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Cell signaling pathways in *Paracoccidioides brasiliensis* - inferred from comparisons with other fungi

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ABSTRACT. The human fungal pathogen *Paracoccidioides brasiliensis* is an ascomycete that displays a temperature-dependent dimorphic transition, appearing as a mycelium at 22°C and as a yeast at 37°C, this latter being the virulent form. We report on the *in silico* search made of the *P. brasiliensis* transcriptome-expressed sequence tag database for components of signaling pathways previously known to be involved in morphogenesis and virulence in other species of fungi, including *Saccharomyces cerevisiae*, *Cryptococcus neoformans*, *Candida albicans*, and *Aspergillus fumigatus.* Using this approach, it was possible to identify several protein cascades in *P. brasiliensis*, such as i) mitogen-activated protein kinase signaling for cell integrity, cell wall construction, pheromone/mating, and osmo-regulation, ii) the cAMP/PKA system, which regulates fungal development and virulence, iii) the Ras protein, which allows cross-talking between cascades, iv) calcium-cal-

modulin-calcineurin, which controls cell survival under oxidative stress, high temperature, and membrane/cell wall perturbation, and v) the target of rapamycin pathway, controlling cell growth and proliferation. The ways in which *P. brasiliensis* responds to the environment and modulates the expression of genes required for its survival and virulence can be inferred through comparison with other fungi for which this type of data is already available.

Key words: Fungi, *Paracoccidioides brasiliensis*, Signaling pathways, Comparison

INTRODUCTION

Signal transduction describes a great number of biochemical events that transmit a signal from the cell exterior, through the cell membrane, and into the cytoplasm. This involves a number of molecules, including receptors, intermediate proteins, and messengers. The signaling pathways are commonly used by an extensive array of biological ligands to modulate various cell processes, such as growth, differentiation, and proliferation. These transduction pathways communicate information about the external environment to the inside of a cell. Signaling systems in fungi also regulate cell polarity, mating, and pheromone control, and hence they play a role in determining cell shape. Some of the known responses include changes in the cell cycle, polarized growth, and modifications to the transcriptional profile of the cell.

Since the 50's, signaling pathways have been investigated by genetic and biochemical experimentation. In a large series of experiments, eukaryotic organisms were studied for their nutritional limitations and for their reactions to various environmental stresses, such as heat, oxidative, osmotic, or ethylic shock (Engebrecht, 2003).

The genomic era changed the perspective of signaling pathway studies. By using a sequence database, such as GeneBank, the screening of potentially transcribed genes in a given cell led to the rapid identification of critical genes. These methods enable researchers to assess genetic diversity or find similarities among cell types.

Pathogenic organisms sense and respond to the harsh conditions imposed by the host-activating components of signaling pathways that culminate in the expression of genes responsible for the virulence and differentiation of the pathogen. Therefore, studies on signal transduction in various fungi may reveal common conserved mechanisms of signal transduction as well as the differences between these organisms. These studies could lead to drug development.

We report the *in silico* search made of the *Paracoccidioides brasiliensis* transcriptome expressed sequence tag database (Felipe et al., 2003) to evaluate the presence of cellular signaling pathway elements (Figure 1) and to compare them with the cascade components in i) the non-pathogenic fungus *Saccharomyces cerevisiae*, ii) the human opportunistic non-dimorphic fungus *Cryptococcus neoformans*, iii) the human opportunistic dimorphic fungus *Candida albicans*, and iv) the human pathogenic fungus *Aspergillus fumigatus.*

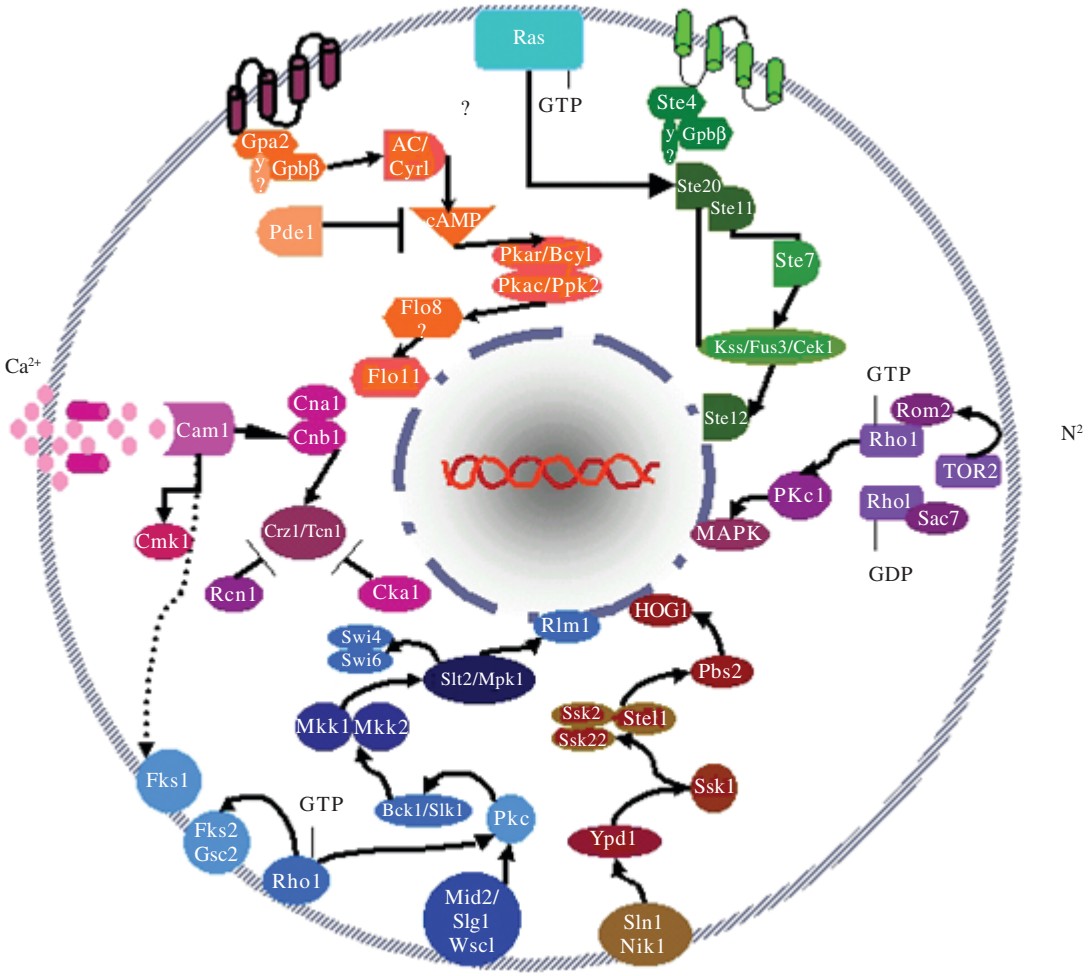

Figure 1. Signal transduction pathways in *Paracoccidioides brasiliensis*. Cell adhesion (orange), pheromone response (green), calcium/calmodulin (pink), cell integrity (blue), high osmotic growth stress response (brown), and TOR (purple) pathways are depicted.

MATERIAL AND METHODS

The identification of putative genes involved in the cellular signaling pathways was performed by the "search by key word" service provided by the bioinformatics group of the PbGenome project (Felipe et al., 2003). The classification of candidates according to the signaling category families was performed by a BLASTx (Zhang, 2003) comparison of sequences against a database with all the signaling protein sequences from Genbank (Benson et al., 2004). The analyzed clusters were assembled by CAP3 software in the sequence-processing pipeline from the PbGenome project. The multiple sequence alignment of the candidates was performed using CLUSTAL W software (Aiyar, 2000).

THE SIGNALING PATHWAYS IN *PARACOCCIDIOIDES BRASILIENSIS*

The mitogen-activated protein kinase cascade

The mitogen-activated protein kinase (MAPK) pathway is a major mechanism for controlling transcription in eukaryotes (Seger and Krebs, 1995). MAPK was originally discovered as an insulin-activated protein-serine kinase, though biochemical studies, reinforced by genetic analysis, indicate a pheromone response in budding yeast.

Fungal cells contain five MAPK cascades that orchestrate responses to different physiological stimuli. One cascade operates in cells undergoing meiosis and regulates spore formation. The other four cascades operate in vegetative, mitotically active cells. Among these cascades, two control developmental events - mating and filamentation. The pheromone response pathway, the cell integrity pathway, and the high osmolarity glycerol (HOG) pathway, can all activate their MAPKs within minutes after initial stimuli (Lengeler et al., 2000).

The pheromone response

Pheromones are ubiquitous in fungal mating systems, and even their general structure is well conserved. Although the pheromone response pathway, and that regulating filamentous growth, share a common MAPK module, their upstream regulators appear to be specific to each of them. Haploid fungal cells respond to pheromones by a MAPK cascade involved in mating.

Normal *S. cerevisiae* cells undergo a vegetative life cycle, but after binding of the appropriate mating pheromone they activate a different developmental pathway that leads to the production of mating filaments. This intercellular communication between the two mating types of cells activates a signal transduction pathway that stimulates the various physiological changes required by the process, such as induction of cell surface agglutinins, cell division arrest at G1, morphogenesis to form a conjugation tube, and cell fusion. The components of this cascade include a G-protein-coupled receptor, several protein kinases, and a pheromone-responsive transcription factor. The molecular mechanisms that transduce the pheromone signal are remarkably similar to the mechanisms of hormone signaling used in multicellular organisms (Konopka and Fields, 1992). The yeast protein kinases encoded by *STE11*, *STE7*, and *FUS3* constitute the kinase cascade in which Ste11p phosphorylates and activates Ste7p, which in turn phosphorylates the MAPK Fus3p (Neiman and Herskowitz, 1994). In this model of signaling, specificity is defined by at least four ramifications of the same signaling pathway. Briefly, Ste12p and Mcm1p activate the transcription factor of the genes that respond to pheromones in haploid cells, whereas in diploid cells, Ste12p and Tec1p activate the genes responsible for filamentation (Lengeler et al., 2000).

In *C. neoformans*, the *STE12* homologue gene, though it has conserved roles in morphogenesis, exists only in mating type (MAT)α cells, and it is directly involved in virulence (Chang et al., 2000). Recently, three genes encoding the MF pheromone were identified in the mating-type locus, and they were found to be transcriptionally induced by limiting nutrients and to co-culture with MATα cells. Overexpression of MFα pheromone enhances haploid budding and the MFα pheromone is not essential for virulence, but it contributes to overall virulence (Wang et al, 2000).

The mating process in *C. albicans* was cytologically described for the first time by Bennett et al. (2003). The cascade consists of Cst20p kinases, which phosphorylate Hst7p and

Cek1p, which are homologous to Fus3p and Kss1p (Lockhart et al., 2002).

The finding of homologue genes suggests the existence of a sexual cycle in *A. fumigatus*. Using the available incomplete genome database, Poggeler and Kuck (2002) deduced that the gene products of the receptor are putative proteins of seven transmembrane domains, which display a high-level amino acid identity with the α-factor receptor Ste3p and the α-receptor Ste2p of *S. cerevisiae*.

The complete pathway from the Skh1p protein (Ste4p), which includes the downstream Ste20p and its targets in tandem Ste11p and Ste7p, was identified in *P. brasiliensis.* The downstream components, such as Kss1p (also called Fus3p/Cek1p), which phosphorylates Ste12p, were also identified in this transcriptome. The proteins *Ste20p*, *Ste11p*, *Ste7p*, and *Kss1p* appear to be constitutively expressed in both mycelial and yeast forms. On the other hand, genes *MOT2* and *MOT3*, which encode nuclear Zn-finger proteins that attenuate the mating pheromone, were not found in the *P. brasiliensis* transcriptome. The function assigned to the whole pathway is to positively regulate growth and cell proliferation (Table 1).

Four mating-type proteins were found through the transcriptome analyses of *P. brasiliensis*: a homologue to the MAT-1 protein of *A. nidulans,* with an E-value of 1e-46, and three PbAESTs, which possibly encode a MAT-2 (Table 1). Despite the presence of the genes involved in the cascade responsive to pheromones, a conclusive observation of the sexual cycle of *P. brasiliensis* is still pending; however, we present evidence that suggests the existence of mating in this organism.

Maintenance of cell integrity

In the budding yeast, *S. cerevisiae*, the MAPK cascade responsible for cell integrity, mediates cell cycle-regulation, cell wall synthesis and responds to various signals, including temperature, changes in external osmolarity, and mating pheromone. Signaling proteins found in *P. brasiliensis* that compose this pathway include GTP binding protein Rho1p, protein kinase C homologue, Pkc1p, MEKK Bck1p (also known as Slk1p), a redundant pair of MEKs, Mkk1p and Mkk2p, MAPK Slt2p (also called Mpk1p), and transcription factor targets Rlm1p and SBF complex (Table 1); the latter is composed of the polypeptides Swi4p and Swi6p (Gustin et al., 1998). There are probably many branches into and out of this pathway.

Rho1p is a small GTP-binding protein of the *Rho* subfamily of Ras-related proteins that is required for cell growth. In *C. neoformans*, a homologue of *S. cerevisiae Mpk1/Slt2* MAPK was identified by Kraus and Heitman (2003). Their work characterized the role of Mpk1p in the maintenance of cell integrity in response to elevated growth temperature and to cell-wall-synthesis inhibitors. The protein encoded by *C. neoformans MPK1* is required for growth at 37°C *in vitro*; this growth defect is suppressed by osmotic stabilization. The cell wall is a fungus-specific dynamic structure essential to almost every aspect of the biology and pathogenicity of *C. albicans*. Its structure confers physical protection and shape to fungal cells, and as the most external part of the fungus, it mediates the interaction with the host, including adhesion to host tissues and modulation of the host anti-*Candida* immune response. Therefore, the search for potential cell wall-related targets can be envisaged as key to understanding fungal pathobiology. Moreno et al. (2003) characterized (*in silico*) a *C. albicans* gene encoding a putative transcriptional factor required for cell wall integrity. This gene codes for a Zn(II) Cys(6) transcriptional factor involved in cell wall architecture.

Table 1. PbAESTs ortholog names, accession number and BLAST e-value.

PbAEST	Ortholog name	Acession number organism	e-value
Calcium/calmodulin-calcineurin			
PbAEST 1629	Camd1	AF156028 *Emericella nidulans* (calmodulin).	3.1e-40
PbAEST 2381	Cna1	AF071751 *Neurospora crassa* (calcineurin B, catalytic subunit).	8e-54
PbAEST 2252	Cnb1	AL513466 *Neurospora crassa* (calcineurin B, regulatory subunit).	5e-84
PbAEST 2171	Cmk1	NP_587941.1 *Saccharomyces cerevisiae* (calcium calmodulin-dependent protein kinase).	1e-165
PbAEST 2415	Crz1/Tcn1	NP_014371.1 *Saccharomyces cerevisiae* (calcineurin-responsive zinc-finger).	4e-41
PbAEST 5082	Cka1	NP_593642.1 *Arabidopsis thaliana* (casein kinase II α subunit).	8.3e-26
PKA/cAMP			
PbAEST 2581	Gpr	sp|P19533 *Emericella nidulans* (G-protein-coupled receptor).	4e-79
PbAEST 595	Gpa2	gi|6323816 *Saccharomyces cerevisiae* (guanine nucleotide-binding regulatory protein).	0.0042
PbAEST 2208	Gpb	AF176775_1 *Aspergillus nidulans* (G protein β-subunit).	5e-88
PbAEST 3426	AC/Cyr1	NP_194335.1 *Arabidopsis thaliana* (adenylate cyclase).	1e-15
PbAEST 5256	Pde1	NP_011266.1 *Saccharomyces cerevisiae* (low-affinity cAMP phosphodiesterase).	1e-10
PbAEST 947	Pkar/Bcy1	AF401202_1 *Aspergillus fumigatus* (regulatory subunit of PKA).	2e-74
PbAEST 1562	Pkac/Tpk1	NP_012371. *Saccharomyces cerevisiae* (catalytic subunit of cAMP-dependent protein kinase).	1.8e-36
PbAEST 3488	Pkac/Tpk2	P34099 *Saccharomyces cerevisiae* (catalytic subunit of PKA).	1e-60
PbAEST 3241	Flo11/Muc1	NP_012284.1 *Saccharomyces cerevisiae* (flocculin: extracellular α-1,4-glucan glucosidase).	2.6e-06
MAPK pheromone regulated			
PbAEST 2475	Mat1-1	AF100925 *Gibberella fujikuroi* (mating-type protein MAT1-1).	1e-16
PbAEST 4188	Mat1-2	AF508279_1 *Emericella nidulans* (mating-type protein MAT1-2).	4e-20
PbAEST 2208	Ste4	AF176775 *Aspergillus nidulans* (GTP-binding protein β subunit of the pheromone pathway).	5e-88
PbAEST 266	Ste20	L04655 *Saccharomyces cerevisiae* (ser/thr protein kinase of the pheromone pathway).	3e-51
PbAEST 659	Ste11	AF034090 *Neurospora crassa* (ser/thr protein kinase of the MEKK family).	e-160
PbAEST 989	Ste7	AJ304830 *Blumeria graminis* (ser/thr/tyr protein kinase of mitogen-activated protein kinase (MAPK) kinase family).	8e-29
PbAEST 1960	Kss1/ Fus3/Cek1	AF268070 *Ustilago maydis* (MAPK).	5e-21
PbAEST 4523	Ste12	AF080600 *Emericella nidulans* (transcriptional activator).	4e-37
MAPK controlling cell wall construction and cell integrity			
PbAEST 1346	Mid2/ Slg1/Wsc1	NP_014650.1 *Saccharomyces cerevisiae* (cell surface sensors for cell integrity signaling during vegetative growth).	4e-11
PbAEST 157	PKC	NC_001148 *Saccharomyces cerevisiae* (ser/thr protein kinase).	2e-17
PbAEST 659	Bck1/Slk1	AF034090 *Neurospora crassa* (ser/thr protein kinase of the MEKK family).	e-160
PbAEST 4403	Mkk1	NC_001148 *Saccharomyces cerevisiae* (ser/thr protein kinase).	2e-60

Continued on next page

Table 1. Continued.

PbAEST	Ortholog name	Acession number organism	e-value
PbAEST 989	Mkk2	AJ304830 *Blumeria graminis* (protein kinase of the MAPK kinase (MEK) family).	8e-29
PbAEST 365	Slt2/Mpk1	AJ304831 *Blumeria graminis* (ser/thr protein kinase of MAPK family).	e-110
PbAEST 1259	Rlm1	NP_015236 *Saccharomyces cerevisiae* (transcription factor of the MADS box family).	9e-26
PbAEST 3256	Swi4	AL513463 *Neurospora crassa* (transcription factor).	4e-08
PbAEST 3615	Swi6	NP_594464.1 *Schizosaccharomyces pombe* (transcription factor).	1.6
PbAEST 1518	Rho1	AY007297 *Aspergillus fumigatus* (GTP-binding protein of the rho subfamily of ras-like proteins).	8e-53
PbAEST 3665	Fks1	AF148715 *Paracoccidioides brasiliensis* (1,3-β-D-glucan synthase, catalytic subunit).	2e-81
PbAEST 4433	Fks2/GSC2	AF148715 *Paracoccidioides brasiliensis* (1,3-β-D-glucan synthase subunit).	e-108
MAPK for high osmotic growth/stress response			
PbAEST 985	Sln1/Nik1	AF435964 *Botryotinia fuckeliana* (putative sensory transduction histidine-kinase).	5e-50
PbAEST 2744	Ypd1	MPR1_SCHPO *Schizosaccharomyces pombe* (phosphorelay intermediate between Sln1p and Ssk1p).	1e-04
PbAEST 913	Ssk1	AF084608_1 *Candida albicans* (two-component signal transducer).	0.046
PbAEST 659	Ssk2	P53599 *Saccharomyces cerevisiae* (MAPK kinase kinase, MAPKKK - suppressor of sensor kinase 2).	1e-38
PbAEST 365	Pbs2	NP_012407 *Saccharomyces cerevisiae (*MAPK kinase - MEK).	3e-31
PbAEST 3218	Sho1	CAC81238 *Candida albicans* (protein osmosensor).	2e-05
PbAEST 1960	Ste50	EAL02925 *Candida albicans* (MAPKKK).	2e-15
PbAEST 356	Hog1	AF184980_1 *Magnaporthe grisea* (osmotic sensitivity MAPK).	1e-73
TOR pathway			
PbAEST 3215	Tor2	CAA50548.1 *Saccharomyces cerevisiae* (protein/ phosphatidylinositol kinase).	1e-15
PbAEST 4664	Rom2	T41524 *Schizosaccharomyces pombe* (rho1 gdp-gtp exchange protein 1).	7e-35
PbAEST 3501	Sac7	CAA20323.1 *Schizosaccharomyces pombe* (RhoGAP GTPase activating protein).	4e-37
PbAEST 70	Cdc55	AAD15987.1 *Neurospora crassa* (protein phosphatase 2A regulatory B subunit).	e-105
PbAEST 5565	Tpd3	AAB03670.1 *Dictyostelium discoideum* (protein phosphatase 2A regulatory A subunit).	2e-35
PbAEST 1737	Sit4	CAB98214.2 *Neurospora crassa* (probable cell shape control protein phosphatase ppe1).	2e-99
PbAEST 1318	Gap1	CAD21063.1 *Neurospora crassa* (amino acid permease NAAP1).	0.0
PbAEST 367	Mkk1-	CAC19662.1 *Blumeria graminis* (MAPK kinase).	e-110
PbAEST 358	Mpk1	AAF09475.1 *Magnaporthe grisea* (osmotic sensitivity MAPK: Hog1p).	1e-73
PbAEST 2132	Npr1	CAA18998.1 (nitrogen permease reactivator/protein kinase).	1e-40
PbAEST 1432	Fpr1	CAA06962.1 (FKBP-type peptidyl-prolyl cis-trans-isomerases).	8e-38

Cell wall integrity in *A. fumigatus* is less well defined, and its role in protecting fungal cells is not clearly understood. In the model yeast *S. cerevisiae*, the cell integrity *Mpk1/Slt2* MAPK and calcineurin pathways monitor the state of the cell wall and promote remodeling under stress conditions. The Rho1p of *P. brasiliensis* bears 86.9% identity with the *S. cerevisiae* counterpart. Rho1p binds to and is required for the activity of Pkc1p *in vivo*, which in turn regulates the MAPK pathway. In addition, Rho1p may participate in the synthesis of α-1,3 glucan, the main structural component of the yeast cell wall in *P. brasiliensis*. The synthesis in *S. cerevisae* occurs at the cell surface by action of two differentially expressed glucan synthases, Fks1p and Fks2p. For Fks1p to be active, Rho1p must be in its GTP-bound form (Gustin et al., 1998).

Osmoregulation by the HOG pathway

To maintain cellular homeostasis, the fungal cells need to have a way to avoid dehydration in conditions of solute excess in the extracellular environment. One strategy is the accumulation of glycerol inside the cell, a compatible substance that avoids cellular water outflow in high osmolarity conditions (Hohmann, 2002).

One system of glycerol production is the HOG pathway. It uses some proteins that are sensitive to differences in the concentration of solutes in the environment and transmit signals to the cell to promote osmoadaptation. The initialization of glycerol production by HOG can be triggered by two membrane osmosensors that detect osmotic alterations and transduce signals to a series of proteins that will activate MAPK *HOG1* (O'Rourke and Herskowitz, 2004).

Sho1p and Sln1p, two osmosensors situated in the membrane, can detect extracellular hyperosmolarity. They transfer signals from the environment through a cascade that will activate Pbs2p (MAPKK), which phosphorylates Hog1p (Marles et al., 2004).

Saccharomyces cerevisiae Sln1p is a histidine-kinase protein that contains 1,220 amino acid residues, with homologues in some fungi, such as *C. albicans* and *P. brasiliensis*. Sln1p is essential for osmoadaptation in the budding yeast *S. cerevisiae*, in which it acts as a negative regulator of the HOG pathway; its deletion in this organism is lethal (Posas et al., 1996). In *C. albicans*, Sln1p is partially required for osmoadaptation, and *sln1* mutants have defective morphogenesis and are less virulent (Nagahashi et al., 1998). At a constant osmotic pressure, Sln1p inhibits the expression of Ssk1p, which is required in one branch of this pathway. Under high extracellular osmolarity, Sln1p is inhibited and Ssk1p is activated, transmitting signals to the other MAPKKs, such as Ssk2p (O'Rourke and Herskowitz, 2004).

The second osmosensor is Sho1p, a protein with 367 amino acid residues, found mainly in polarized growth regions of *S. cerevisiae* cells. Under conditions of high osmolarity, Sho1p transfers the signal to Ste20p, a PKA that activates MAPKKK Ste11p, which is required for activation of Pbs2p.

Glycerol production is triggered when the Hog1p is phosphorylated by Pbs2p and translocated to the nucleus, where it promotes the activation of several genes involved in the production of glycerol, which accumulates inside the cell (O'Rourke and Herskowitz, 2004).

The *in silico* analysis of PbAESTs describes the components of the HOG pathway in *P. brasiliensis* (Table 1). The comparison of the Pb HOG proteins with their homologues in *C. albicans* and *S. cerevisiae*, as well as the multiple sequence alignment in Figure 2, which shows the high conservation of the MAPK domain and the kinase-associated domain 1 of Hog protein, all suggest that *P. brasiliensis* is able to use this same strategy to avoid cell dehydration.

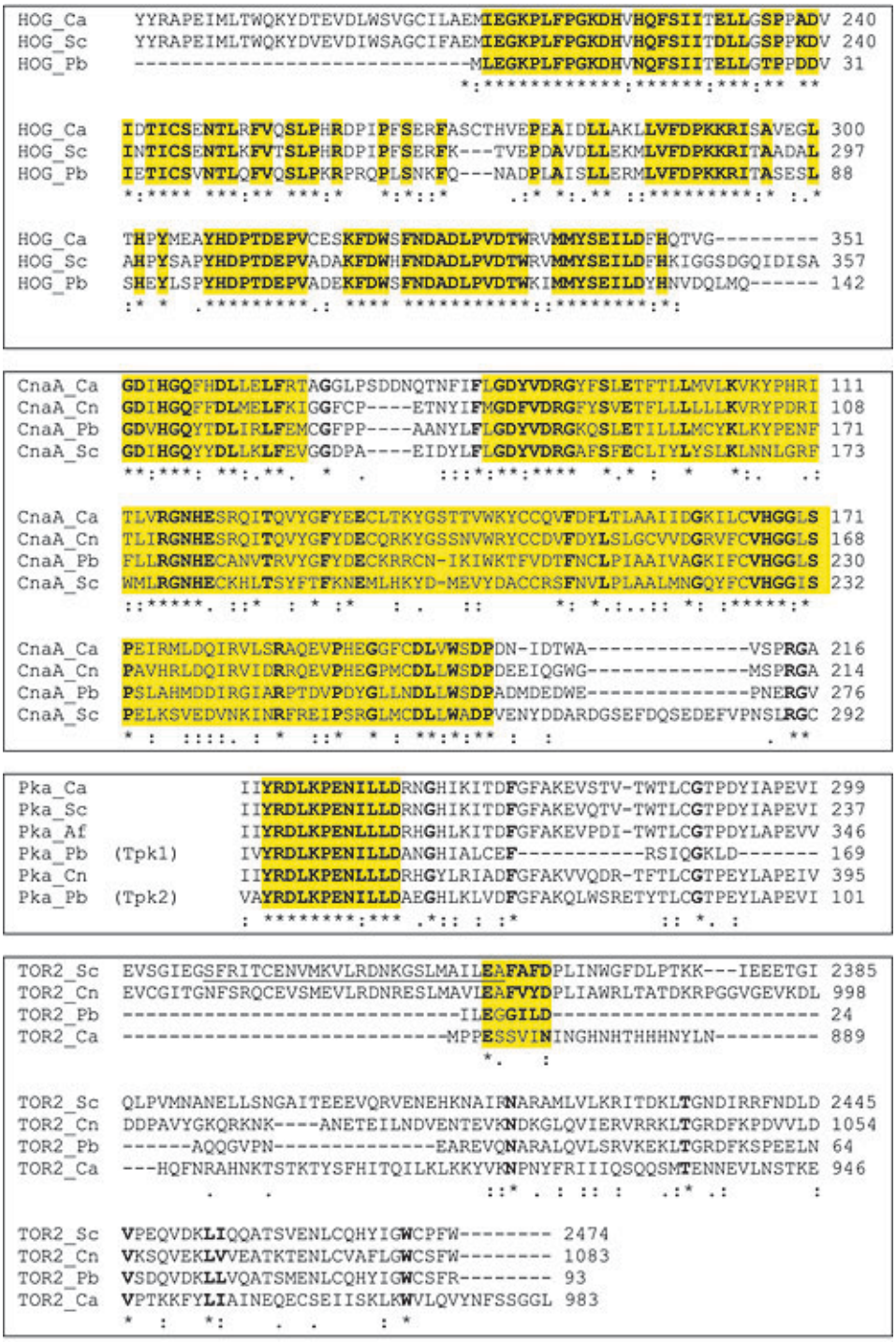

Figure 2. Multiple sequence alignment of the common core of HOG, calcineurin (CnaA), Pka and TOR from *Candida albicans* (Ca), *Saccharomyces cerevisiae* (Sc), *Aspergillus fumigatus* (Af), *Cryptococcus neoformans* (Cn), and *Paracoccidioides brasiliensis* (Pb), as performed in the CLUSTAL W server (Aiyar, 2000). The common domains identified by MIPS (http://mips.gsf.de/proj/yeast/CYGD/db/) are in yellow. The amino acids that are identical within and outside the MIPS domain are in bold.

cAMP/PKA pathway

Cyclic AMP (cAMP) is the regulatory component of a well-characterized signaling pathway implicated in a variety of cellular process among fungal species. Various components of the cAMP signaling pathway were identified through transcriptome analysis of *P. brasiliensis*, including adenylate cyclase Cyr1p, two cAMP-dependent protein kinase catalytic subunits, Tpk1p and Tpk2p, cAMP-dependent protein kinase regulatory subunit Bcy1p, and a low affinity phosphodiesterase, Pde1p (Table 1).

cAMP/protein kinase A (PKA) pathway elements have been dissected in various fungi. In *C. neoformans*, the cAMP signaling cascade is necessary for production of the capsule, mating filaments and melanin, all of them considered virulence factors for this organism. Mutants of the *CAC1* gene for adenylate cyclase in *C. neoformans* are defective in capsule production and melanin synthesis; they fail to produce mating filaments and were found to be avirulent in a mouse model of cryptococcosis (Pukkila-Worley and Alspaugh, 2004). In *C. albicans*, adenylate cyclase - Ca*CDC35*, PKA catalytic subunits (Tpk1p and Tpk2p) and Efg1p (hypha-specific transcriptional activator), all of them elements of the cAMP cascade, are involved in the regulation of vegetative growth and are essential for the serum-induced switch of yeast to hyphae, which has to do with the virulence of this fungus (Rocha et al. 2001; Cloutier et al., 2003). Mutants of Ca*CDC35* are unable to switch from yeast to hyphae, and they are unable to establish vaginal infection in a mouse model of candidiasis; they are also avirulent in systemic disease (Rocha et al., 2001). In the human pathogen *A. fumigatus*, elements of the cAMP pathway, such as ACYA (adenylate cyclase), PKAC and PKAR (PKA catalytic and regulatory subunits, respectively) have recently been shown to regulate the virulence gene pksP (Liebmann et al., 2003). The Δ*acyA* mutants of *A. fumigatus* have severely impaired sporulation and growth (Liebmann et al., 2003) and are more susceptible to being killing by human monocyte-derived macrophages.

The yeast prototype, *S. cerevisiae*, in which the cAMP signaling pathway elements are best known. This cascade is activated by nitrogen starvation, and it transduces the signals that control the phenotypic switch in haploid cells that promote invasive growth. In diploid cells, these signals transform budding yeast into elongated forms that constitute a chain known as a pseudohypha. In addition, cAMP controls cell growth, metabolism, stress resistance, and agar adherence (Lengeler et al., 2000). The regulation of adenylate cyclase activity differs among the species. In *S. cerevisiae*, Ras proteins and the Gα protein *GPA2* activate adenylyl cyclase in response to nutrient conditions and intracellular acidification (Colombo et al., 1998); however, in *Schizosaccharomyces pombe*, adenylate cyclase is regulated by Gpa2p, and Ras1p has no effect (Fukui et al., 1986). In *C. neoformans* and *A. fumigatus*, *GPA1* and *GPAB* were identified; both of their Gα protein homologues are involved in the activation of this pathway (Madhani et al., 1999; Liebmann et al., 2003). The G-protein-coupled-receptor (*Gpcr*), the upstream component responsible for sensing the nitrogen limitations in *S. cerevisiae* (Pan et al., 2000), was also identified in *P. brasiliensis*, although it is still not described in *C. neoformans*, *C. albicans* and *A. fumigatus*. In *S. cerevisiae*, *Cyr1p* (adenylate cyclase) is an essential gene responsible for the progression of cells through the G1 phase (Mosch et al., 1999), while in *C. albicans*, *A. fumigatus* and *C. neoformans*, it is not an essential gene.

Despite the high degree of conservation of the signaling cascade elements among fungi, their specific functions differ greatly. The multiple sequence alignment in Figure 2 shows that *P.*

brasiliensis has two different catalytic PKA subunits, defined as Tpk1p or *TPK1* (PBAEST1562) and Tpk2p or *TPK2* (PBAEST3488), with a very conserved serine/threonine protein kinase catalytic domain. The enzymatic activity of these protein kinases is controlled by the phosphorylation of specific residues in the activation segment of the catalytic domain.

The differences in the roles of adenylate cyclase can be explained by the fact that the cAMP pathway in *S. cerevisiae* is primarily involved in mediating nutritional signals to the cell cycle machinery, whereas in pathogenic or opportunistic fungi, the main function of these components is to sense and transduce stress signals from the host environment to the morphogenetic machinery that controls virulence and pathogenicity factors, thus establishing infection and allowing survival of the pathogen.

In the dimorphic human pathogen *Histoplasma capsulatum*, it has been shown that during the transition from budding yeast to mycelium, which is dependent on a temperature shift from 37° to 25°C, there is an increase in intracellular levels of cAMP associated with this dramatic morphological switch. In addition, exogenous cAMP and phosphodiesterase inhibitors allow the Y-M transition, even at 37°C (Sacco et al., 1981).

In our biological model, *P. brasiliensis*, evidence that cAMP has a role in the dimorphic transition, which is closely related to virulence, was first reported in 1985 by Paris and Duran, who observed that exogenous cAMP inhibits rather than induces the process of filamentation in this organism. The identification of cAMP/PKA signaling pathway components suggests a possible mechanism for cAMP involvement in the temperature-dependent dimorphic transition from mycelium to yeast. However, the complexity of the pathogenesis regulation in *P. brasiliensis* requires further studies of each signaling element.

The roles of Ras

RAS proteins belong to the *Rho* family - a superfamily of GTPases. It is a small guanine nucleotide-binding protein bound to the plasma membrane and involved in the regulation of several cell functions. It hydrolyses GTP; the GTP-bound form is active and GDP is inactive (Waugh et al., 2002). In human cells, this class of proteins plays a role in transmitting growth regulation signals from membrane-localized tyrosine-kinase receptors to different pathways, and ultimately to the nucleus. Some mutations in the RAS1 gene have been associated with unchecked cell growth, resulting in cancer (Barbacid, 1987). In *S. cerevisiae*, the Ras proteins regulate pseudohyphal growth and act upstream of the MAPK and cAMP/PKA pathways (Mösch et al., 1999). In *C. neoformans*, Ras1 protein is involved in filamentation, mating and high-temperature growth. The *ras1* mutants are avirulent in meningitis animal models; they have a growth defect at 37°C that arrests them as large unbudded cells with depolarized actin and loss of cytoskeletal asymmetry. They are unable to produce filaments (Waugh et al., 2002). A homologue of RAS was also identified in *P. brasiliensis* (Table 1) and in *C. albicans*, where it is not an essential gene, but it is involved in hyphal growth, since *ras1/ras1* mutants have a severe defect in filamentation in response to serum (Leberer et al., 2001). In *C. albicans*, Ras1p is also implicated in the cross talk between the MAPK and cAMP/PKA systems for the activation of the hypha-specific transcriptional factor Efg1, which is involved in the dimorphic transition from yeast to hyphae. The *RAS* gene should be further investigated in *P. brasiliensis* to elucidate its function in the Y-M transition and its possible involvement in pathogenicity.

Calcium-calmodulin-calcineurin dealing with stress

Among the diverse activities performed by cells, adaptation to changes in the environment is crucial for their viability. Calcium signaling is important in eukaryotes in numerous cellular processes, including the alteration of gene expression in response to external stimuli. Two important mediators of calcium signals in eukaryotic cells are the Ca^{2+}-binding protein calmodulin (Camp) and the Ca^{2+}/calmodulin-dependent phosphatase calcineurin.

Calmodulin is present in all eukaryotic cells, including *P. brasiliensis* (Table 1). In the cytoplasm, it has been implicated in the regulation of protein kinases, protein phosphatases, transcription factors, motor proteins, and cytoskeletal components. Calmodulin is usually genetically represented by a single copy gene that varies from 1 to 15 kb (de Carvalho et al., 2003; Kraus and Heitman, 2003). Inhibitors of the calmodulin pathway are able to impair the transition from mycelium to yeast cells in *P. brasiliensis* (de Carvalho et al., 2003).

In *S. cerevisiae* studies using temperature-sensitivity mutants of Camp, it has been found that this is an essential protein for cytoskeletal actin organization, endocytosis, and nuclear division (Desrivieres et al., 2002). In *C. albicans*, calcium signaling via calmodulin is important for the transition from the yeast to the filamentous form. Camp, which regulates cell proliferation and mediates the secondary messenger functions of Ca^{2+}, was also identified in several fungi, including *Phycomyces blakesleeanus*, *Neurospora crassa* and *A. nidulans*. Recent studies reported that Camp plays a vital role in chitin synthase activation by mediating phosphorylation of specific microsomal protein kinases during chitin formation in *N. crassa* (Suresh and Subramanyam, 1997).

Calcineurin is a serine-threonine-specific phosphatase that is conserved among eukaryotes. It consists of a catalytic subunit (CnaAp) and a Ca^{2+}-binding regulatory subunit (CnaBp), and the association of the two subunits is essential for activity (Watanabe et al., 1996). Activation of calcineurin occurs when Ca^{2+}/calmodulin binds to the C-terminal region of CnaAp, resulting in a conformational change that releases the active site from an auto-inhibitory domain. In *C. neoformans*, it is required for mating and haploid fruiting, for growth at elevated temperature and for virulence. Calcineurin participates in the morphogenesis of *S. pombe* by altering septal positioning and aberrant spindle body organization (Yoshida et al., 1994; Odom et al., 1997). Calcineurin was identified in *P. brasiliensis* transcriptome analysis and aligns well with its known homologues (Figure 2), which is also true for the catalytic serine/threonine domain specific to 2A phosphatases.

In *S. cerevisiae*, the expression of the truncated CnaAp resulted in cell elongation with a unipolar budding pattern (Mendoza et al., 1996). In *C. neoformans*, calcineurin plays a role in hyphal elongation, observed in mating and in the survival of the heterokaryon produced by cell fusion. It is also required for hyphal elongation in diploid strains and during asexual haploid budding of MATα cells in response to nitrogen limitation (Cruz et al., 2001).

Activated calcineurin dephosphorylates and activates the transcriptional factor Crz1p/Tcn1p, which enters the nucleus and, in turn, activates a set of responsive genes by binding to calcineurin-dependent responsive elements. It has been well documented that Crz1p/Tcn1p mediates most of the transcriptional responses driven by the activation of calcineurin under stress conditions (Viladevall et al., 2004). The genes that are controlled by Cna1p/Crz1p encode products that promote cell survival under stress. Crz1p is the best-characterized substrate of calcineurin in yeast, although recent studies on the identification of new substrates promise to

provide novel insights into additional functions of calcineurin *in vivo* (Cyert, 2003). Most Tcn1p-dependent genes can be differentially induced based on mechanisms of sensitivity to Ca^{2+} signals and other regulatory inputs (Matheos et al., 1997).

Calcium-mediated signaling mechanisms are used by virtually every eukaryotic cell to regulate a wide variety of cellular processes, including the maintenance of cell integrity under various types of stress. *Paracoccidioides brasiliensis* may use this machinery to protect itself against the harsh environment of the host.

TOR signaling pathway

TOR (target of rapamycin) is a phosphatidylinositol kinase-related protein kinase that controls cellular functions necessary for cell growth and proliferation in response to nutrients (Helliwell et al., 1998). In a recent study, it was reported that TOR acts within a growth regulatory network mediated by nutrient availability. This network affects all aspects of gene expression, including transcription, translation, and protein stability (Powers et al., 2004). In addition, the TOR pathway regulates the developmental program of pseudohyphal differentiation in concert with highly conserved MAPK and PKA signaling cascades (Rohde and Cardenas, 2004).

In *S. cerevisiae* and *S. pombe*, there are two TOR proteins, Tor1p and Tor2p; however, other organisms, such as *C. albicans* and *C. neoformans*, have only one TOR homologue (Cruz et al., 1999). The function of Tor1p is conserved throughout evolution; it is involved in translation initiation, transcription, ribosome biogenesis and tRNA synthesis (Schmelzle et al., 2004). In addition, Tor2p has two functions: one that is redundant to that of Tor1p and another that is unique, controlling the organization of the actin cytoskeleton during the cell cycle. Tor2p is not sensitive to rapamycin, being inhibited only by nitrogen starvation (Rohde and Cardenas, 2004).

Tor2p activates Rho1p GTPase, exchanging the GDP of the inactive form for GTP. This exchange is achieved by Rom2p protein, a guanidine nucleotide exchange factor, whereas Sac7p is a GTPase-activating protein. Rho1p phosphorylates *Pkc1p*, which activates the MAPK cascade Bck1p, Mkk1/2p and Mpk1p (Hay and Sonenberg, 2004).

In the course of *P. brasiliensis* transcriptome analysis, *TOR2*, *ROM2*, *RHO1*, *SAC7*, *PKC1*, *BCK1*, *MKK1*, *MPK1*, and *FPR1* homologues were identified (Table 1). Although Tor1p was not identified, some components of its signaling pathway, such as Cdc55p, Tpd3p and Sit4p are present in *P. brasiliensis*. Therefore, Tor1p may be present in the *P. brasiliensis* genome, unless Tor2p fulfils both functions in *P. brasiliensis*. Multiple sequence analysis of Tor2p revealed a kinase domain (Figure 2). Tor protein has a C-terminal region homology with the catalytic domain of phosphatidylinositol 3-kinase and phosphatidylinositol 4-kinase. This domain is characteristic of the TOR family and is essential to its function (Crespo and Hall, 2002).

CONCLUSIONS

Transcriptome analyses are a powerful tool to investigate organisms such as *P. brasiliensis*, in which the direct genetic analysis has many drawbacks and for which there is no efficient method of direct genetic analysis. By this comparative approach, it was possible to identify several components of signaling pathways in *P. brasiliensis* known to regulate cell events, such as morphogenesis and virulence. These components were found to be similar to

those of other fungi. We suggest that *P. brasiliensis* has co-opted a conserved signaling pathway to regulate phenotypes required for virulence, as have the other pathogenic microorganisms. However, there are still regulatory connections that need to be understood, in particular the broad transcriptional effects caused by the loss of the aforementioned cascades. The identification of pathogen-specific signaling events will help us understand *P. brasiliensis* pathogenesis, which could lead to the development of new antifungals.

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