Medical Mycology June 2008, **46**, 385–388

informa
healthcare

Expression of a kexin-like gene from the human pathogenic fungus *Paracoccidioides brasiliensis* in *Saccharomyces cerevisiae*

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Kexin-like proteins are proteinases belonging to the subtilase family which are involved in the processing of pro-proteins to their active forms. In fungi, kexin-like proteins are involved in several important cellular processes, including mating and dimorphism. *Paracoccidioides brasiliensis*, the causative agent of paracoccidioidomycosis undergoes a thermo-regulated dimorphic transition which is essential for the establishment of the infection. Although the molecular mechanisms which rule this process are still unknown, several genes identified in *P. brasiliensis* have been implicated in dimorphism, including *kex2*, a kexin-like protein. In this work we have used the baker's yeast *Saccharomyces cerevisiae* as a host to perform heterologous expression analysis of the *P. brasiliensis kex2* gene. Our data shows that *kex2* can complement the functions of a *S. cerevisiae kex2* mutant strain and could therefore be considered its functional homologue.

Keywords *kex2*, kexin-like protein, dimorphic fungus, *Paracoccidioides brasiliensis*

Introduction

Many secreted proteins and peptides are initially synthesized as high molecular mass precursors which require proteolytic cleavage in order to be fully functional. Kexin-like proteins are Ca^{2+}-dependent transmembrane serine proteinases which belong to the subtilase family. These proteinases recognize and cleave after dibasic residues in the amino-terminal portion of pro-proteins thus leading to their active forms [1]. In fungi, kexin-like proteinases are known to be involved in the processing of many proteins like the *Saccharomyces cerevisiae* mating pherormone (α-factor) which is processed by Kex2p [2].

The dimorphic fungus *Paracoccidiodes brasiliensis* is the aetiological agent of paracoccidioidomycosis, an endemic human systemic mycosis. The disease normally starts after inhalation of fungal propagules which can differentiate to the yeast form inside the human host. The mycelium to yeast transition can be triggered solely by a temperature shift from 26–36°C which may be sufficient for the establishment of the infection [3]. The molecular mechanisms involved in this process are largely unknown although several differently expressed genes have been isolated [4]. Disruption of kexin-like genes in *Candida albicans* and *Yarrowia lipolytica* affect hyphae production and induce morphological cell defects suggesting a possible role of these proteinases in the dimorphic transition in human pathogenic fungi [5,6]. At present, the role of kexin-like proteins in the *P. brasiliensis* dimorphic transition can not be assessed by similar genetic analyses since this fungus is multinucleated and molecular genetic tools are still being developed. Alternatively, the use of other genetic systems, such as in *S. cerevisiae*, may represent one of the few options to perform *in vivo* functional analysis. The *P. brasiliensis kex2* gene, hereafter called *Pbkex2*, has been isolated and characterized [7]. Due to the relevant role of this gene in dimorphism in other pathogens we sought the

Received 16 April 2007; Accepted 12 December 2007
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DOI: 10.1080/13693780701875827

functional analysis of *Pbkex2* in a heterologous system. Here we present the functional analysis of *Pbkex2* in *S. cerevisiae* by *trans*-complementation.

Materials and methods

Strains and media

Paracoccidiodes brasiliensis Pb01 (ATCC MYA-826) and *Saccharomyces cerevisiae* S228C (*MATα SUC2 mal mel gal2 CUP1*) were used as a source of genomic DNA for PCR. *Escherichia coli* strains DH5α and XL1 Blue were routinely used for cloning and plasmid amplification and were grown in LB medium (0.5% yeast extract, 1% peptone, 1% NaCl). Yeast media used included: YPD/Gal (1% yeast extract, 2% peptone and 2% glucose/galactose); SD (6.7% yeast nitrogen base plus ammonium sulphate, without amino acids, 2% dextrose 2% agar supplemented with the required amino acids); and SPO (1% potassium acetate, 0.1% yeast extract, 0.05% glucose, 2% agar).

Molecular cloning and yeast transformation

All DNA manipulations were carried out as detailed elsewhere [8]. Restriction endonucleases were obtained from New BioLabs and GE Healthcare. Automated DNA sequencing was performed in a MegaBACE 1000 sequencer (GE Healthcare) using universal primers. The *KEX2* genes from *S. cerevisiae* (*ScKEX2*) and *P. brasiliensis* (*Pbkex2*) were obtained by PCR. Primers used were 5KEX2 (5′-GGATCCCATTATCAGATGAAAGTGAGG) and 3KEX2 (5′-GGATCCTCACGATCGTCCGGAAGATGG) for *ScKEX2* and 5PBKEX (5′-CGGGATCCGAGGCAACATGAAACTTCTGAG) and 3PBKEX2 (5′-CGGGATCCCTAGGTCGTCTCACTCACAC) for *Pbkex*. PCR was carried out using 10 ng genomic DNA, 10 pmoles of each primer, 2.5 mM $MgSO_4$, 1U Platinum Taq (Invitrogen) in a final volume of 50 µl. The system was submitted to 30 amplification cycles of 94°C/30 s, 50°C/30 s, 72°C/2.5 min and a final elongation step of 72°C/5 min. PCR products were cloned into pGEM-T (Promega) and the presence of inserts was checked by BamH I digestion following automated DNA sequencing. After verification of insert orientation by restriction analysis, the resulting plasmids were digested with Kpn I and Not I for directional cloning into the yeast galactose-inducible vector pYES2 (Invitrogen) linearized with the same enzymes. Yeast transformation was performed by the lithium acetate procedure [9].

Yeast mating and sporulation assays

Yeast strains of opposite mating types, BJ5459 (*MAT*a *ura3-52 trp1 lys2-801 leu2Δ1 his3Δ200 pep4::HIS3 prb1Δ1.6R can1* GAL) and JB1-3B (*MATα kex2Δ::kanR ura3 leu2 his3 trp1*), were grown in 5 ml of YPD/Gal at 30°C for 16 h. In a 1.5 ml tube, 200 µl of each culture were mixed following incubation at 30°C for 3–4 h. The mating system was collected by centrifugation, washed, and 10 µl were spotted on SD media supplemented with leucine and tryptophan. Plates were incubated at 30°C until colonies were visible. Sporulation was performed by plating putative diploid cells on SPO medium and incubating at 30°C for 7–10 days. Tetrads were visualized under a light microscope.

Halo assay for α-factor secretion

The halo assay was essentially performed as described elsewhere [10]. *S. cerevisiae* strains hypersensitive to α-factor were W303 (*MAT*a *ade2 his3 trp1 ura3 can1 sst1::LEU2*) or M298-12A (*MAT*a *his3 sst1::LEU2 sst2::LEU2 kex2::URA3*) [11]. *MATα* cells (JBI-3B) transformed with plasmids expressing *KEX2* were grown for 24 h in YPD/Gal at 30°C and spotted onto a plate containing a lawn of hypersensitive cells. Plates were incubated at 30°C for 48 h. Halos around tested colonies are the result of the inhibition of the tester cells in response to secreted active α-pheromone.

Results

Molecular cloning of the KEX2 genes from S. cerevisiae and P. brasiliensis

In order to perform *trans*-complementation of *Pbkex2* gene from *P. brasiliensis* in *S. cerevisiae*, the complete *Pbkex2* ORF was amplified by PCR. As a control used in all experiments, the *ScKEX2* ORF was also cloned by PCR. The ~2.6 kb *Pbkex2* amplicon included a 93-pb intron, and *ScKEX2* resulted in a ~2.4 kb intronless amplicon. After cloning and sequence analysis, both genes were transferred to the yeast expression vector pYES2 resulting in plasmids pYESKEX2Pb and pYESKEX2Sc.

Mating assay

KEX2 is involved in yeast mating since it is required for the correct processing of α-factor. *MATα* strains mutant for *KEX2* are unable to mate with *MAT*a cells and are therefore sterile. Plasmids pYESKEX2Pb, pYESKEX2Sc (positive control) and pYES2 (negative control) were used to transform JB1-3B, a *MATα* strain

which is sterile due to insertional inactivation of *KEX2*. Transformed cells were grown in YPD/Gal in order to induce *KEX2* expression and then co-cultivated with BJ5459, a *MAT*a strain. Mating was assessed by complementation of nutritional deficiencies of the cells being mated in selective plates lacking uracil and histidine to ensure that only diploid cells could be selected. A single colony from each mating system was plated on SPO and tetrads were visualized (data not shown) suggesting that they represented authentic diploids.

α-Factor hypersensitivity assay

To further extend our analysis of the functional complementation of *Pbkex2* in *S. cerevisiae*, we carried out a hypersensitivity assay using two *MAT*a tester strains which are defective in *SST1*, the barrier protease that inactivates α-factor allowing cells to recover from α-factor-induced cell cycle arrest [12]. In this assay, a lawn of hypersensitive tester cells is spotted with a *MATα* strain and an inhibition halo is formed around it if functional α-factor is produced. We tested a lawn of two different hypersensitive strains, W303 and M298-12A, with a spot of JBI-3B transformed with pYESKEX2Pb or pYESKEX2Sc and grown in the presence of galactose. The negative control (non-transformed JBI-3B) produced no halo while a $Kex2^+$ control strain (YIP48-*MATα ura3-52 lys2-801 ade1 ade2 his7 trp1Δ1*) was able to inhibit growth of the tester strains (Fig. 1). Both clones expressing *Pbkex2* or *ScKEX2* produced an inhibition halo showing that the cloned *KEX2* genes were able to produce functional α-factor.

Discussion

With the conclusion of two *P. brasiliensis* transcriptome projects [4,13] several new genes have been identified as well as homologues to other known genes which require functional analysis in order to establish their role in cell metabolism. However, in *P. brasiliensis*, functional analysis is hampered by the absence of specific expression vectors despite the fact that transformation protocols have become available recently [14]. Because of its powerful genetic tools, *S. cerevisiae* is an interesting alternative to perform functional analysis of genes isolated from *P. brasiliensis* [15]. In this work we have provided evidence that *Pbkex2* is able to functionally complement a *S. cerevisiae kex2* mutation. Despite the low similarity between the proteins coded by *Pbkex2* and *ScKEX2* (~41% identity), they share the same functional domains found in other fungal kexin-like proteinases [7]. Using molecular yeast genetics tools *Pbkex2* was expressed in a haploid yeast strain (JBI-3B) with a disrupted non-functional copy of *KEX2*. This strain is considered sterile since it is unable to process the Kex2p precursor. Phenotypic rescue may be accomplished by expressing an exogenous version of *KEX2*. We have complemented the loss of Kex function in JBI-3B by expressing *ScKEX2* (positive control) or *Pbkex2* in the yeast expression vector pYES2. Since *ScKEX2* has no introns, it was PCR-amplified directly from the yeast genome [8]. Several attempts were made to clone *Pbkex2* by RT-PCR with no success. We speculate that under the conditions that *P. brasiliensis* is typically grown *Pbkex2* mRNA might be poorly represented. In fact, even after the analysis of more than 6000 transcripts (~80% predicted genome), no ESTs representing *Pbkex2* cDNA sequences were identified [4].

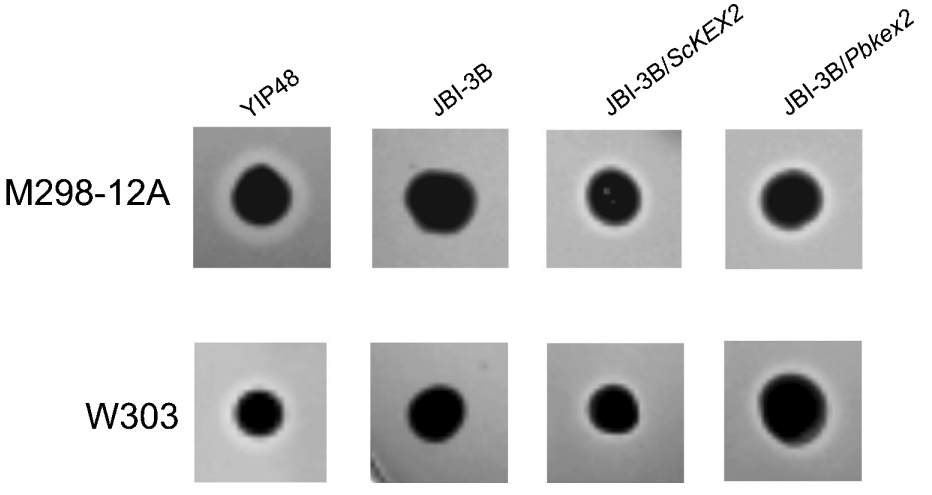

Fig. 1 Halo plate assay for α-pheromone activity. A lawn of α-factor hypersensitive cells (M298-12A and W303) was spotted with JB1-3B transformed with plasmids expressing either *ScKEX2* or *Pbkex2*. The halo surrounding cells is caused by growth inhibition of hypersensitive cells by functional α-factor. YIP48 cells were used as positive controls whereas non-transformed JB1-3B cells were used as negative controls.

One of the basic functions of *KEX2* is to process the α-factor pheromone precursor which can then trigger the first steps of mating in *MAT*a cells. When expressing *PbKex2* or *ScKEX2* sterile JBI-3B cells were able to recover the ability to mate when placed in contact with *MAT*a cells and the resulting diploid sporulated when plated in SPO medium. This is supporting evidence that *Pbkex2* mRNA was being correctly processed by the *S. cerevisiae* splicing apparatus and that the encoded kexin was functionally complementing the absence of Kex activity in JBI-3B yeast cells. Finally, to further show that *Pbkex2* was functionally expressed in *S. cerevisiae* we performed a hypersensitivity test which is usually employed to assess Kex2p activity in yeast. In this assay, tester cells (*MAT*a *sst1*) are placed in contact with a *MATα* strain expressing *KEX2*. If functional Kex2p is produced, α-factor is correctly processed thus causing growth arrest in *sst1* cells. The results of this assay using *Pbkex2* expressed in JBI-3B cells confirmed that α-factor was correctly processed because of the inhibiting halo formed around the tester strain (Fig. 1).

This work represents the second report of a functional analysis of a *P. brasiliensis* gene in *S. cerevisiae*. Now that the function of *Pbkex2* has been tested in a heterologous system it remains to determine the role of this gene in the dimorphic transition of *P. brasiliensis*. One promising tool that could address the role of *PbKex2* in dimorphism is RNA interference which has successfully been used in other fungi [16]. Until molecular genetics in *P. brasiliensis* becomes fully developed, more genes can be tested by *trans*-complementation in the *S. cerevisiae* system taking advantage of the large available collection of mutant strains deleted for specific genes.

Acknowledgements

Yeast strains W303 and M298-12A used throughout this work were a kind gift of Dr Malcon Whiteway. Rosemary Vilaça had a fellowship from CNPq (Brazil).

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