutants in both *MATα* and *MAT**a*** strains. Deletion of the *KlMFα1* gene from the typical *K. lactis MATα* cells did not affect the efficiency of mating (Fig. 3). In contrast, mating of the *MAT**a*** cells in which the *KlMFα1* gene had been deleted was much lower than that of the parental strain. Therefore, the *KlMFα1* gene is essential for mating of *MAT**a*** cells in *K. lactis*. On the other hand, deletion of the *KlMFA1* gene solely affected mating of *MATα* cells, and had no effect on the mating of *MAT**a*** cells (Fig. 3), indicating that the *KlMFA1* gene is specifically required for the mating of *MATα* cells. The mating defects of disruptant *MAT**a*** and *MATα* strains were reversed by transfection with plasmidic copies of the wild-type *KlMFα1*

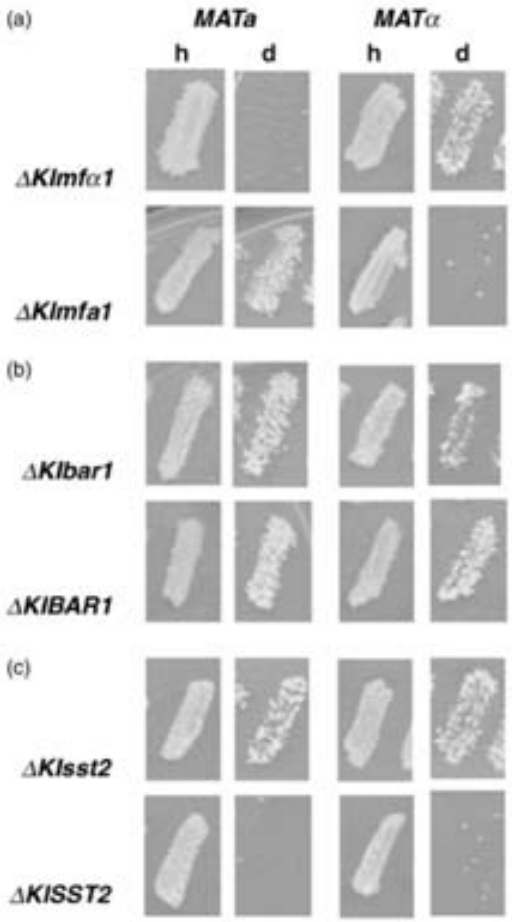

Fig. 3. Effect of disruption of KlMFα1, KlMFA1, *KlBAR1* and *KlSST2* genes on mating. Mating was done by replica plating. Patches of strains to be tested were streaked on selective medium and replicated onto YPD plates containing a lawn of the wild-type tester strain, and this was followed by incubation overnight (a, b) or for 2 h (c) at 30 °C. Diploid selection was done by replica plating onto SD. Plates were photographed 48 h later. h, haploid strains; d, diploid strains.

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and *KlMFA1* genes (not shown). This analysis also supports the suggestion that α-factor and **a**-factor in *K. lactis* are encoded by single-copy genes, whereas in *Sa. cerevisiae*, each pheromone is encoded by two different genes (Kurjan & Herskowitz, 1982; Singh *et al.*, 1983; Michaelis & Herskowitz, 1988). All of the above observations are in agreement with the fact that *K. lactis MAT*α cells express the KlSte2p receptor, whereas the *MAT**a*** cells express the KlSte3p receptor (Torres-Quiroz *et al.*, 2007).

Biological activity of α-factor

In *Sa. cerevisiae*, the α-factor is known to induce growth arrest and morphologic changes (shmoo morphology) in haploid *MAT**a*** cells (Kurjan & Herskowitz, 1982) that are characteristic of the mating process. The appearance of morphologically abnormal cells allows a sensitive method for the assay of α-factor activity. The tridecapeptide WSWITLRPGQPIF was synthesized *in vitro* and added at different concentrations to wild-type *K. lactis* cells of both mating types, and growth arrest and induction of shmoo morphology were looked for. Wild-type cells of both mating types failed to show any response to synthetic α-factor, even at concentrations as high as 100 $\mu g\,mL^{-1}$.

These observations indicate that *K. lactis* wild-type cells are refractory to synthetic α-factor. An additional observation supporting the above result is that, in contrast to what happens in *Sa. cerevisiae*, where mating products can be obtained in a short time (2 h can be sufficient to obtain good efficiencies), efficient mating of *K. lactis* sexual partners is obtained after long incubation periods (over 8 h). Although it is reasonable to assume that mating partners should go through the shmoo stage during mating, microscopy examinations of mating mixtures at different times of incubation did not show obvious shmoo morphology (not shown), suggesting that this stage is quite transient in *K. lactis*.

The molecular basis for the pheromone-resistance phenomenon can be explained, at least in part, by two different mechanisms. First, *Sa. cerevisiae* cells of mating type **a** secrete a protease (Bar1p) that hydrolyzes the α-factor (Ciejek & Thorner, 1979). Mutants deficient in this 'barrier activity' (*bar1* mutants) are highly sensitive to α-pheromone (Chan & Otte, 1982). This protease was thought to be involved in the recovery of yeast cells from the pheromone-induced cell cycle arrest that is part of the premating response. Second, cells exposed to mating pheromones become desensitized after a period of time by different feedback events, including negative regulation of the pheromone response by the activity of Sst2p. Sst2p belongs to the designated RGS family of proteins (regulators of G-protein signaling). Sst2p physically interacts with Gpa1, the α subunit of the heterotrimeric G-protein that is involved in the pheromone response, and induces its intrinsic GTPase activity (Dohlman *et al.*, 1996). Mutants defective in Sst2p activity show high sensitivity to pheromone, and fail to resume growth after exposure to pheromone (Dohlman *et al.*, 1996).

Taking into account the above observations, we reasoned that the natural resistance of *K. lactis* wild-type cells to α-pheromone can be suppressed by inactivating either *KlBAR1* or *KlSST2*.

KlBar1p contains 511 amino acid residues, showing a moderate degree of identity (35%) with ScBar1p (Table 1). By analogy to what is known in *Sa. cerevisiae*, KlBar1p should catalyze the proteolytic cleavage of the L6–R7 bond of the *K. lactis* α-pheromone. KlBar1p conserves the cysteine residues characteristic of the pepsin family that allow the formation of three disulfide loops, and has a very well-conserved active site (ALLDSGSTIM) containing the aspartate residue that is necessary for catalytic activity (Dunn, 2002).

The putative KlSst2p is a predicted 715-residue polypeptide that shows overall 41% identity with ScSst2p (Table 1). Sst2p is a GTPase-accelerating protein that promotes GTP hydrolysis by the α subunit of heterotrimeric G-protein, thereby inactivating the G-protein and rapidly switching off G-protein-coupled receptor signaling pathways (De Vries *et al.*, 2000). ScSst2p contains an 'RGS box' (or RGS domain), which is required for activity, and at least one DEP domain, required for membrane targeting (Burchett, 2000). A putative RGS domain is found in the C-terminal end of KlSst2p (residues 474–715) that shows only 36% identity with the corresponding RGS domain in ScSst2p, suggesting a high specificity of binding and activity over its cognate Gα subunit. The predicted DEP domain found in KlSst2p (residues 297–381) reaches 60% of identity with its counterpart in *Sa. cerevisiae*.

We cloned and disrupted the putative *KlBAR1* and *KlSST2* genes from *K. lactis* wild-type cells of both mating types, determined their mating efficiency, and assayed the biological activity of the synthetic pheromone on these mutants. Deletion of *KlBAR1* did not affect the mating capacity of **a** and α strains, but a slight delay in mating was observed in α cells (Fig. 3). It has been reported that *bar1* α cells in *Sa. cerevisiae* also mate less efficiently with **a** cells in a mass mating mixture (Jackson & Hartwell, 1990). The Δ*Klsst2* mutant, however, showed a significant increase in its mating capacity, seen in our experiments by the high efficiency of mating at 2 h of incubation with the mating partner (Fig. 3). In this time period, wild-type strains mated sporadically and never reached the diploid density observed with the *Klsst2* mutant. The effect of *Klsst2* mutation was independent of the mating type, indicating that KlSst2p plays the same role in **a** and α cells.

Finally, we tested the activity of the synthetic α-factor on cell suspensions of *Klbar1* and *Klsst2* mutants. For this, cells were suspended in YPD medium, and α-pheromone was added to a final concentration of 100 $\mu g\,mL^{-1}$. We observed

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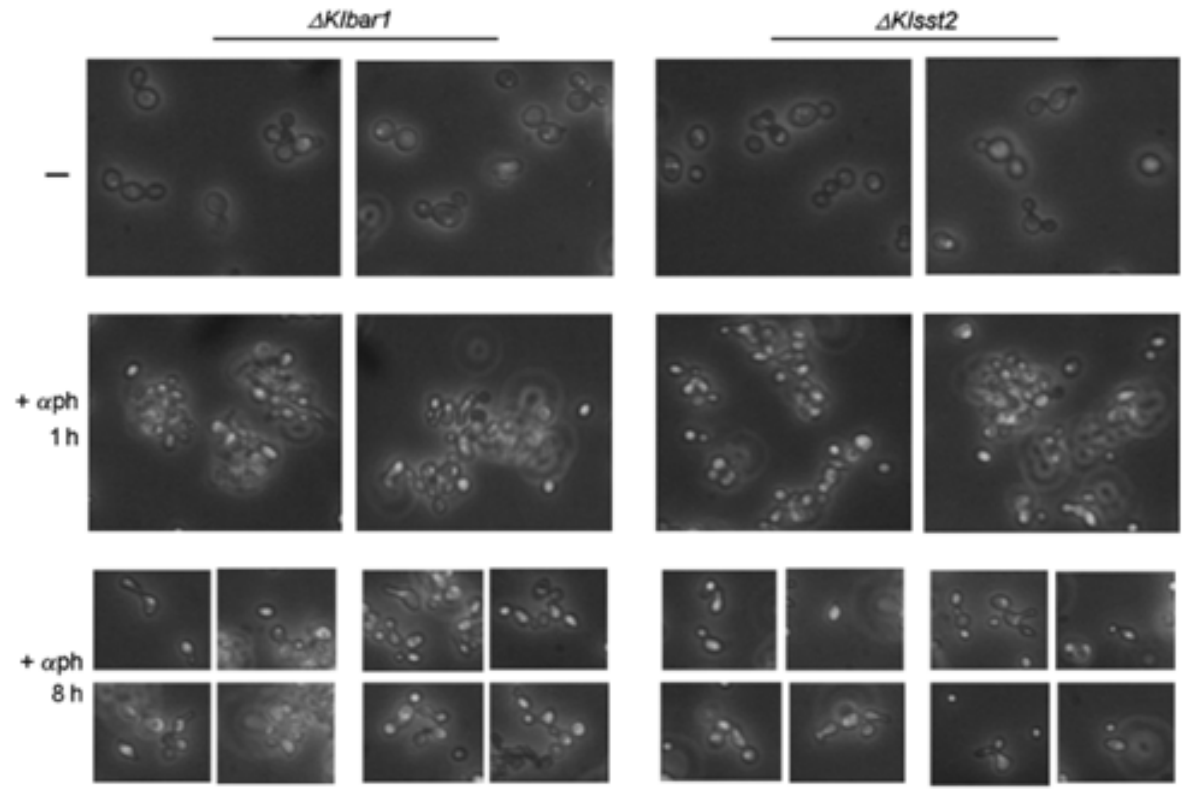

Fig. 4. Effect of synthetic α-pheromone on the morphology of *MAT*α *Klbar1* and *Klsst2* mutants. Upper photographs show mutant strains without α-pheromone. α-Pheromone was added to cell suspensions at a final concentration of 100 μg mL^{-1}, and the suspensions were observed at different times. For simplicity, only images taken at 1 h and 8 h are shown. Photographs were obtained by phase-contrast microscopy.

that only *Klbar1* and *Klsst2* mutants of the *MAT*α type responded to α-factor (Fig. 4), whereas *MAT***a** mutants remain unresponsive. In this experiment, both *Klbar1* and *Klsst2* mutants showed enhanced agglutinability after 1 h of exposure to pheromone. This phenomenon is not observed in wild-type mating mixtures, and has not been reported so far, so we believe that it is due to the artificially high concentration of the peptide pheromone added to the cell suspension. After 4 h of treatment, most cells stopped dividing (determined by the high proportion of unbudded cells, 70–80%, compared to untreated cells, 30–40%), and produced polarized projections that closely resembled the shmoo cells induced by mating pheromones in *Sa. cerevisiae* (Fig. 4). Although individual polarized cells were easily observed after 8 h of treatment (Fig. 4), most cells were agglutinated, rounded and smaller in size (half-size in average) as compared with untreated cells. Prolonged treatment with α-pheromone caused a decrease in agglutination, but cell morphology remained unchanged.

We have identified the genes encoding the sexual pheromones in *K. lactis*, and shown that mutations in *KlBAR1* and *KlSST2* induce sensibility to synthetic α-pheromone. The amino acid sequences of both the α-pheromone and **a**-pheromone are related to those of *Sa. cerevisiae*, and are expected to follow similar processing pathways. Further information on the mechanisms of pheromone activity can be obtained by mutagenic studies oriented towards the natural substitutions that evolution has caused in the pheromones of these two yeast species. This report supports the idea that the general characteristics of pheromone signaling have been closely conserved between yeasts.

Acknowledgements

We thank Guadalupe Codiz and Minerva Mora (Molecular Biology Unit) for technical assistance, and the staff of the Computer Unit at IFC, UNAM. We also thank Marisela Bolaños for technical assistance. This work was partially funded by grants 44178 from CONACyT, and PAPIIT IN211906 from DGAPA, UNAM.

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JUSTIFICACIÓN

En nuestro laboratorio hemos encontrado diferencias en la activación del sistema de respuesta a feromona sexual de la levadura *Kluyveromyces lactis* con respecto a otras levaduras ya descritas. Sin embargo, hasta el momento no se han descrito los receptores del sistema y no sabemos cual es su papel en la activación del sistema. Por otro lado, se sabe que elementos de las vías de transducción de señales se pueden compartir y esto implica mecanismos de regulación para la activación específica de las vías. Estos mecanismos de regulación aun no se han estudiado en *K. lactis*, que es un modelo atractivo de estudio, ya que contamos con el genoma secuenciado y es fácil de manipular genéticamente. Por lo que creemos que el estudio de vías de transducción de señales nos puede ayudar a entender nuevos procesos de formas de regulación en células eucariontes.

OBJETIVOS GENERALES

Identificar los receptores y los componentes de la vía de transducción de señales del sistema de respuesta a feromona sexual de la levadura *Kluyveromyces lactis*.

Determinar si el sistema de respuesta a feromona sexual y el sistema de respuesta a estrés osmótico de la levadura *Kluyveromyces lactis* comparten elementos.

OBJETIVOS ESPECÍFICOS

Clonar e interrumpir los genes que codifican para los receptores de la vía de respuesta a feromona sexual.

Definir si estos receptores pertenecen a la familia de genes sexo específico.

Identificar si existe interacción entre los receptores y las subunidades α y β de la proteína G heterotrimérica del sistema de respuesta a feromona sexual.

Investigar los fenotipos de las mutantes que codifican para cinasas del sistema de respuesta a feromona en condiciones de estrés osmótico.

MATERIALES Y METODOS

Cepas

Escherichia coli: DH5α. *supE*44 Δ*lac*U169(ϕ80 *lacZ*ΔM15) *hsd*R17 recA1 *end*A1 *gyrA*96 *thi*-1 *relA*1 Gm33. *λ-in* (*rrnD-rrnE*)1 *dam*-3 *sup*-85

Saccharomyces cerevisiae: W303-A. *MAT**a**, ade1, ura3, his3, leu2, trp1*.

Kluyveromyces lactis: 155. *MATα, ade2, his3, ura3.*

12/8.*MAT**a**, lysA, ura3, argA.*

Identificación y clonación de genes

Los genes *KlSTE2* y *KlSTE3* se clonaron mediante una búsqueda por BLAST en la secuencia parcial del genoma de *K. lactis* del Proyecto Genolevure I (http://cbi.labri.fr/Genolevures/elt/KLLA). Donde se identificaron dos ORFs con una identidad de 47% y 52.6% con los genes de *S. cerevisiae STE2* y *STE3,* respectivamente. Utilizando los oligonucleótidos descritos en la Tabla 1, se amplificó DNA genómico por medio de PCR, de la cepa silvestre 155. Los productos de PCR se marcaron con [α-^{32}P]dCTP con el sistema de Random Primers (Rediprime™ II, Amersham Biosciences) para utilizarse como sondas en hibridaciones tipo Southern utilizando DNA genómico digerido con varias enzimas. Las digestiones con EcoRI hibridaron en el rango de 2.1 kpb para *KlSTE2* y 3.5 kpb para *KlSTE3*. Se utilizaron fragmentos de estos rangos para ser clonados independientemente en el plásmido pBlueScript. Se hizo una búsqueda por tamizaje para encontrar clonas con el ORF completo de cada uno de los genes y se comprobaron mediante digestión y secuenciación.

Para clonar el resto de los genes que se utilizaron en este trabajo, que corresponden a las cinasas de la cascada de señalización de apareamiento y de estrés osmótico, se hizo una búsqueda por BLAST en la base de datos del genoma de *K. lactis* del Proyecto Genolevure II. Donde se obtuvo la secuencia de genes putativos que codifican para genes ortólogos correspondientes a las proteínas cinasas de S. cerevisiae. Se amplificaron mediante PCR utilizando los oligonucleótidos de la Tabla 1 marcados como c y d de cada gen y se utilizó DNA genómico de una cepa silvestre (155) como templado. Los productos fueron clonados en el vector pGEM®-T Easy Vector (Promega) y secuenciados. Para producir a KlSte7p con una marca de seis histidinas en su extremo carboxilo terminal [KlSte7p(His_6)] se utilizaron los oligonucleótidos Ste7c y Ste7e (Tabla 1). Para generar las mutantes se clonaron los genes en el vector de interrupción YIp352 y se digirieron con enzimas de restricción como se describe en Kawasaki et al., 2008; Torres-Quiroz et al., 2007 excepto con *KlSTE2* que se utilizaron los oligonucleótidos b y c (**Tabla 1)** utilizando como templado el gen clonado en YIp352.

Interacción de Proteínas

Se utilizó el sistema de LexA-B42 de doble híbrido descrito en Kawasaki et al., 2005. Se utilizaron los oligonucleótidos marcados como b y c para poder clonar los genes en el plásmido correspondiente del sistema de doble híbrido (**Tabla1**), a excepción de *KlSTE3* donde se cortó con la enzima de restricción SspI para utilizar sólo el extremo carboxilo terminal (Torres-Quiroz et al., 2007).

Localización de KlSte3p

El gen que codifica para la proteína verde fluorescente (GFP) se fusionó al extremo carboxilo terminal de *KlSTE3* digiriendo con la enzima de restricción ClaI en la posición 1189pb. Este híbrido se subclonó en el vector de expresión YEpKDGal, que permite controlar la transcripción con galactosa (pKlSTE3-GFP). Se transfectó en células Δ*Klste3* y se incubó con 2.5 μg/ml de 4',6-diamidino-2-fenilindol (DAPI) por 2 horas a temperatura ambiente. Las células

se fijaron con el medio para fluorescencia DAKO® (DakoCytomation) y la la fluorescencia de la GFP se observó a través de microscopía confocal.

Ensayos de apareamiento.

Las células a probar se estriaron en cajas con medio selectivo y se incubaron a 30°C durante 24 horas. La cepa testigo se creció como cesped en cajas de medio YPD y se incubaron bajo las mismas condiciones. Posteriormente, las dos cajas fueron replicadas por terciopelo en una caja de YPD a 30°C durante 8 horas, para permitir el apareamiento. Finalmente, se replicaron las células a una caja de medio mínimo SD, con los aminoácidos necesarios para seleccionar diploides. Se crecieron durante 24 horas a 30°C.

Ensayos de estrés osmótico.

Las cepas a probar se crecieron durante toda la noche en medio selectivo. Posteriormente las células fueron lavadas con agua y resuspendidas en YPD a una DO_{600nm} de 0.1 para ser incubadas. Una vez que los cultivos llegaron a una DO_{600nm} de 0.5, se hicieron diluciones seriadas y se gotearon en YPD conteniendo 0.5 M KCl o 1 M D-sorbitol. Las cepas se incubaron a 30ºC. Las cajas se fotografiaron después de 48hr de incubación.

Oligo	Secuencia
Ste2a	(+371) ACAAACATCTTTACGGT (+387)
Ste2b	(+777) TGGACCTATTAATGTC (+762)
Ste2c	(+400) TTAGAGTCACGGCACC (+385)
Ste2d	(+905) CACCAAGTGCCAGTAC (+920)
Ste3a	(+258) TGTTGCCAATATCGCAT (+274)
Ste3b	(+999) TTGAGGGTTGTTAGGTAG (+982)
Ste50a	(+88) CTAGATAGAAccCGgGAGAACAAGATA (+114) SmaI
Ste50b	(+741) TTGATCTCCAAAGCtTATCACTAAAAC (+715) HindIII
Ste50c	(–19) ATAGAAACAAAAACtcgagATGAGCGGTAC(+11) XhoI
Ste50d	(+929) AAGTGTTTTTTCtcGagGATGAAGGAGGAG (+899) XhoI
Ste20a	(–14) TTTGTCTCCtcgagATGACGGATACTGGAT (+16) XhoI
Ste20b	(+3002) TAAATTCATGTAcTGCaGTAAAACAGAAAT (+2972) PstI
Ste5a	(+1) ATGTCTAGAGGTAATAC (+17)
Ste5b	(+2300) GTTTAGAGAGAGAACGG (+2284)
Ste11a	(+360) GCAAAGAAAGcTtACGTTAACGGGT (+385) HindIII
Ste11b	(+1210) TGGATTCTGAGGTACCAG (+1192) Asp718
Ste11c	(–19) TTTTTAAGATCTctcgAgATGAGCAGTGAC (+12) XhoI
Ste11d	(+2222) AGTAACAATACCTCGAGACTGAAAACGGC (+2194) XhoI
Ste7a	(+232) GAGAAGCTTCCGGTCC (+247) HindIII
Ste7b	(+1303) CTTTCAAAGAgGATCcTCCTCTCTCCTC (+1275) BamHI
Ste7c	(–18) AGATTGACTTAActcgAgATGATTACACGT (+12) XhoI
Ste7d	(+1543) CTAATATAGTGCtcgagCTTAATTTATTTC (+1514) XhoI
Ste7e	(+1513) cccgggTCAgtggtggtggtggtggtgTCGCCTTGTACT (+1501) SmaI
Fus3a	(+498) ACAGATGGCGgTaCCCATGTTGACG (+523) Asp718
Fus3b	(+1171) TGATATAATAaGCTtGTGGTTACTTAG (+1144) HindIII
Pbs2a	(–21) GGCGATTAAGTGgtaCCAATTATGAGT (+6) Asp718
Pbs2b	(+2293) GGCGAGGCAAAGgaTccCTTACAATCCGCC (+2264) BamHI
Hog1a	(+490) CTTGCTAGGATcCAAGATCCTCAAATG (+517) BamHI
Hog1b	(+1366) CTTCTGTCAAGCttATTTCATTACATTG (+1339) HindIII
Hog1c	(–18) TTTTCCAATTGAGAATTCATGTCGAATGAG (+12) EcoRI
Hog1d	(+1397) TATACTTCTGTTCCTCGAGATTTCATTAC (+1343) XhoI

Tabla 1. Lista de oligonucleótidos que se utilizaron en este trabajo. Los oligonucleótidos marcados con a y b se utilizaron para realizar interrupciones del gen correspondiente y los oligos c y d se clonaron en plásmidos de doble híbrido. Las excepciones están indicadas en el texto.

Ensayos de Fosforilación.

Para determinar el grado de fosforilación de KlHog1p y el total de ésta proteína, en la cepa silvestre, *ΔKlpbs2* y *ΔKlste7*, cultivos de 5 ml en fase exponencial se centrifugaron y resuspendieron en YPD o YPD con KCl 0.5 M durante diferentes tiempo. Las células se centrifugaron y se resuspendieron en Buffer de carga (SDS 5%, Tris-HCl [pH 7.5] 0.1 M, glicerol 5%, 2-mercaproetanol 0.07 M, azul de bromofenol 0.02 mM), hirviéndolos durante 5 min. Las muestras se centrifugaron y el sobrenadante se tranfirió a tubos limpios. Una alícuota de la muestra fue cargada en geles de electroforesis de SDS-poliacrilamida al 10% y fueron transferidas a una membrana de transferencia Immobilon-P™ (Millipore Corporation).

Inmunodetección.

Las membranas se incubaron con buffer de bloqueo (1X PBS, 1% albumina, 0.05% Tween 20) durante una hora a 37ºC. Después fueron incubados en el mismo buffer con anticuerpo policlonal de conejo anti-Hog1p (Santa Cruz Biotechnology) o con el anticuerpo monoclonal de conejo anti-p38-fosforilado (Cell Siganling Technology) durante una hora a temperatura ambiente. Para detectar el anticuerpo, se incubó durante 40 min con el anticuerpo de cabra anti-inmunoglobulina G de conejo acoplado a peroxidasa (Zymed) y se reveló por medio del kit de quimioluminiscencia para peroxidasa LumiGLO® (Millipore Corporation).

Ensayo de Inmunoprecipitación

Células transformadas con el plásmido pPHO o pPHO-KlSte7(His_6) se incubaron a 30ºC en 50 ml de medio selectivo hasta una DO_{600nm} 0.25. Se les agregó KH_2PO_4 a una concentración final de 10 mM y se incubaron durante 2 h a 30ºC. La mitad del cultivo se trató con KCl 0.5 M durante 10 min. Las células fueron centrifugadas y resuspendidas en 300μl de buffer TEA (Tris [pH 7.4] 10 mM, EDTA 1 mM, PMSF 100 mM, NaN_3 20 mM y el inhibidor de proteasas Complete™ (Roche) 1 X). Se extrajo proteína rompiendo las células con perlas de vidrio, se ajustó el volumen de las muestras a 800μl con buffer TEA y se agregaron 10μl de anticuerpo anti-His_6 acoplado a peroxidasa (Roche), se incubaron durante toda la noche a 4ºC en agitación constante. Se agregaron 60μl de perlas de agarosa acopladas a proteína G (Upstate) e incubaron durante 2 h. Las perlas se lavaron lavadas tres veces con 1.0 ml de buffer TNTE frío (Tris [pH 7.4] 50 mM, NaCl 150 mM, Triton X-100 0.1% y EDTA 1 mM). Se agregaron 30μl de buffer de carga y se hirvieron durante 5 min, posteriormente se enfriaron en hielo durante 5 min y se centrifugaron. 20μl de sobrenadante de cada muestra se utilizó para hacer un inmunoblot contra Hog1p como se mencionó antes. La inmunoprecipitación recíproca se hizo con 5μl de anticuerpo anti-Hog1p (Santa Cruz Biotechnology) para precipitar y el blot se reveló con el anticuerpo anti-His_6 acoplado a peroxidasa.

RESULTADOS

“The KlSTE2 and KlSTE3 genes encode MATalpha- and MATa-specific G-protein-coupled receptors, respectively, which are required for mating of Kluyveromyces lactis haploid cells”

Yeast
Yeast 2007; **24**: 17–25.
Published online in Wiley InterScience
(www.interscience.wiley.com) **DOI:** 10.1002/yea.1432

Research Article

The *KlSTE2* and *KlSTE3* genes encode *MAT*α- and *MAT*a-specific G-protein-coupled receptors, respectively, which are required for mating of *Kluyveromyces lactis* haploid cells

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Received: 29 May 2006
Accepted: 28 October 2006

Abstract

Mating in yeast is initiated by binding of pheromone to G-protein-coupled receptors expressed in haploid cells. We analysed the role of KlSte2p and KlSte3p receptors in the *Kluyveromyces lactis* mating pathway. By sequence analysis, KlSte2p and KlSte3p are the homologues of the *Saccharomyces cerevisiae* α-pheromone and a-pheromone receptors, respectively. However, by expression experiments, we determined that *KlSTE2* gene is expressed in the cells typified as *MAT*α and therefore is the receptor for the *K. lactis* a-pheromone and *KlSTE3* gene is expressed in the *MAT*a cells and binds the α-pheromone. The *KlSTE2* gene is silent in *MAT*a cells, while it is highly expressed in *MAT*α cells, and conversely the *KlSTE3* gene is expressed in *MAT*a cells and repressed in *MAT*α cells. Disruption mutants of both genes were found to be sterile, and this defect is reversed by plasmidic copies of each gene. The cytoplasmic C-terminus of KlSte3p interacts strongly with the KlGpa1p (Gα) subunit, which is involved in the transduction of the pheromone stimulus to induce mating. Remarkably, this same domain does not interact with a constitutive active allele of the Gα subunit, indicating that the C-terminus is able to discriminate between the active (GTP-bound) and inactive (GDP-bound) forms of the Gα subunit.
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Keywords: KlSte3p; KlSte2p; GPCR; G protein; pheromone receptors; *Kluyveromyces lactis*

Introduction

Mating in *Saccharomyces cerevisiae* is initiated by the binding of sexual pheromone to a seven-transmembrane G-protein-coupled receptor (known as serpentine receptor or GPCR) located in the plasma membrane of haploid cells. In this yeast, the α-pheromone receptor is designated Ste2p, while the **a**-pheromone receptor is known as Ste3p. Activated receptors catalyse the exchange of GDP for GTP in the α-subunit of the heterotrimeric G protein, which induces the release of the stimulatory Gβγ dimer. The liberated Gβγ directly associates with a scaffold protein, Ste5p, and a p21-activated kinase (PAK), Ste20p, thus activating the MEKK Ste11p, which activates the MEK Ste7p, and the latter activates the redundant MAPKses, Fus3p and Kss1p. Finally, transcription factors are activated. These factors induce growth arrest, shmoo morphology, expression of genes required

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for membrane fusion, and diploid formation (Herskowitz, 1995).

Although the primary sequences of the pheromone receptors do not show similarity, all GPCRs share parallelism in their topological arrangement in the plasma membrane, consisting of seven hydrophobic and potentially α-helical segments that span the lipid bilayer (Dohlman and Thorner, 2001). The N-terminus of these receptors is located extracellularly and, along with portions of extracellular loops, forms the binding pocket for pheromone (Naider and Becker, 2004). The long C-terminus extends into the cytoplasm. In the case of the yeast α-pheromone receptor, it extends at least 130 amino acid residues out from the membrane and physically interacts with both the α- and β-subunits of its associated heterotrimeric G protein (Bourne, 1997; Dosil *et al*., 2000). The receptor's C-terminus has been implicated in desensitization after pheromone treatment (Konopka *et al*., 1988; Reneke *et al*., 1988), in the formation of a preactivation complex with its associated G protein (Dosil *et al*., 2000), and plays an active role in the coupling with the G protein (Durán-Avelar *et al*., 2001).

Besides *S. cerevisiae*, GPCRs have been identified in a number of fungal species. Examples of well-characterized pheromone receptor genes, some of which control processes needed for virulence, have been characterized in *Candida albicans* (Tzung *et al*., 2001; Bennett *et al*., 2003), *Cryptococcus neoformans* (Chung *et al*., 2002), *Neurospora crassa* (Kim and Borkovich, 2004), *Coprinus cinereus* (O'Shea *et al*., 1998), *Schizophyllum commune* (Fowler *et al*., 1999), and *Ustilago maydis* (Bolker *et al*., 1992). In the fission yeast *Schizosaccharomyces pombe*, GPCRs have been also described (Tanaka *et al*., 1993; Kitamura and Shimoda, 1991) and receptor mutants exhibiting constitutive activity have been isolated (Ladds *et al*., 2005).

Interest in developing a detailed understanding of the yeast G-protein pathways comes from several areas of biology, one of which is related to the fact that many pharmaceutical drugs act on GPCRs (Davey, 2004). During the last decade various GPCRs have been successfully expressed in *S. cerevisiae* (Minic *et al*., 2005). Yeast is an attractive expression system because it offers the genetic engineering tools typical of a microorganism, while possessing an eukaryotic type of secretory pathway and post-translational machinery. The recent interest in *K. lactis* seems to come particularly from its ability to express and secrete high molecular weight proteins, even if such capacity is not a unique characteristic of this species.

Kluyveromyces lactis is a heterothallic budding yeast, able to use lactose as a sole source of carbon and energy (Wésolowski-Louvel *et al*., 1996). Its life cycle is similar to that of *S. cerevisiae*. Haploid *MAT***a** and *MAT*α cells undergo mating when they are mixed together in the same medium. In this process it has been shown that both the Gα (Saviñón-Tejeda, *et al*. 2001) and the Gβ (Kawasaki *et al*., 2005) subunits of the heterotrimeric G protein transmit the pheromone signal to downstream effectors. Moreover, a constitutively active allele of Gα1 is able to rescue *ΔGα1* cells from sterility and induces moderate growth arrest in wild-type cells (Lloret *et al*., 2003). Function of both G protein subunits in the mating pathway occurs via the KlSte12p transcription factor. This factor is thought to bind to pheromone response elements located in promoter regions of genes required for mating (Yuan *et al*., 1993).

Although information has been obtained regarding G protein activity in the *K. lactis* pheromone response pathway, nothing is known about the role that pheromone receptors may play in this budding yeast. In this work we describe the characterization of receptors and their role in the mating process of *K. lactis*.

Materials and methods

Strains and media

Yeast strains used in this work were: *K. lactis*, 155 (*MAT*α*, ade2, his3, ura3*) and 12/8 (*MAT***a**, *lysA, argA, ura3*); *S. cerevisiae*, W303-1A (*MAT***a**, *ade1, ura3, his3, leu2, trp1, can1-100*); *Escherichia coli* strains, DH5α and Gm33 were used to propagate plasmids. YPD medium consisted of 1% yeast extract, 2% bacto-peptone and 2% glucose. YPGal was the same, except for the substitution of glucose by galactose. SD minimal medium consisted of 0.67% yeast nitrogen base without amino acids (Difco) and 2% glucose. SGal medium was the same, except for the substitution of glucose by galactose. SD and SGal minimal media were supplemented with the required amino acids

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DOI: 10.1002/yea

(50 μg/ml) and nitrogen bases. LB + ampicillin (100 μg/ml) was used to propagate recombinant plasmids.

Identification and cloning of *KlSTE2* and *KlSTE3* genes

BLAST searching of the partial *K. lactis* genome sequence (Genolevures project I: **http://cbi.labri.fr/Genolevures/elt/KLLA**), allowed the identification of putative ORFs encoding the homologues Ste2p and Ste3p pheromone receptors. Oligodeoxynucleotides were designed from position +371 to +387 (forward primer) and from position +762 to +777 (backward primer) of the putative *KlSTE2* ORF and from positions +258 to +274 (forward primer) and from +999 to +982 (backward primer) of the putative *KlSTE3* sequence. These primers were used in a polymerase chain reaction (PCR) to amplify fragments of these genes using chromosomal DNA of *K. lactis* (strain 155) as template. The PCR products labelled with [α-^{32}P]dCTP by the Random Prime Labelling System (Rediprime™ II, Amersham Biosciences) were used to probe chromosomal DNA digested with several enzymes. *Eco*RI DNA fragments in the range of 2.1 kb (for *KlSTE2*) and 3.5 kb (for *KlSTE3*), which cross-reacted with the labelled probes, were used to construct independent pBlueScript-based libraries. Screening of libraries was done by colony hybridization and selected clones containing the full ORF of both genes were analysed with restriction enzymes and sequenced in full.

Gene disruptions

KlSTE2 and *KlSTE3* gene disruptions were achieved by homologous recombination. The full *KlSTE2* ORF was introduced into the YIp352 vector, then divergent primers were designed at positions +400 to +385 and +905 to +920 of the coding sequence and used to generate a linear PCR product which contained the full YIp352 plasmid flanked by 430 bp and 429 bp *KlSTE2* ends. For *KlSTE3*, the original PCR fragment was introduced into YIp352 opened at the *Eco*RI restriction site. The resulting plasmid was opened in the *KlSTE3* sequence at the natural occurring *Bcl*I sites, generating an integrating molecule that contains the full YIp352 vector flanked by 396 bp and 366 bp *KlSTE3* ends. YIp–*KlSTE2* and YIp–*KlSTE3* constructs were used to transfect strains 155 and 12/8 of *K. lactis*. Both *Klste2* and *Klste3* disruption mutants were confirmed by Southern hybridization.

Mating assays

A patch of cells of the strain to be tested was grown on a plate of selective medium for 24 h. The tester strain was grown as a lawn on an YPD plate for 24 h. Both strains were replica-plated onto YPD plate and incubated for 8 h at 30 °C to allow cells to mate. Diploids were selected on SD medium by replica-plating.

KlSte3p–G protein interactions

Assays of physical interaction were performed with the LexA-B42 two-hybrid system as described (Ongay-Larios *et al*., 2000). The fragment encoding the C-terminus tail of KlSte3p was digested with the restriction enzyme *Ssp*I and cloned in-frame into pJG4-5. Genes encoding Gα1 (KlGpa1p), Gα2 (KlGpa2p) and Gβ (KlSte4p) subunits were subcloned into the plasmid pEG202 (Kawasaki *et al*., 2005). Strain W303-1A was transfected with two-hybrid plasmids and grown overnight in SGal medium at 30 °C. Protein interactions were determined by the ability of hybrid proteins to induce expression of the *LACZ* reporter gene, located into the pSH18-34 plasmid.

KlSte3p localization

A portion of the green fluorescent protein (GFP) was fused in-frame to the COOH-terminus of the KlSte3p receptor. This fusion protein was made by placing 717 kb of the gene encoding the GFP into the *KlSTE3* ORF opened with *Cla*I at position 1189 bp. This hybrid gene was then subcloned into the YEpKDGal vector, which allows control of transcription by galactose, rendering plasmid pKlSTE3–GFP. Transfected *ΔKlste3* cells expressing the fusion protein were incubated with 2.5 μg/ml of 4′,6-diamidino-2-phenylindole (DAPI) for 2 h at room temperature. Cells were fixed and observed under confocal microscopy.

Other

For expression experiments, total RNA was isolated by the standard hot-acidic phenol protocol. Northern and Southern blot analysis and recombinant DNA technology were performed as described

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by Sambrook *et al*. (1989). Standard yeast genetics procedures were performed as described by Sherman *et al*. (1986).

Results

Gene fragments with homology to *ScSTE2* and *ScSTE3* were identified from the *K. lactis* sequencing genome project Genolevures I (Bolotin-Fukuhara *et al*., 2000). Oligodeoxynucleotides were designed from these sequences and used to amplify *KlSTE2* (403 bp) and *KlSTE3* (761 bp) fragments. The PCR products were then used as probes to screen mini-libraries of genomic DNA fragments that cross-reacted with the radio-labelled PCR products. In the case of *STE2*, a 1.9 kb clone was selected and sequenced in both directions revealing an ORF of 1.311 kb including the stop codon. For *STE3*, an *Eco*RI 3.5 kb fragment was selected and sequenced, revealing an ORF of 1.308 kb. The selected clones carried upstream sequences which in further studies showed promoter activity. The gene clones were designated *KlSTE2* and *KlSTE3*, respectively, because of their significant homology with *STE2* and *STE3* from *S. cerevisiae*. Subsequent to this finding the entire genome sequence of *K. lactis* was reported (**http://cbi.labri.fr/Genolevures/elt/KLLA**). *KlSTE2* and *KlSTE3* genes correspond to entries KLLA0F25102g and KLLA0A06534g in this database and encode predicted proteins belonging to class D of GPCRs (Kolakowski, 1994). The deduced primary structure of KlSte2p contains 436 amino acid residues with a predicted molecular weight of 48.4 kDa, while KlSte3p is a protein of 435 residues long with a predicted molecular weight of 50 kDa. Analysis of the primary sequence of these proteins using the Kyte–Dolittle algorithm predicts the seven transmembrane domains characteristic of G-protein coupled receptors (Figure 1). Direct comparison of KlSte2p and KlSte3p receptors did not show significant homology (20% identity and 33% similarity). When comparing *K. lactis* receptors with their *S. cerevisiae* homologues, most conservation occurs in residues that nucleate transmembrane helices (Figure 1). Mostly, key amino acids that have been involved in interhelical contacts, in resting and active states of the receptor, are preserved in both proteins (Eilers *et al*., 2005). Regarding the extra-membrane segments, the region most highly conserved is the third cytoplasmic loop in both proteins, 17 out of 21 for the Ste2p receptors, and 31 out of 36 for the Ste3p receptors (Figure 1). The third cytoplasmic loop is involved in the

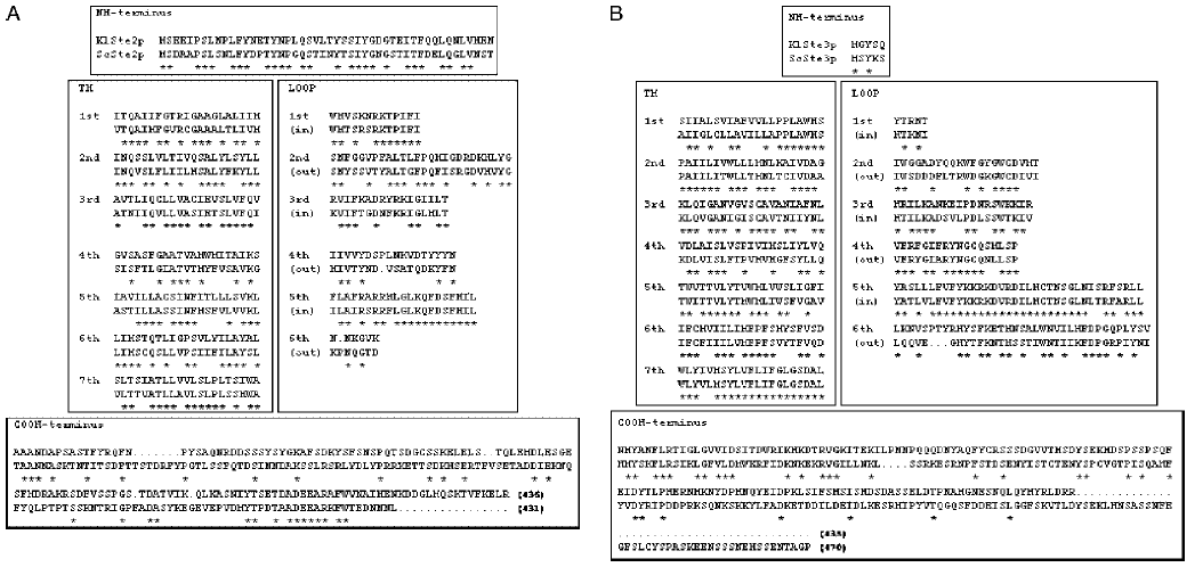

Figure 1. Alignment of amino acid sequences of *K. lactis* and *S. cerevisiae* G-protein-coupled receptors arranged according to transmembrane and extramembrane motifs. (A) Ste2p homologues; (B) Ste3p homologues. *, identical amino acids; ●, gaps; TM, transmembrane segments; (in), cytoplasmic loop; (out), extracelullar loop

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DOI: 10.1002/yea

regulation of the G-protein activation/inactivation cycle. Overall, the homology of KlSte2p and ScSte2p receptors reaches 48% identity and 65% similarity, while KlSte3p and ScSte3p share 51% identity and 71% similarity.

To determine their functions in *K. lactis*, we generated mutants with the *KlSTE2* and *KlSTE3* genes deleted. Both genes were replaced by the *URA3* gene using the integrative vector YIp352 in *MAT***a** and *MAT*α wild-type strains. After a double recombination process, the *URA3*$^+$ segregants had lost most of the coding region for both proteins. The *ΔKlste2* and *ΔKlste3* deletion mutants were identified and confirmed by DNA gel blotting hybridization. Both deletion mutants were viable and their growth rates on YPD plates were similar to those of the wild-type strains.

Figure 2 shows mating phenotype of wild-type and disruption strains. While wild-type strains of both mating types mate normally, disruption of the *KlSTE2* locus produced sterility only in the *MAT*α strain, while disruption of the *KlSTE3* locus produced sterility only in the *MAT***a** strain. This indicates that the product of the *KlSTE2* gene is required for *MAT*α mating but dispensable in the *MAT***a** strain, and conversely the expression of the *KlSTE3* gene is required for proper mating of *MAT***a** strains but is dispensable in the *MAT*α cells. Expression of plasmidic copies of both *KlSTE2* and *KlSTE3* genes are able to reverse their respective disruption mutants (not shown).

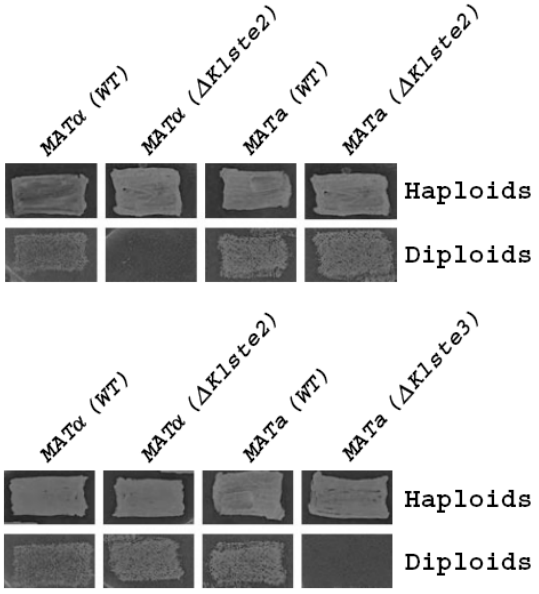

Figure 2. Effect of disruption of GPCRs on mating. Mating was done by replica-plating. Patches of wild-type strains (WT) of both mating types and their *Klste2*- and *Klste3*-disrupted mutants were streaked on selective medium and replicated onto YPD plates containing a lawn of tester cells, followed by incubation overnight at 30°C, and diploid selection by replica-plating onto SD. The plates were photographed 48 h later

To confirm this observation, we determined the transcription product of both *KlSTE2* and *KlSTE3* genes by means of Northern blotting techniques. Blots of total RNA were probed separately under high-stringency conditions with internal fragments of the *KlSTE2* and *KlSTE3* encoding regions (Figure 3). As can be observed, only *MAT*α cells showed a transcript that cross-reacted with the radio-labelled *KlSTE2* probe, while the *KlSTE3* probe showed a signal in the RNA obtained from *MAT***a** wild-type cells. None of the disruption mutants of either mating type showed a signal in the hybridization experiment with their respective probes.

Our findings provide evidence that *KlSTE2* is a *MAT*α-specific gene while *KlSTE3* is a *MAT***a**-specific gene, and that their inactivation produces complete sterility, indicating that these receptors participate in the mating pheromone response pathway in haploid cells. Thus, we conducted further experiments with the KlSte3p receptor. Initially we determined whether KlSte3p is able to physically interact with the G protein involved in transducing the pheromone signal during the mating response of haploid cells (Saviñón-Tejeda

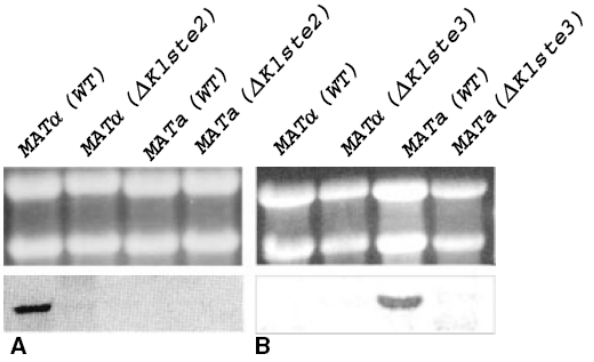

Figure 3. Northern analysis of *KlSTE2* and *KlSTE3* transcripts. Total RNA was isolated from mid-log cultures of the indicated strains. The RNA was electrophoresed, transferred to nylon membrane and probed with radio-labelled fragments of *KlSTE2* (A) and *KlSTE3* (B)

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et al., 2001, Kawasaki *et al.*, 2005). Previously, we had shown that the cytoplamic C-terminus of the ScSte2p receptor interacts with both the α- and the β-subunits of the heterotrimeric G protein (Duran-Avelar *et al.*, 2001), and that these interactions are required for proper coupling and transmission of the pheromone stimulus (Ongay-Larios *et al.*, 2000). Furthermore, this property was used as practical interaction screening for cloning the Gpa2-coupled receptor in *S. cerevisiae* (Cheol-Won *et al.*, 1997). Interaction of the C-terminus of KlSte3p with the various G protein subunits was achieved by the two-hybrid system (Ongay-Larios *et al.*, 2000). In this experiment we measured the β-galactosidase activity induced by the magnitude of the interaction between proteins (Figure 4). As a positive control we included the activity induced by the interaction between the Gα (KlGpa1p)- and Gβ (KlSte4p)-subunits, which have shown strong association in previous reports (Kawasaki *et al.*, 2005). Interaction of the KlGpa1p-subunit with the KlSte3p pheromone receptor is three-fold stronger than with the KlGpa2p-subunit, which is involved in regulating cAMP levels upon glucose stimulus in *K. lactis* (Saviñon-Tejeda *et al.*, 1996). Accordingly, an active form of the KlGpa1p-subunit ($KlGpa1^{Q298L}$), which induces constitutive mating (Lloret *et al.*, 2003) showed weak interaction with the receptor, indicating that this portion of the receptor retains the structural information to recognize between GDP- and GTP-bound Gα-subunits. This finding is striking and confirms that some of the basic forces that regulate G-protein activity reside in the C-terminus of the pheromone receptors.

Finally, with the purpose of localizing the pheromone receptor in *K. lactis* haploid cells, we fused the green fluorescent protein (GFP) to the C-terminus of the KlSte3p receptor. Cells expressing this fusion were observed under confocal microscopy. The fluorescence emitted by the GFP was located mostly dispersed in the cytoplasm of budding cells (Figure 5A). The same cells were stained with DAPI, which is a blue fluorescent nucleic acid dye that preferentially stains double-stranded DNA, to contrast with the nucleus, where no green fluorescence was observed. Control cells transfected with the GFP alone failed to show this distribution. The GFP–KlSte3 fusion protein is able to reverse the mating deficiency of a *ΔKlste3* mutant (Figure 5B), indicating that the addition of the GFP to the COOH-terminus of the pheromone receptor did not affect its functionality and targeting at the plasma membrane. The high level of fluorescence observed in the cytoplasm was probably due to excess of expression of GFP–KlSte3p.

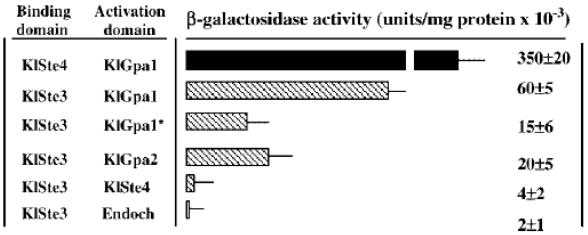

Figure 4. Physical association of KlSte3p to G protein subunits. The activation domain corresponds to B42-fused proteins cloned into pJG4-5. The binding domain corresponds to LexA-fused proteins cloned into pEG202. KlGpa1*, is the constitutive active allele $KlGpa1^{Q298L}$. KlSte4 is the Gβ subunit. 'Endoch' is a portion of the *CTS1* gene which encodes for endochitinase and is used as control for negative interaction. Strain W303-1A transfected with indicated constructions was grown until mid-exponential phase in SD selective medium; 5×10^7 cells were transferred to liquid SGal medium and incubated at 30°C for 15 h. The β-galactosidase activity corresponds to the average value of three independent clones

Discussion

The *Kluyveromyces lactis* genome encodes two putative pheromone receptors, KlSte2p and KlSte3p, which show on average 50% identity with their *S. cerevisiae* homologues. Conserved amino acids are mostly located at transmembrane regions and at the second and third intracellular loops. Remarkably, the amino acid sequence of the third intracellular loop is the most highly conserved (around 85% between species). This loop plays a key role in maintaining a productive G protein coupling (Ćelić *et al.*, 2003). Although GPCRs have widely divergent amino acid sequences, they retain a common architecture and some key residues that play important roles in structure and function are conserved between species. For instance, strong polar amino acids that mediate helix interactions in transmembrane segments, and weak polar residues that participate in helix packing (Eilers *et al.*, 2005), are mostly conserved between *K. lactis* and *S. cerevisiae* receptors.

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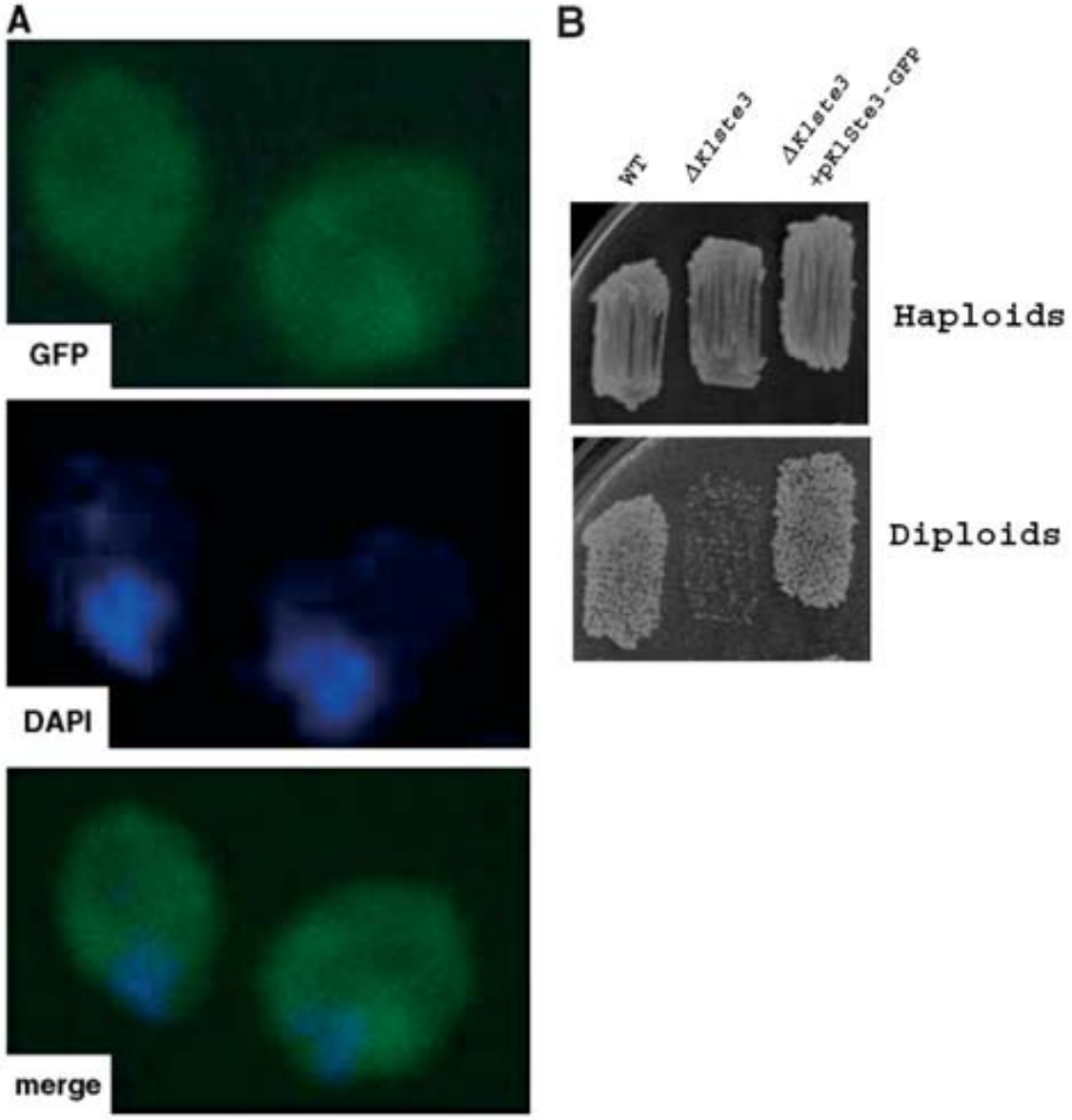

Figure 5. Distribution of KISte3p–GFP fusion protein and its effect on mating. (A) Confocal microscopy images. Cells transfected with pKISTE3–GFP were grown until mid-log phase in medium containing 2% galactose for transcription induction. The cells were washed twice, collected and incubated with 2.5 μg/ml DAPI for 2 h at room temperature, then fixed and observed under confocal microscopy. GFP, DAPI and merged images are shown. The merge image was obtained with software from the manufacturer (Olympus Fluoview FV100 V. 1.4). (B) Mating of transfected cells expressing KISte3–GFP fusion protein. Patches of *MAT***a** wild-type strain, *Klste3* mutant carrying vector alone, and *Klste3* mutant expressing the KISte3–GFP fusion protein were streaked on SGal medium and replicated onto YPGal plates containing a lawn of tester cells, followed by incubation overnight at 30 °C, and diploid selection by replica onto SD. The plates were photographed 48 h later

Studies using chimerical receptors and pheromones from *S. cerevisiae* and *S. kluyveri* indicated that amino acids 47, 48 and 49, located at the boundary of the N-terminus and the first transmembrane segment, are important elements for pheromone recognition (Naider and Becker, 2004). In *S. cerevisiae*, these residues are S, T, and V, while in the KISte2p receptor they are

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E, N and I. Aromatic residues located in TM5 (F204) and in TM6 (Y266) have been postulated as interaction points of the ScSte2p receptor with aromatic residues (W1 and W3) of the α-pheromone (Lin *et al.*, 2003; Naider and Becker, 2004). Aromatic residues are also present in these positions in the KlSte2p receptor (Y in both positions) and may have a role in making contact with putative W residues of the *K. lactis* sexual pheromone (Coria *et al.*, 2006).

We have analysed the role of both KlSte2p and KlSte3p in the mating pathway. By sequence analysis, KlSte2p is the homologue of the *S. cerevisiae* α-pheromone receptor, while KlSte3p is the homologue of the **a**-pheromone receptor. By expression experiments, however, the *KlSTE2* gene is expressed in *K. lactis* cells typified as *MAT*α and therefore is the receptor for the *K. lactis* **a**-pheromone (which corresponds to the *S. cerevisiae* α-pheromone), while *KlSTE3* is expressed in the *MAT***a** cell and therefore is the receptor for the *K. lactis* α-pheromone (which corresponds to the *S. cerevisiae* **a**-pheromone). As expected, disruption of both genes resulted in sterility in a mating type-dependent manner.

Although it has been shown that the C-terminal domain of the α-factor receptor is unlikely to play an essential role in G-protein activation, since truncated receptors lacking this region remain responsive to agonist (Konopka *et al.*, 1988; Reneke *et al.*, 1988), the results here described indicate that the C-terminus tail of the receptor, when present, plays an active role in signal transduction and may regulate G protein activation. The wild-type form of the KlGpa1p-subunit exhibits strong interaction with the C-terminus of the KlSte3p receptor, while its active form (KlGpa1^{Q298L}), which induces constitutive mating (Lloret *et al.*, 2003) shows weak interaction. This indicates that this portion of the receptor retains the structural information to discriminate between GDP- and GTP-bound Gα-subunits. This finding is striking and confirms that some of the basic forces that regulate G-protein activity reside in the carboxyl-tail of the pheromone receptors. Crystal structures of heterotrimeric G protein as well as biochemical and mutagenic studies indicate that the C-terminal tail of the Gα-subunit interacts directly with the receptor, perhaps in a domain formed by transmembrane regions 3, 6 and 7 (Bourne, 1997; Conklin *et al.*, 1993). In this model, the receptor's carboxy-terminal tail may accommodate into the interface between Gα and G$\beta\gamma$, interacting with both subunits. In fact, point mutations in residues located at the C-terminus of the ScSte2 receptor have been implicated in physical association with the G protein α- and β-subunits (Durán-Avelar *et al.*, 2006). However, the two-hybrid assays performed in this study failed to show interaction between Gβ (KlSte4p) and the C-terminus of the KlSte3p receptor, which suggest that some interactions of GPCRs with their associated G-protein may not be universal.

Acknowledgements

We are grateful for technical assistance from Laura Ongay, Guadalupe Codiz and Minerva Mora (Molecular Biology Unit), Gerardo Coello and Ana María Escalante (Computer Unit), and the staff of the Microscopy Unit at IFC, UNAM, and also for the technical assistance of Marisela Bolaños. This work was partially funded by Grant 44178 from CONACyT, México, and Grant PAPIIT IN211906 from DGAPA, UNAM.

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Yeast
Yeast 2008; **25**: 241.
Published online 7 February 2008 in Wiley InterScience
(www.interscience.wiley.com) **DOI:** 10.1002/yea.1584

Erratum

The *KlSTE2* and *KlSTE3* genes encode *MATα*- and *MAT*a-specific G-protein-coupled receptors, respectively, which are required for mating of *Kluyveromyces lactis* haploid cells.

Francisco Torres-Quiroz, Laura Kawasaki, Miriam Rodríguez-González, Araceli Patrón-Soberano and Roberto Coria.

Yeast 2007; **24**: 17–25

The right mating type of strains used in the above article is: *MAT*a for strain 155 and *MATα* for strain 12/8. The right assignment of mating type implicates that *KlSTE2* encodes the *MAT***a** receptor while *KlSTE3* encodes the *MATα* receptor. These changes do not alter the results shown in this work.

The authors would like to apologize for this error and for any confusion caused.

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“Protein kinases involved in mating and osmotic stress in the yeast Kluyveromyces lactis”

EUKARYOTIC CELL, Jan. 2008, p. 78–85 Vol. 7, No. 1
1535-9778/08/$08.00+0 doi:10.1128/EC.00362-07
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Protein Kinases Involved in Mating and Osmotic Stress in the Yeast *Kluyveromyces lactis*▽

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Received 2 October 2007/Accepted 29 October 2007

Systematic disruption of genes encoding kinases and mitogen-activated protein kinases (MAPKs) was performed in *Kluyveromyces lactis* haploid cells. The mutated strains were assayed by their capacity to mate and to respond to hyperosmotic stress. The *K. lactis* Ste11p (KlSte11p) MAPK kinase kinase (MAPKKK) was found to act in both mating and osmoresponse pathways while the scaffold KlSte5p and the MAPK KlFus3p appeared to be specific for mating. The p21-activated kinase KlSte20p and the kinase KlSte50p participated in both pathways. Protein association experiments showed interaction of KlSte50p and KlSte20p with Gα and Gβ, respectively, the G protein subunits involved in the mating pathway. Both KlSte50p and KlSte20p also showed interaction with KlSte11p. Disruption mutants of the *K. lactis PBS2* (Kl*PBS2*) and Kl*HOG1* genes of the canonical osmotic response pathway resulted in mutations sensitive to high salt and high sorbitol but dispensable for mating. Mutations that eliminate the MAPKK KlSte7p activity had a strong effect on mating and also showed sensitivity to osmotic stress. Finally, we found evidence of physical interaction between KlSte7p and KlHog1p, in addition to diminished Hog1p phosphorylation after a hyperosmotic shock in cells lacking KlSte7p. This study reveals novel roles for components of transduction systems in yeast.

Cellular signaling transduction networks continuously sense extracellular cues and transduce signals from the cell surface to the interior of the cell. The cell responds to these signals through changes in gene expression and protein activity to yield specific phenotypes.

G-protein signaling pathways in fungi are used to regulate development and virulence and to detect nutrients and other environmental signals. Some of these pathways contain mitogen-activated protein kinase (MAPK) cascades that are highly conserved in metazoans (12, 18). The yeast pheromone signaling, for example, occurs through the action of a G-protein-coupled receptor (GPCR) and the associated G protein in order to activate a MAPK cascade that conducts the signal to the nucleus. Besides G-protein-activated signal transduction pathways, there are at least three more pathways involving MAPK components or their presumed upstream regulators that modulate responses to several stimuli (10).

The best-characterized transduction system in eukaryotic organisms is the *Saccharomyces cerevisiae* pheromone response pathway. This pathway is initiated by the binding of peptide pheromones to a GPCR (Ste2p in *MAT***a** cells and Ste3p in *MAT*α cells) and the dissociation of the heterotrimeric G protein into Gα (Gpa1p) and the stimulatory Gβγ (Ste4p/Ste18p) subunits. The liberated Gβγ dimer directly associates with a scaffold protein, Ste5p, and with a p21-activated kinase (PAK), Ste20p, which is essential for activating the MAPK kinase kinase (MAPKKK) Ste11p. Activation of Ste11p is also promoted by action of the Ste50 protein kinase. Ste11p in turn, activates the MAPKK Ste7p. Downstream from Ste7p, Fus3p and Kss1p, two partially redundant MAPKs, induce the activation of transcription factors, Ste12p among others, that regulate the mating process (2).

Some components of the pheromone response pathway participate in other MAPK cascades. For example, Ste20p, Ste50p, and Ste11p play a role in the high-osmolarity glycerol (HOG) pathway, helping the cell to survive osmotic stress (24), and Ste20p, Ste50p, Ste11p, and Ste7p are used by both the pseudohyphal development pathway and the cell wall integrity pathway (8, 13).

The HOG pathway in *S. cerevisiae* is regulated by two different inputs that converge to activate the MAPKK Pbs2p. These two branches are composed of membrane-spanning proteins (Sln1p and Sho1p) that respond to variant conditions of external osmolarity.

Under isosmotic conditions, the protein histidine kinase, Sln1p, acting through a phosphorelay reaction with Ypd1p and Ssk1p, keeps the partially redundant MAPKKKs Ssk2p and Ssk22p in an inactive state (27). High external osmolarity blocks Sln1p function, allowing activation of Ssk2p and Ssk22p, which, in turn, phosphorylate Pbs2p. On the other hand, Sho1p uses Ste20p and Ste50p to activate Ste11p (22, 26) in response to high-solute concentrations. Sho1p binds Ste11p and Pbs2p, resulting in activation by phosphorylation of Pbs2p (26, 37). Activation of Pbs2p by the Sln1p and Sho1p branches stimulates phosphorylation and nuclear translocation of Hog1p, triggering expression of a number of genes and production of glycerol to prevent dehydration (13, 33).

While a large amount of information has accumulated on signal transduction cascades in *S. cerevisiae* and in many other fungi (18), a critical question concerns which features of the

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▽ Published ahead of print on 16 November 2007.

transduction systems are generic and which are species specific. For example, in the budding yeast *Kluyveromyces lactis*, the signal transduction system that mediates the mating response is triggered by both the Gα (*K. lactis* Gpa1p [KlGpa1p]) (31) and Gβ (KlSte4p) (14) subunits of the heterotrimeric G protein. There are two documented differences between the G protein pheromone response pathways of *S. cerevisiae* and *K. lactis*: first, while disruption of *GPA1*, the gene encoding the G protein α-subunit in *S. cerevisiae*, leads to permanent growth arrest and therefore to lethality in haploid cells (7), inactivation of Gα (KlGpa1p) in *K. lactis* does not affect cell viability but produces sterility (31); second, overexpression of the Gβ (Ste4p) subunit induces growth arrest and mating in *S. cerevisiae* (34) but has no effect in *K. lactis* (14). Although it has been shown that both Gα and Gβ trigger the mating response by activating the transcription factor KlSte12p (14, 19), nothing is known about the elements that connect the G protein with the transcription factor in *K. lactis*. We have therefore investigated the phenotypic characteristics of disruption mutants in components of the pheromone response pathway, and in order to elucidate the overlapping of protein function with other transduction systems, we assayed the disruption mutants for their capacity to respond also to osmotic stress.

The budding yeast *K. lactis* provides an attractive model system for studying cellular differentiation processes in response to environmental cues. It is a unicellular and essentially aerobic and heterothallic organism with a conventionally organized cell cycle. Its entire genome sequence is now available (http://cbi.labri.fr/Genolevures), and it is easily subjected to genetic analysis.

MATERIALS AND METHODS

Strains, media, and genetic techniques. The yeast strains used in this work were *K. lactis* 155 (*MATa ade2 his3 ura3*) and 12/8 (*MATα lysA argA ura3*). *S. cerevisiae* strain EGY48 (*MATα his3 trp1 ura3-52 leu2::pLeu-LexAop6*) was used for two-hybrid assays (23). *Escherichia coli* strains DH5α and Gm33 (for preparation of unmethylated DNA) were used to propagate plasmids. YPD medium consisted of 1% yeast extract, 2% Bacto-peptone, and 2% glucose. Synthetic dextrose (SD) minimal medium consisted of 0.67% yeast nitrogen base without amino acids (Difco) and 2% glucose. SD medium was supplemented with the required amino acids and nitrogen bases (50 μg/ml). SGal medium was the same as SD medium except for the substitution of glucose for galactose. Selective SD medium containing 10 mM KH_2PO_4 was used for induction of pPHO constructs. LB medium plus ampicillin (100 μg/ml) was used to propagate recombinant bacteria. Standard protocols were followed for Southern blot analysis, recombinant DNA technology, and yeast manipulation.

Gene identification and cloning. A BLAST search of the *K. lactis* genome database, Genolevures project II (http://cbi.labri.fr/Genolevures/elt/KLLA), allowed the identification of putative open reading frames (ORFs) encoding the orthologues of *S. cerevisiae* protein kinases. Pairs a and b of the oligodeoxynucleotides listed in Table 1 were used for PCR amplification. Chromosomal DNA from *K. lactis* strain 155 was used as a template. Standard PCRs were carried out to amplify the desired gene fragments. The PCR products were cloned into the pGEMTeasy vector (Promega) and sequenced in full. Plasmid pPHO (containing the *PHO5* promoter from *S. cerevisiae* and a *K. lactis* replication origin) was obtained from Hiroshi Fukuhara. A six-histidine tag was fused in frame to the C terminus of KlSte7p [KlSte7(His$_6$)] by PCR-mediated amplification, employing primers Ste7c and Ste7d (Table 1). An XhoI-SmaI fragment of the KlSte7(His$_6$) construct was Klenow filled and subcloned into pPHO previously digested and filled in at EcoRI. Recombinant plasmid containing the KlSte7(His$_6$) gene under the control of the *PHO5* promoter was identified by restriction analysis.

Gene disruptions. Gene disruptions were achieved by homologous recombination using the integrative vector YIp352 (11). Disruption mutants were selected by their capacity to grow in Ura-deficient medium. All gene fragments were obtained from the pGEMTeasy clones. For *K. lactis STE50* (*KlSTE50*), a SmaI-HindIII 634-bp fragment was introduced into the integrative vector YIp352 previously digested with EcoRI (Klenow filled) and HindIII. The resulting plasmid was digested with EcoRI and SacI (natural restriction sites of *KlSTE50* ORF located at positions 298 and 438, respectively). This yields a linear YIp352 plasmid flanked by 298 bp and 196 bp of *KlSTE50* recombinant ends. For *KlSTE20*, a 1,046-bp fragment obtained by EcoRI and SalI digestion was subcloned into YIp352 digested with the same enzymes. The resulting integrative plasmid was opened with BglII, which cuts the *KlSTE20* ORF at position 268. The resulting construct contains recombinant ends of 267 bp and 779 bp. *KlSTE5* was obtained as a 1,266-bp EcoRI fragment and ligated into YIp352 opened with the same enzyme. The resulting construct was digested with HpaI and SpeI, yielding an integrating molecule with 573 bp and 140 bp of recombinant ends. A *KlSTE11* 850-bp fragment obtained with HindIII-Asp718 was introduced into YIp352 digested with the same enzymes. This plasmid was opened with SpeI, yielding an integrating fragment containing 190 bp and 275 bp of recombinant ends. For *KlSTE7*, a 1,071-bp fragment was obtained by HindIII-BamHI digestion and ligated into YIp352 digested with the same enzymes. This construct was digested with XbaI and BglII, leaving recombinant ends of 333 bp and 338 bp. For *KlFUS3*, a 670-bp EcoRI fragment was subcloned into YIp352 vector prepared with the same enzyme. A linear molecule was generated by digesting the resulting plasmid with SmaI and PstI. This yields an integrating molecule containing 227 bp and 277 bp of recombinant ends. A 967-bp KpnI-PstI fragment of *KlPBS2* was subcloned into YIp352 opened with the same enzymes. The YIp352-

TABLE 1. Primers used for PCR amplification

Primer name	Sequence[a]
Ste50a	(+88) CTAGATAGAAccCGgGAGAACAAGATA (+114) SmaI
Ste50b	(+741) TTGATCTCCAAAGCttTATCACTAAAAC (+715) HindIII
Ste50c	(−19) ATAGAAACAAAAACtcgagATGAGCGGTAC(+11) XhoI
Ste50d	(+929) AAGTGTTTTTTCtcGagGATGAAGGAGGAG (+899) XhoI
Ste20a	(−14) TTTGTCTCCtcgagATGACGGATACTGGAT (+16) XhoI
Ste20b	(+3002) TAAATTCATGTAcTGCaGTAAAACAGAAAT (+2972) PstI
Ste5a	(+1) ATGTCTAGAGGTAATAC (+17)
Ste5b	(+2300) GTTTAGAGAGAGAACGG (+2284)
Ste11a	(+360) GCAAAGAAAGcTtACGTTAACGGGT (+385) HindIII
Ste11b	(+1210) TGGATTCTGAGGTACCAG (+1192) Asp718
Ste11c	(−19) TTTTTAAGATCTctcgAgATGAGCAGTGAC (+12) XhoI
Ste11d	(+2222) AGTAACAATACCTCGAGACTGAAAACGGC (+2194) XhoI
Ste7a	(+232) GAGAAGCTTCCGGTCC (+247) HindIII
Ste7b	(+1303) CTTTCAAAGAgGATCcTCCTCTCTCCTC (+1275) BamHI
Ste7c	(−18) AGATTGACTTAActcgAgATGATTACACGT (+12) XhoI
Ste7d	(+1543) CTAATATAGTGCtcgagCTTAATTTATTTC (+1514) XhoI
Ste7e	(+1513) ccccgggTCAgtggtggtggtggtggtgTCGCCTTGTACT (+1501) SmaI
Fus3a	(+498) ACAGATGGCGgTaCCCATGTTGACG (+523) Asp718
Fus3b	(+1171) TGATATAATAaGCTtGTGGTTACTTAG (+1144) HindIII
Pbs2a	(−21) GGCGATTAAGTGgtaCCAATTATGAGT (+6) Asp718
Pbs2b	(+2293) GGCGAGGCAAAGgaTccCTTACAATCCGCC (+2264) BamHI
Hog1a	(+490) CTTGCTAGGATcCAAGATCCTCAAATG (+517) BamHI
Hog1b	(+1366) CTTCTGTCAAGCttATTTCATTACATTG (+1339) HindIII
Hog1c	(−18) TTTTCCAATTGAGAATTCATGTCGAATGAG (+12) EcoRI
Hog1d	(+1397) TATACTTCTGTTCCTCGAGATTTCATTAC (+1343) XhoI

[a] Numbers in parentheses correspond to coordinates considering A in the translation start codon as the +1 position. Nucleotide changes introduced to generate restriction sites or tag sequences are indicated by lowercase letters.

KlPBS2 construct was digested with XbaI and BstEII, yielding a linear molecule containing 431 bp and 313 bp of recombinant ends. For *KlHOG1*, a BamHI-HindIII 876-bp fragment was subcloned into YIp352 digested with the same enzymes. BglII digestion of YIp352-*KlHOG1* yielded a linear molecule flanked by recombinant ends of 331 bp and 350 bp. When required, YPD medium containing 1 mg/ml of 5-fluoroorotic acid (5-FOA) was used for negative selection of the *URA3* cassette.

Protein interactions. Assays of physical interaction were done with a LexA-B42 two-hybrid system as described previously (14). The *KlGPA1* and *KlSTE4* genes were subcloned into pEG202 as previously reported (14, 31). *KlGPA1* was also subcloned into pJG4-5 as reported previously (14). Genes of interest (with the exception of *KlSTE20* and *KlPBS2*) were amplified by PCR employing specific c and d primers (Table 1) and ligated in frame into two-hybrid plasmids. The full *KlSTE50* gene was ligated into pJG4-5 as an XhoI fragment. *KlSTE11* was subcloned into pEG202 as a 2,215-bp XhoI fragment. The *KlSTE7* gene was obtained as a 1,536-bp XhoI fragment and ligated into pJG4-5. The gene encoding KlPbs2p was subcloned into pEG202 as a 2,285-bp Asp718-BamHI fragment. *KlHOG1* was subcloned in both pEG202 and pJG4-5 as a 1,364-bp EcoRI-XhoI fragment. The *KlSTE20* gene was obtained as a 2,987-bp fragment from the pGEMTeasy clone by XhoI digestion and subcloned into pJG4-5 digested with the same enzyme. *S. cerevisiae* endochitinase gene (*CTS1*) cloned into either plasmid pEG202 or pJG4-5 (14) was used as a negative interaction control for each interacting couple. All recombinant genes were sequenced in full. *S. cerevisiae* strain EGY48 was transfected with two-hybrid plasmids and grown overnight in SD medium at 30°C. Induction of genes subcloned into pJG4-5 was done by shifting cells to SGal medium 4 h prior to harvesting. Protein interactions were determined by the ability of hybrid proteins to induce blue colonies by expression of the *LACZ* reporter gene located in the pSH18-34 plasmid and by reversion of the *leu2* deficiency.

Mating assays. A patch of cells of the strain to be tested was grown on a plate of selective medium for 24 h. The tester strain was grown as a lawn on a YPD plate for 24 h. Both strains were replica plated onto a YPD plate and incubated for 8 h at 30°C, allowing cells to mate. Diploids were selected on SD medium by replica plating.

Osmotic stress assays. Strains to be tested were grown overnight on SD medium. Cells were washed, suspended in YPD medium at an optical density at 600 nm of 0.1, and grown to mid-exponential phase. Serial dilutions of cells were spotted on YPD medium containing 0.5 M KCl or 1 M D-sorbitol and incubated at 30°C. Plates were photographed 48 h later.

Phosphorylation assays. To estimate the cellular content of both total and phosphorylated KlHog1p in wild-type, Δ*Klpbs2*, and Δ*Klste7* strains, exponentially growing cells in 5 ml of YPD medium were pelleted and suspended in YPD medium or YPD medium plus 0.5 M KCl for different times. Cells were concentrated by centrifugation, and the supernatant was removed by aspiration. Cells were resuspended in loading buffer (5% sodium dodecyl sulfate [SDS], 0.1 M Tris-HCl [pH 7.5], 5% glycerol, 0.07 M 2-mercaptoethanol, 0.02 mM bromophenol blue) and boiled for 5 min. Samples were centrifuged, and the supernatants were transferred to a clean tube. An aliquot of the samples was subjected to SDS-polyacrylamide gel electrophoresis on an 10% gel and transferred to polyvinylidene difluoride membrane (Millipore Corporation).

Immunoblotting. Blots were incubated with blocking buffer (1× phosphate-buffered saline, 1% albumin, 0.05% Tween 20) for 1 h at 37°C. Blots were incubated in the same buffer containing rabbit polyclonal anti-Hog1p (Santa Cruz Biotechnology) or rabbit monoclonal anti-phospho-p38 antibody (Cell Signaling Technology) for 1 h at room temperature. Filter-bound antibodies were detected with horseradish peroxidase-conjugated secondary goat anti-rabbit immunoglobulin G antibody (Zymed) and visualized with chemiluminescent horseradish peroxidase substrate (Millipore Corporation).

Immunoprecipitation assays. Cells bearing pPHO or pPHO-KlSte7(His_6) were grown at 30°C to mid-log phase in 50 ml of selective medium. At mid-log phase, KH_2PO_4 was added at a final concentration of 10 mM, and cells were incubated for another 2 h at 30°C. Half of the culture was treated with 0.5 M KCl for 10 min. Cells were collected by centrifugation and resuspended in 300 μl of TEA buffer containing 10 mM Tris (pH 7.4), 1 mM EDTA, 100 mM phenylmethylsulfonyl fluoride, 20 mM NaN_3, and 1× complete protease inhibitor cocktail (Roche). Sterile acid-washed glass beads were added, and cell disruption was performed by vortexing for 5 min at 4°C. Lysates were cleared in a microcentrifuge (3 min at 2,000 rpm), and the supernatant was adjusted to an 800-μl final volume with TEA buffer. Ten microliters of anti-His_6-peroxidase antibody (Roche) was added and incubated at 4°C overnight with gentle shaking. Sixty microliters of protein G-agarose beads (Upstate) was added, and incubation continued for 2 h. The beads were washed three times with 1.0 ml of cold TNTE buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.1% Triton X-100, 1 mM EDTA). Thirty microliters of loading buffer was added, followed by 5 min of boiling. Samples were chilled on ice and centrifuged briefly. Twenty microliters of each sample was used for immunoblotting. Blots were probed with anti-Hog1p as described above. Reciprocal immunoprecipitation was done in the same way except that 5 μl of anti-Hog1p (Santa Cruz Biotechnology) antibodies was used for precipitation, and blots were probed with anti-His-peroxidase antibodies.

TABLE 2. Proven components of *K. lactis* mating and osmoresponse pathways

Gene name	Mutant phenotype[a]		*S. cerevisiae* homologue[b]		Reference or source
	Mating	High osmolarity	% Identity	E value	
KlSTE2	Sterile	ND	48	5e-92	32
KlSTE3	Sterile	ND	51	1e-131	32
KlGPA1	Sterile	ND	62	8e-154	31
KlSTE4	Sterile	ND	52	1e-119	14
KlSTE18	Fertile	ND	53	3e-24	6
KlSTE20	Sterile	Sensitive	50	0.0	This work
KlSTE50	Sterile	Sensitive	41	9e-60	This work
KlSTE5	Sterile	Resistant	27	3e-56	This work
KlSTE11	Sterile	Sensitive	58	1e-60	This work
KlSTE7	Sterile	Sensitive	52	1e-130	This work
KlFUS3	Sterile	Resistant	69	1e-150	This work
KlSTE12	Sterile	ND	35	1e-107	19
KlPBS2	Fertile	Sensitive	57	0.0	This work
KlHOG1	Fertile	Sensitive	80	0.0	This work

[a] ND, not determined.
[b] Identity values were determined by BLAST using the BLOSUM62 matrix.

RESULTS

In previous work, we have demonstrated that both the Gα (KlGpa1p) and Gβ (KlSte4p) subunits of the heterotrimeric G protein trigger the mating pheromone response pathway in *K. lactis* (6, 14, 31). Additionally, we have found that this activation requires the transcription factor KlSte12p (14, 19). In order to elucidate further the pathway that promotes mating in *K. lactis*, we constructed disruption mutants of genes encoding putative intermediates between the G protein and the KlSte12p transcription factor. We therefore inactivated the genes encoding components of the MAPK cascade, and we also inactivated the genes for KlSte50p and KlSte20p protein kinases, two putative inputs of the MAPK module. To investigate the overlapping of protein function with other transduction systems, we assayed the disruption mutants for their capacity to respond to osmotic stress, and for this purpose we added to this screening mutants of genes encoding KlPbs2p and KlHog1p, which have been shown to be key elements in the osmotic adaptation response in other yeast species (10, 15). Table 2 shows relevant features of the studied genes and their products as well as the degrees of homology with their *S. cerevisiae* counterparts.

Because each particular protein may modulate responses in different degrees and its inactivation may have several effects, detailed studies on the structure and the role that each protein may have in different cell functions will be described elsewhere.

All disruption mutants were constructed by integrating a *URA3* cassette in haploid cells of both mating types; therefore, transformants were selected by their capacity to grow in Ura-deficient medium, and the disrupted alleles were confirmed by

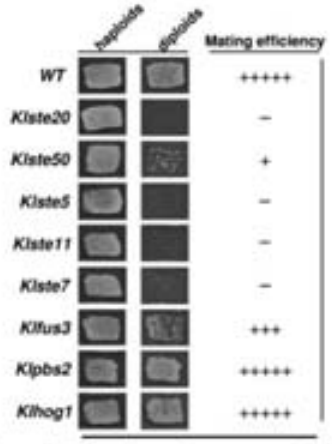

FIG. 1. Mating of *MAT*α disruption mutants. Mating was done by replica plating. Patches of strains to be tested were streaked on selective medium and replicated onto YPD plates containing a lawn of the *MATa* wild-type tester strain, followed by incubation overnight at 30°C. Diploid selection was done by replica plating onto SD medium. Plates were photographed 48 h later. WT, wild type.

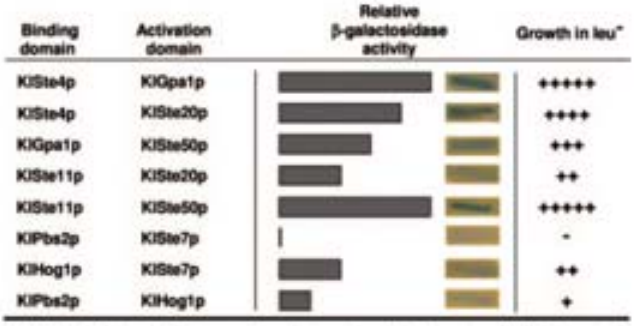

FIG. 2. Protein interactions determined by the two-hybrid system. The binding domain corresponds to LexA-fused proteins cloned into pEG202. The activation domain corresponds to B42-fused proteins cloned into pJG4-5. Two-hybrid plasmids were introduced into strain EGY48. β-Galactosidase activity was determined by the relative intensity of blue staining in cells growing on SGal plates containing 1 mg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), pH 7.0. Growth in SD medium without leucine (leu⁻) was determined by relative size of the colony after a 24-h incubation at 30°C. The *S. cerevisiae* endochitinase gene (*CTS1*) cloned either into pEG202 or pJG4-5 was used as negative interaction control for each interacting couple.

Southern blot. In all cases, plasmidic gene copies reversed the phenotypic defects of 5-FOA-resistant strains to almost wild-type levels (data not shown). Since our results suggest that gene disruptions seem to have the same phenotypic effects in *MATa* and *MAT*α cells, results described in this work refer solely to *MAT*α strains.

All mutant strains were viable, and they exhibited normal growth; however, when assayed in sexual crosses with a tester strain, they showed (with the exception of Kl*hog1* and Kl*pbs2* mutants) mating defects (Fig. 1). The hierarchy of defects in mating was determined as follows: Kl*ste5* = Kl*ste20* = Kl*ste11* = Kl*ste7* > Kl*ste50* > Kl*fus3* (Fig. 1). The strongest effect on mating was observed when the scaffold protein KlSte5p and the KlSte20p, KlSte11p, and KlSte7p kinases were inactivated, indicating that all these proteins play essential roles in haploid mating. KlSte5p shows a moderate degree of identity with its counterpart in *S. cerevisiae* (27% identity) (Table 2). *S. cerevisiae* Ste5p (ScSte5p) has no catalytic activity but acts as a scaffold, organizing and forming a complex with the MAPK module composed by ScSte11p, ScSte7p, and ScFus3p (4, 35). The KlSte20p is a member of the PAK family (17), which is 50% identical to ScSte20p (Table 2) and shows high degree of conservation in the CRIB domain, needed for Cdc42p binding (a small Rho-like G protein) (1), and in the Gβ and kinase domains (16). KlSte11p is 58% identical to ScSte11p (Table 2), which in *S. cerevisiae* also binds to Gβ and is phosphorylated and activated by the ScSte20p kinase (28). ScSte11p has an essential role in mating since it appears to be an essential bridge between proteins acting as inputs of the MAPK module and the G protein (10). KlSte7p is 52% identical to ScSte7p (Table 2). In the *S. cerevisiae* pheromone response pathway, Ste7p is required for efficient activation of the MAPK Fus3p via its recruitment to the scaffold protein Ste5 and its activation by Ste11p (10, 20). Disruption of the Kl*STE50* gene affected mating with moderate strength. Comparison of the primary structure of KlSte50p with its *S. cerevisiae* homologue showed overall 41% identity (Table 2). The SAM (sterile alpha motif) domain, involved in Ste11p binding, and the Ras association domain (30) are the most conserved regions within the primary structure of Ste50 proteins. Inactivation of ScSte50p leads to attenuation of pheromone signaling and to sterility in *S. cerevisiae* (36). Finally, inactivation of KlFus3p yielded the weakest sterile phenotype. The *K. lactis* and *S. cerevisiae* Fus3 proteins share a relatively high degree of identity (69%) (Table 2). In *S. cerevisiae* inactivation of Fus3p leads to partial sterility (21) due to the fact that it can be replaced by ScKss1p, the MAPK involved in pseudohyphal development (5). The *K. lactis* genome contains an ORF (KLLA0A02497) that encodes a putative KlKss1p. Although we have not investigated the role of this kinase, we assume, by analogy to *S. cerevisiae*, that KlKss1p can replace KlFus3p in the mating pathway, leading to the observed weak sterile phenotype of the Δ Kl*fus3* mutant.

To determine the connection between the heterotrimeric G protein and the MAPK module, we assayed protein-protein interactions of KlSte50p and KlSte20p with the MAPKKK KlSte11p and with the Gα (KlGpa1p) and Gβ (KlSte4p) subunits of the heterotrimeric G protein. Although it should be confirmed that protein interactions have a functional meaning, as a first approach we chose the two-hybrid system to measure interactions because its reliability has been proved in a number of cases involving components of the mating pathway. We used the LexA-B42-based two-hybrid system in which protein association leads to both activation of the reporter *LACZ* gene located in plasmid pSH18-34 and activation of the chromosomal *LEU2* gene, controlled by several *lexA-op* sites, in the host strain (23). The KlSte50p and KlSte20p kinases were the focus of this analysis because we have observed that they are implicated in the pheromone response pathway, possibly acting as effectors of the G protein (data not shown). The two-hybrid assay detected that KlSte50p makes a moderate interaction with KlGpa1p, the Gα subunit of the heterotrimeric G protein, while the KlSte20p kinase interacts strongly with the Gβ subunit KlSte4p (Fig. 2). These assays also revealed that both

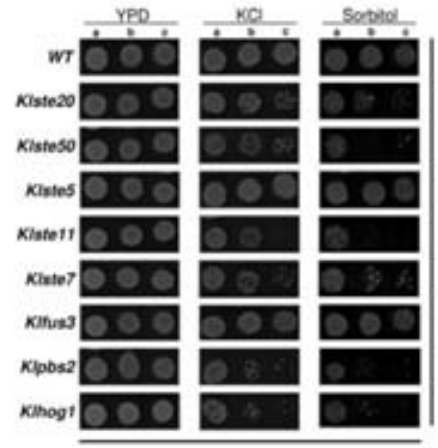

FIG. 3. Effect of high osmolarity on the growth properties of disruption mutants. Tenfold serial dilutions of the indicated strains were spotted onto plates containing YPD medium alone, YPD medium with 0.5 M KCl, and YPD medium with 1.0 M sorbitol and incubated at 30°C for 48 h. Lanes a, 10^6 cells; lanes b, 10^5 cells; lanes c, 10^4 cells. WT, wild type.

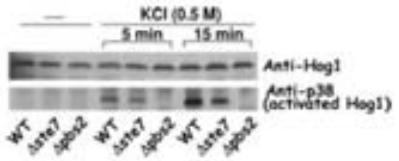

FIG. 4. Phosphorylation of KlHog1p in wild-type (WT), ΔKl*ste7*, and ΔKl*pbs2* strains. Cells were grown to mid-exponential phase in YPD medium and not treated or treated with 0.5 M KCl. After the indicated times, the cells were lysed as described in Materials and Methods. Samples of the resulting extracts were analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting with anti-Hog1 or anti-p38.

the KlSte50p and the KlSte20p kinases associate with the MAPKKK KlSte11p. The KlSte50p-KlSte11p interaction is stronger than that of KlSte20p with KlSte11p (Fig. 2), most probably due to the presence of the strongly interacting SAM domains in KlSte50p and KlSte11p (30). These findings suggest that KlSte50p and KlSte20p may serve as links between the G protein and the MAPK module in the transmission of the pheromone stimulus in *K. lactis*.

Several elements of the yeast pheromone response pathway play a role in other signal transduction cascades (10, 13, 20). Concerning the high-osmolarity response pathway in *S. cerevisiae*, at least three elements of the pheromone pathway are needed to regulate the osmoadaptation program: Ste50p, Ste20p, and Ste11p, which function as upstream elements of the scaffold and kinase protein Pbs2p and the MAPK Hog1p in one of the branches dedicated to adaptation of yeast to high osmolarity (13). All these proteins are components of the so-called HOG pathway. We assayed the *K. lactis* disrupted strains under conditions that produced osmotic (high K^+ and high sorbitol) stress (Fig. 3). In this set of experiments we found increased sensitivity to sorbitol and KCl of mutant strains ΔKl*Pbs2* and ΔKl*Hog1* compared to the wild-type strain, which is in agreement with their expected roles in osmoadaptation. The strong sensitivity of mutant ΔKl*Ste11* to high osmolarity was also evident, indicating some parallelism of KlSte11p function with Ste11p from *S. cerevisiae* (26). Surprisingly, in this set of experiments we observed that the ΔKl*Ste7* mutant showed sensitivity to stress conditions, as indicated by its marked growth delay in 0.5 M KCl and 1 M sorbitol compared to the growth of wild-type cells (Fig. 3). This finding is striking and suggests that the MAPKK KlSte7p is involved in a signal transduction cascade related to the osmotic response. Inactivation of the KlSte50p and KlSte20p kinases produced severe sensitivity to high osmotic stress, confirming that these kinases play overlapping roles in the propagation of different signals (Fig. 3). As expected, the ΔKl*ste5* and ΔKl*fus3* mutants were insensitive to hyperosmotic stress (Fig. 3).

In *S. cerevisiae*, Pbs2p acts as a MAPKK in phosphorylating Hog1p, and in addition to its catalytic activity, it also serves as a scaffold, binding Sho1p, Ste11p, and Hog1p (9). Comparison of the primary sequences of ScPbs2p and KlPbs2p indicates that KlPbs2p conserves the kinase domain which in *S. cerevisiae* has been shown to phosphorylate ScHog1p (3), suggesting that KlPbs2p may acts as a MAPKK over KlHog1p. Since the sensitivity to high osmolarity of the ΔKl*ste7* mutant is not as strong as the one of ΔKl*pbs2* (Fig. 3), we hypothesize that either KlPbs2p lacks a kinase activity and KlSte7p may replace it or that the propagation of the osmoresponse requires both proteins acting in parallel. In order to gain some light on the role that KlSte7p plays in the osmotolerance process, we determined the KlHog1p phosphorylation levels in wild-type, ΔKl*ste7*, and ΔKl*pbs2* backgrounds (Fig. 4). Dual phosphorylation of Hog1p (Thr173 and Tyr175 in *K. lactis*) can be detected by immunoblotting with an antibody raised against activated mammalian p38, as previously reported (24). Upon exposure for 5 min of wild-type cells to 0.5 M KCl, KlHog1p was phosphorylated. By 15 min, the amount of activated KlHog1p increased 1.4-fold (Fig. 4). KlHog1p phosphorylation levels were 30% (5 min) and 60% (15 min) lower in the ΔKl*ste7* mutant than in the wild-type strain even though the amount of total KlHog1p did not show significant variation, as determined by an anti-Hog1 antibody (Fig. 4). No detectable phosphorylation of KlHog1p was observed in the ΔKl*pbs2* mutant, even at 15 min of exposure to KCl. These results suggest that KlSte7p may partially activate KlHog1p in response to hyperosmotic stress and that this activation is totally dependent on KlPbs2p.

The above observations suggest that KlSte7p and KlHog1p are able to physically associate with each other. By means of the two-hybrid assay, we observed that KlSte7p made a significant interaction with KlHog1p but failed to associate with KlPbs2p (Fig. 2). KlPbs2p and KlHog1p showed very weak interaction in the two-hybrid assay. Confirmatory data were obtained by immunoprecipitation assays. We transformed a 5-FOA-resistant ΔKl*ste7* strain with the pPHO (phosphate-dependent promoter) plasmid carrying the gene encoding KlSte7p tagged with six His residues at its C terminus. This plasmid reverses the sterile phenotype of the 5-FOA-resistant mutant. Under phosphate induction conditions, KlHog1p was immunoprecipitated by anti-His antibodies in the presence of KlSte7(His_6)p (Fig. 5) and, vice-versa, the KlSte7(His_6) protein was immunoprecipitated by anti-Hog1p antibodies in the presence of KlHog1p (Fig. 5). Coimmunoprecipitation was

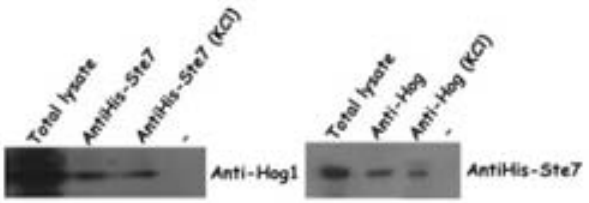

FIG. 5. Physical interaction between KlSte7p and KlHog1p. KlSte7p and KlHog1p coimmunoprecipitation from cells extracts of the Δ*Klste7* strain carrying the pPHO-KlSte7(His_6) plasmid. Cells were grown to mid-exponential phase in selective medium and not treated or treated with 0.5 M KCl for 10 min. Cells were lysed as described in Materials and Methods. KlSte7(His_6)p was precipitated with anti-His antibodies, and the recruited KlHog1p was detected by immunoblotting using an anti-Hog1 antibody. KlHog1p was precipitated with anti-Hog1, and the recruited KlSte7p was detected by immunoblotting using an anti-His antibody.

independent of hyperosmotic shock induced with 0.5 M KCl. Anti-Hog1p antibodies were able to precipitate KlSte7(His_6)p at different phosphate concentrations which induce variable expression levels of KlSte7(His_6)p (data not shown). These findings suggest that KlSte7p makes physical contact with both activated and inactivated KlHog1p and suggest a model whereby KlSte7p is able to phosphorylate KlHog1p during the response to high-osmolarity conditions only when KlHog1p has been recruited by KlPbs2p.

DISCUSSION

In *S. cerevisiae*, the Gβγ complex of the heterotrimeric G protein transmits the pheromone signal to different effectors, two of which are the Ste20p kinase and the Ste5p/Ste11p complex. The PAK kinase Ste20p is essential for Ste11p activation (2), and Ste50p has been implicated as an adaptor protein linking the G-protein-associated Cdc42p-Ste20p kinase complex to Ste11p via its SAM domain, thus modulating the pheromone response pathway (30). In *K. lactis* disruption of Kl*STE20* produced complete sterility while disruption of Kl*STE50* reduced but did not eliminate mating. Our experiments reveal that the MAPK cascade that drives mating in *K. lactis* is composed of the proteins KlSte5p, KlSte11p, KlSte7p, and KlFus3p and suggest that this MAPK module is triggered by KlSte50p and KlSte20p kinases in response to activation of the heterotrimeric G protein. It is reasonable to assume that the *K. lactis* MAPK module is formed by KlSte11p (MAPKKK), KlSte7p (MAPKK), and KlFus3p (MAPK), anchored by KlSte5p (scaffold). However, detailed epistasis experiments should be performed to confirm this arrangement. Our preliminary studies indicate that both KlSte50p and KlSte20p interact with KlSte11p, thus activating the MAPK cascade that elicits mating in *K. lactis*. The actual picture of the pheromone response pathway in *K. lactis* points to a model where activation of G protein by binding of pheromone to GPCR (32) triggers two branches that converge in the MAPKKK KlSte11p; one is essential for mating and is formed by Gβ-KlSte20p, and the second is dispensable and is formed by Gα-KlSte50p. Additional support for this model is given by the disruption phenotypes of mutants that inactivate the G protein subunits. While inactivation of KlSte4p (Gβ) leads to complete incapacity to mate (14), inactivation of KlGpa1p (Gα) only reduces mating but does not eliminate it (31). The physical association between Gα and KlSte50p opens a new window for the study of new functional relationships of G protein signaling components.

Participation of Ste50p, Ste20p, and Ste11p in the Sho1p branch of the HOG pathway has been extensively documented in *S. cerevisiae* (12, 15). In a brief view of the pathway, the osmosensor Sho1p recruits Pbs2p to the membrane during signaling. Both Sho1p and Pbs2p can bind Ste11p. Ste11p is activated by phosphorylation, which is mediated by the Ste20p and Cla4p kinases and requires Ste50p (30). Ste11p activates Pbs2p, which in turn activates Hog1p (15). The high-osmolarity response pathway in *S. cerevisiae* is also regulated by the Sln1p branch, which consists of the Sln1p-Ypd1p-Ssk1p phosphorelay system. Sln1p is an osmosensor histidine kinase, Ypd1p is a phosphotransfer protein, and Ssk1p is a response regulator (25). Hyperosmotic shock deactivates Sln1p, leading to activation of Ssk2p and Ssk22p (two redundant kinases) via the Ssk1p response regulator. Ssk2p and Ssk22p activate the MAPKK Pbs2p, which in turn activates, by phosphorylation, the MAPK Hog1p (22). The two branches for Hog1p activation are not redundant, since the Sln1p-Ssk1p branch has a more prominent role than the Sho1p-Ste11p branch in intermediate osmolarity (0.1 M KCl), but both branches function at high osmolarity (24). At present, it is not known if these two branches are active in *K. lactis*; however, the phenotype of strong sensitivity to high sorbitol (1.0 M) and high salt (0.5 M KCl) displayed by mutants Δ*KlSte50*, Δ*KlSte20*, and Δ*Klste11*, strongly suggests that the Sho1p pathway has remarkable participation in osmoadaptation to high-stress conditions. Moreover, disruption of Kl*STE11* induces severe growing defects under high osmotic stress in *K. lactis*, while in *S. cerevisiae* cells, inactivation of Ste11p induces sensitivity to hyperosmotic conditions only in Δ*ssk1* or Δ*ssk2* Δ*ssk22* backgrounds (22, 24). In addition, it is worth noting that the Sln1p branch of the HOG pathway in *K. lactis* lacks the homologue Ssk22p (15) although we actually do not know the functional meaning for the absence of this MAPKKK.

In addition to the interaction detected between components of the MAPK module and scaffold proteins, two-hybrid studies indicate that some of the protein kinases associate with each other independently of their association with the scaffold protein. In particular, both ScSte7p and ScSte11p interact with the MAPKs ScFus3p and ScKss1p, independently of Ste5p (4, 29). However, due to the sensitivity to conditions of high osmotic stress and to the lower KlHog1p phosphorylation level displayed by the Δ*Klste7* strain, we believe that the interaction observed between KlSte7p and KlHog1p is significant and might play an important role in osmoadaptation. In *S. cerevisiae*, the Ste7p kinase has been shown to participate not only in mating but also in filamentous and invasive growth (20); however, a mutant strain lacking Ste7p shows normal osmotolerance, which indicates that this kinase does not participate in the HOG response pathway in this yeast species. We were unable to find physical interaction between KlSte7p and KlPbs2p, and the interaction between KlPbs2p and KlHog1p was rather weak. It is well known that failure to find an association by the two-hybrid system can occur for many reasons; therefore, association between these proteins should be tested by different techniques.

Sensitivity of the ΔKl*ste7* mutant to high osmotic stress and association of KlSte7p with KlHog1p in *K. lactis* are, at least, intriguing observations. Either KlPbs2p lacks a kinase activity and KlSte7p is able to replace it, or KlHog1p can be phosphorylated by redundant MAPKK activities in the HOG pathway. Our results discarded the first hypothesis since KlHog1p phosphorylation is still observed in ΔKl*ste7* mutant cells. Although we have no direct evidence that KlSte7p phosphorylates KlHog1p, we found that the amount of phosphorylated KlHog1p diminished in cells lacking the KlSte7p kinase. This may affect the growing rate of the Kl*ste7* mutant under hyperosmotic conditions. Additionally, our results showed that KlHog1p phosphorylation is completely dependent on KlPbs2p, suggesting that KlHog1p needs to be recruited by the scaffold activity of KlPbs2p in order to be phosphorylated by KlSte7p; i.e., KlSte7p fails to phosphorylate soluble KlHog1p.

We have provided evidence that in addition to KlSte50p, KlSte20p, and KlSte11p, KlSte7p also participates in both the pheromone response pathway and in the high-osmolarity response pathway in *K. lactis*. Despite the common use of protein kinases in both pathways, our observations also indicate that there is no cross talk between these two signaling systems; i.e., sexual pheromone does not induce a high-osmolarity response and conditions of high osmotic stress do not induce mating (data not shown).

The composition of the G protein signaling pathway for mating, the participation of the KlSte7p in the HOG pathway, and the strong sensitivity phenotype of the ΔKl*ste11* mutant to hyperosmotic stress indicate that signaling systems in *K. lactis* follow, at least in part, mechanisms different from those of *S. cerevisiae*. The perspective ahead is to understand further the organization and functional interactions of the elements belonging to the intracellular MAPK signaling pathways in *K. lactis*. The results will be of interest for the understanding of related problems in other organisms.

ACKNOWLEDGMENTS

We thank Guadalupe Codiz and Minerva Mora (Molecular Biology Unit) for technical assistance and the staff of the Computer Facility at Instituto de Fisiología Celular, Universidad Nacional Autónoma de México. We also thank Marisela Bolaños for technical assistance.

This work was partially funded by grants 44178 from CONACyT, México, and IN211906 from PAPIIT, DGAPA, Universidad Nacional Autónoma de México.

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DISCUSIÓN

Sistema de Respuesta a feromonas en K. lactis.

El sistema de respuesta a feromonas de *K. lactis*, a pesar de utilizar la misma cascada de MAP cinasas que *S. cerevisiae*, presenta diferencias importantes en la manera de transducir la señal.

Los receptores de feromona, tanto KlSte2p como KlSte3p, contienen regiones sumamente conservadas como los dominios transmembranales, la segunda y tercer asa intracelular, llegando a tener hasta un 86% de identidad en ésta última.

El extremo carboxilo terminal de Ste2p es necesario para la regulación y la estabilidad de la proteína, ya que mutantes que carecen de esta región tienen defectos en la formación y orientación de las proyecciones que forma la célula al estar en presencia de feromona, no se adaptan a la respuesta y su internalización es más lenta (Konopka et al., 1988; Reneke et al., 1988; Vallier et al., 2002). Ste2p se regula por medio de su secuencia SINNDAKSS, ya que al ser fosforiladas estas serinas (S), se puede ubiquitinar alguna de las siete lisinas (K) de su extremo carboxilo terminal, para activar la endocitosis del receptor; también se regula por un mecanismo independiente de ubiquitina por medio de la secuencia GPFAD (Hicke and Riezman, 1996; Hicke et al., 1998; Rohrer et al., 1993; Terrell et al., 1998). Sin embargo la identidad entre el extremo carboxilo terminal de Ste2p y KlSte2p es sólo del 21%, siendo la región menos conservada de la proteína. En KlSte2p no se pudo localizar una secuencia semejante a SINNDAKSS de Ste2p, por lo que aun queda por explorar los sitios de regulación de este receptor.

En Ste3p se ha identificado la secuencia NPFSTD como señal de internalización independiente de ubiquitina (Tan et al., 1996). También se sabe que la región del residuo 414 al 451 es ubiquitinada principalmente en K^{432} aunque en ausencia de ésta K^{424} o K^{453} pueden utilizarse para regular su internalización (Roth and Davis, 1996; Roth and Davis, 2000; Roth et al., 1998). Al igual que como sucede con Ste2p, el extremo carboxilo de Ste3p y KlSte3p tiene poca similitud, sólo de 22%. No es posible encontrar secuencias parecidas a las ya descritas como importantes para su regulación. Ste3p tiene 17 lisinas en la parte que corresponde al extremo carboxilo terminal, a diferencia de KlSte3p que sólo tiene 7, por lo que es difícil pensar cuales serían sus posibles puntos de regulación para la endocitosis del receptor. Esto sugiere que los mecanismos de regulación de los receptores para feromona en *K. lactis*, son distintos a los de *S. cerevisiae*.

Los genes de los receptores son sexo específicos, es decir *KlSTE2* solamente se expresa en las células *Mat***a** y *KlSTE3* sólo se expresa en células *Mat*α, teniendo como ligando a la feromona α y **a** respectivamente. Los receptores son necesarios para que la respuesta a feromonas se active, ya que las mutantes tienen fenotipo de esterilidad. En *S. cerevisiae*, se ha reportado que la tercer asa intracelular es importante para acoplar la proteína G heterotrimérica (Celic et al., 2003; Stefan and Blumer, 1994), al igual que el extremo carboxilo terminal donde se ha demostrado que hay interacciones tanto con la subunidad α, como con la subunidad β (Duran-Avelar et al., 2001). Sin embargo, en *K. lactis* el extremo carboxilo terminal del receptor sólo es capaz de interactuar con la subunidad α y esta interacción es dependiente de la activación del sistema, ya que la afinidad del receptor por la mutante de α KlGpa1p^{Q298L} que está constitutivamente activa es menor que por KlGpa1p. Así que el extremo carboxilo terminal del receptor es capaz de discriminar entre una subunidad α unida a GDP o GTP.

Esto implica que el acoplamiento de la proteína G heterotrimérica al receptor es distinta en las dos especies, posiblemente por la diferencia que existe entre el extremo carboxilo terminal de los receptores de ambas especies.

Como ya había mencionado anteriormente en *K. lactis* tanto KlGpa1p (Gα) como KlSte4p (Gβ) son efectores de la vía. Una mutante Δ*Klste4* es estéril lo que indica que es esencial para la transducción de la señal. Por otro lado una mutante Δ*Klgpa1* baja la eficiencia del apareamiento, lo que nos sugiere que KlGpa1 tiene efectores y en ausencia de la señal tiene consecuencias en la transducción de la señal dentro de la vía (Kawasaki et al., 2008; Lloret et al., 2003; Savinon-Tejeda et al., 2001).

En *S. cerevisiae*, una vez activado el sistema Ste4p recluta a Ste20p y Ste5p, interactuando con el extremo carboxilo terminal del primero (Leeuw et al., 1998) y utilizando el dominio RING-H2 del extremo carboxilo terminal del segundo, que es una proteína de andamiaje, aunque Ste5p también requiere de una α hélice amfipática del extremo amino terminal, que es afín a la membrana plasmática (Inouye et al., 1997; Whiteway et al., 1995; Winters et al., 2005). Ste20p del complejo Cdc42p-Ste20p es la cinasa que activa a Ste11p (Wu et al., 1995) y requiere de la proteína adaptadora Ste50p, debido a que Cdc42p tiene afinidad por el dominio RAD (Ras associated domain) de Ste50p y ésta a su vez interactúa con Ste11p por medio de los dominios SAM de ambas proteínas, de esta manera Ste20p está cerca de Ste11p gracias a Ste50p y Cdc42p (Ramezani-Rad, 2003; Tatebayashi et al., 2006; Truckses et al., 2006). Además, Ste5p interactua con Ste11p, de esta manera se forma un gran complejo de señalización donde se encuentran todas las moléculas involucradas en la trasducción de la señal (Choi et al., 1994; Inouye et al., 1997).

En *K. lactis* nosotros generamos las mutantes de los genes que codifican para los elementos de la cascada de señalización de respuesta a feromona y comprobamos que son estériles al igual que en *S. cerevisiae*, a excepción de la

mutante Δ*Klste50* que puede formar diploides, pero con menor eficiencia que la cepa silvestre. Esto significa que no es indispensable en la transducción de la señal y que otra molécula podría seguir con la señalización aun en la ausencia de Klste50p. La secuencia de KlSte50p tiene una identidad del 41% con su homólogo en *S. cerevisiae*, el dominio SAM sólo tiene una identidad del 35%, a diferencia del dominio RAD que tiene una identidad del 74%. La función de Ste50p es facilitar que la distancia entre Ste20p y Ste11p sea lo suficientemente corta para que la primera fosforile a la segunda, mediante la unión Ste50p-Cdc42p. Sin embargo en *K. lactis* parece tener un papel adicional, ya que por ensayo de doble híbrido encontramos interacción entre KlSte50p y KlGpa1, además de la interacción esperada de KlSte50p con KlSte11p. Anteriormente ya se había reportado en *S. cerevisiae* que Ste50p puede llegar a tener una interacción débil con Gpa1 (Xu et al., 1996). Además el fenotipo de la mutante Δ*Klste50* es semejante a Δ*KlGpa1*, con una baja en la eficiencia de apareamiento, así que la transducción de la señal que depende de KlGpa1p podría involucrar su interacción con KlSte50p. Con los resultados obtenidos por las mutantes y las interacciones de proteínas con el sistema de doble híbrido podemos proponer el siguiente modelo, donde tendríamos dos ramas que convergen en la activación de KlSte11p, la primera rama sería activada por Klste4 y activaría al módulo de MAP cinasas formado por KlSte11p (MAPKKK), KlSte7p (MAPKK), KlFus3p (MAPK) y KlSte5p como proteína de andamiaje (**Figura 5**), utilizando la interacción KlSte50p-KlSte20p como intermediario para fosforilar a KlSte11, aunque KlSte50p sería dispensable y el reclutamiento de KlSte20p al módulo podría ser mediante la proteína de andamiaje KlSte5 o alguna otra proteína, aunque aun hace falta realizar más experimentos para probar esta hipótesis. La otra rama estaría activada por KlGpa1p y ésta mediante su unión con KlSte50p promovería la activación de KlSte11p, tal vez activada por KlSte20p reclutada por KlSte50. Este estudio nos conduce al estudio de una forma de señalización mediada por proteínas G que no se ha descrito anteriormente.

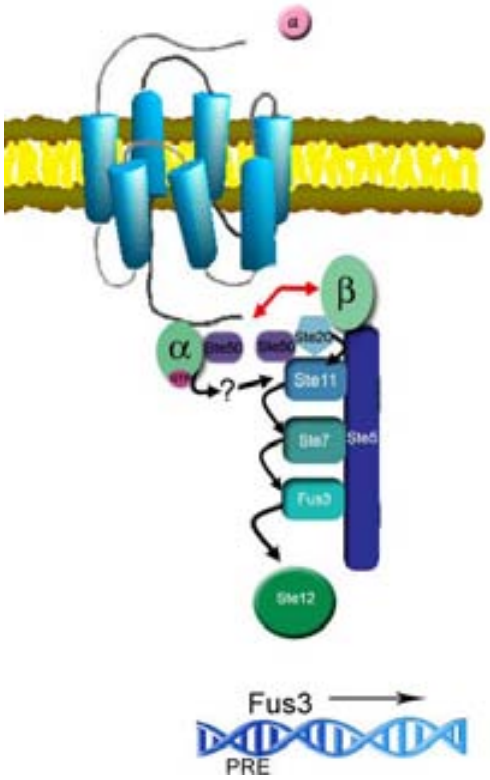

Figura 5. Modelo de la vía de respuesta a feromona sexual en *K. lactis*. KlGpa1p (Gα) interacciona con KlSte50p y posiblemente mediante esta interacción se transduce la señal vía KlGpa1p, de tal manera que tanto la subunidad Gα (KlGpa1p), como la subunidad Gβ (KlSte4p) convergen en la activación de la MAPKKK KlSte11p y de esta manera su activa el módulo de MAP cinasas que tiene como consecuencia la activación de genes necesarios para el apareamiento (como KlFus3p) por medio del factor transcripcional KlSte12p.

Sistema de respuesta a estrés osmótico en K. lactis.

Algunos de los elementos de la vía de respuesta a feromona están involucrados en la respuesta a estrés osmótico. Se sabe que en *S. cerevisiae* estos elementos son Ste50p, Ste20p y Ste11p (Posas et al., 1998; Raitt et al., 2000). La manera en que participan estas proteínas es mediante la proteína transmembranal Sho1p que tiene un dominio SH3 en su región citoplásmica mediante el cual puede unirse al complejo Ste11p-Ste50p y a Pbs2p (Maeda et al., 1995; Tatebayashi et al., 2006), además Sho1p activa a Cdc42p, que a su vez estimula a Ste20 para fosforilar a Ste11p, que fosforila a Pbs2p y que éste

finalmente fosforile a la MAP cinasa Hog1p (Raitt et al., 2000). En esta activación la interacción Ste50p-Cdc42p también es importante para acercar a Ste20p a Ste11p. Además existe otra rama involucrada en respuesta osmótica que está regulada por el sistema de fosforrelevo Sln1p-Ypd1p-Ssk1p. El sensor Sln1p es la cinasa de histidinas, Ypd1p la proteína que transfiere el fosfato y Ssk1p el regulador de la respuesta (Posas et al., 1996). Un choque hiperosmótico inactiva a Sln1 que tiene como consecuencia la activación de las proteínas redundantes Ssk2p-Ssk22p mediante Ssk1p. Ssk2p y Ssk22p activan a Pbs2p para que fosforile a Hog1p (Posas and Saito, 1998). Las dos ramas responden a estrés hiperosmótico, aunque la rama de SLN tiene un umbral más bajo y responde desde concentraciones bajas (0. 0625 mM de KCl) (O'Rourke and Herskowitz, 2004).

En *K. lactis* hasta el momento no se había descrito que elementos participan en respuesta a estrés osmótico, sin embargo las mutantes Δ*Klste50*, Δ*Klste20* y Δ*Klste11* tienen un fenotipo de sensibilidad en condiciones hiperosmóticas tanto con sorbitol 1M como con KCl 0.5M, lo que nos indica que el papel de la rama de SHO es más importante que el que pudiera tener la rama de SLN que actualmente se esta estudiando en nuestro laboratorio, ya que en *S. cerevisiae* la ausencia de Ste11p sólo presenta fenotipo en un fondo genético Δ*ssk1* o Δ*ssk2* Δ*ssk22* (Posas and Saito, 1997). Lo que nos deja por explorar si la rama de SLN juega algún papel menor en estrés osmótico en *K. lactis* o responde a otro tipo de respuesta.

Lo que es de resaltar es que la mutante Δ*Klste7* tiene un fenotipo de sensibilidad a estrés osmótico, lo que nos indica que juega un papel en esta vía de señalización. Se sabe que en *S. cerevisiae* Ste7p no participa en la vía de HOG. Mediante el ensayo de doble híbrido encontramos interacción entre KlSte7p y KlHog1, sin embargo no pudimos detectar interacción con KlPbs2p, lo que nos llevó a plantear un papel funcional de ésta cinasa en la activación de KlHog1, teniendo dos posibilidades: KlPbs2p no tiene actividad de cinasa y

KlHog1p es fosforilada por KlSte7p, o KlHog1p puede ser fosforilada por las dos MAPK cinasas (**Figura 6**). La primera hipótesis se descartó debido a que en la mutante Δ*Klste7* se sigue fosforilando KlHog1p, pero baja el nivel de fosforilación. Ahora bien, la coinmunoprecipitación nos confirmó que existe una interacción directa entre KlSte7p y KlHog1p que es independiente de la activación de la vía. Estos datos nos llevan a proponer que KlSte7p interactúa con KlHog1p y puede tener un efecto aditivo en la fosforilación de KlHog1p, pero necesita de la proteína de andamiaje KlPbs2.

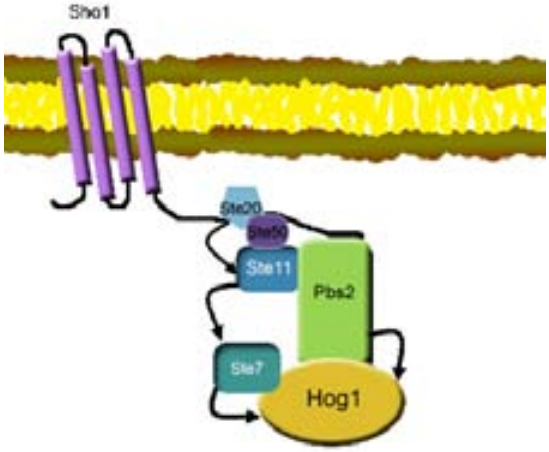

Figura 6. Modelo de la vía de estrés osmótico en *K. lactis*. KlSte7p participa en la vía, interacciona con KlHog1p y aumenta su nivel de fosforilación una vez activada la vía. De esta manera tanto KlPbs2p como KlSte7p contribuyen a la activación de KlHog1p.

Nosotros encontramos que además de KlSte20p, KlSte50p y KlSte11p, KlSte7p participan tanto en la vía de respuesta a feromona, como en la vía de respuesta a estrés osmótico en *K. lactis*. Se sabe que en *S. cerevisiae* por compartir elementos que participan en distintas vías la activación de una de éstas puede activar otras (O'Rourke and Herskowitz, 1998), sin embargo en *K. lactis* este fenómeno no se presenta entre la vía de respuesta a feromona y estrés osmótico, ya que inducir con feromona y después someter a la célula a estrés osmótico o viceversa no le confiere a la célula ninguna resistencia a

cambios osmóticos o capacidad para aparearse como sucede en *S. cerevisiae*, lo que nos indica que los mecanismos de regulación de las dos vías son distintos en estas dos especies, de manera que a pesar de compartir elementos se conservan específicos para su ruta de señalización. Posiblemente esta especificidad esté regulada por las proteínas de andamiaje como se ha propuesto (Good et al., 2009; Harris et al., 2001; Park et al., 2003), sin embargo a diferencia de lo que ocurre en *S. cerevisiae* en *K. lactis* existiría un mecanismo que compromete a los complejos de señalización ya ensamblados a ser específicos solo para una determinada vía de transducción de señales, de tal manera que una vez comprometido el complejo de cinasas para una determinada vía, las demás no podrían utilizarlo.

En resumen la señalización mediada por la proteína G del sistema de respuesta a feromonas en *K. lactis* donde KlGpa1 transduce la señal posiblemente mediante KlSte50p, la participación de KlSte7p en respuesta a estrés omsótico y el fenotipo acusado de los elementos de la rama SHO en respuesta a estrés osmótico (KlSte20p, KlSte50p y KlSte11p), nos demuestran que estos dos sistemas son diferentes. Llegar a entender los mecanismos de regulación nos ayudaría a entender el funcionamiento de sistemas de transducción de señales en otros organismos.

CONCLUSIONES

Los receptores transmembranales KlSte2p y KlSte3p son los receptores para las feromonas α y **a** respectivamente, del sistema de respuesta a feromona sexual.

El extremo carboxilo terminal del receptor KlSte3p se asocia a la subunidad α (KlGpa1p) de la proteína G y esta unión es dependiente de la activación del sistema.

Las proteínas KlSte20p, KlSte50p, KlSte5p, KlSte11p, KlSte7p y KlFus3p participan en el sistema de respuesta a feromona sexual.

La proteína KlSte50p es un posible efector de la subunidad α (KlGpa1p) de la proteína G en el sistema de respuesta a feromona sexual.

Las proteínas KlSte20p, KlSte50p, KlSte11p, KlSte7p, KlPbs2p y KlHog1p participan en el sistema de respuesta a estrés osmótico.

La MAPK cinasa KlSte7p se asocia a la MAP cinasa KlHog1p y aumenta su nivel de fosforilación en respuesta a estrés osmótico.

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