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Screening for glycosylphosphatidylinositol-anchored proteins in the *Paracoccidioides brasiliensis* transcriptome

Nadya da Silva Castro, Zilma Alves Maia, Maristela Pereira and Célia Maria de Almeida Soares

Laboratório de Biologia Molecular, Instituto de Ciências Biológicas, Universidade Federal de Goiás, 74001-970 Goiânia, GO, Brasil
Corresponding author: C.M.A. Soares
E-mail: celia@icb.ufg.br

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ABSTRACT. Open reading frames in the transcriptome of *Paracoccidioides brasiliensis* were screened for potential glycosylphosphatidylinositol (GPI)-anchored proteins, which are a functionally and structurally diverse family of post-translationally modified molecules found in a variety of eukaryotic cells. Numerous studies have demonstrated that various GPI anchor sequences can affect the localization of these proteins in the plasma membrane or the cell wall. The GPI anchor core is produced in the endoplasmic reticulum by sequential addition of monosaccharides and phospho-ethanolamine to phosphatidylinositol. The complete GPI anchor is post-translationally attached to the protein carboxyl-terminus by GPI transamidases. Removal of this GPI lipid moiety by phospholipases generates a soluble form of the protein. The identification of putative GPI-attached proteins in the *P. brasiliensis* transcriptome was based on the following criteria: the presence of an N-terminal signal peptide for secretion and a hydrophobic region in the C-terminus presenting the GPI-attachment site. The proteins that were identified were in several functional categories: i) eight proteins were predicted to be enzymes (Gel1, Gel2, Gel3, α-amylase, aspartic proteinase, Cu-Zn

SOD, DFG5, PLB); ii) Ag2/PRA, ELI-Ag1 and Gel1 are probably surface antigens; iii) Crh-like and the GPI-anchored cell wall protein have a putative structural role; iv) ECM33 and Gels (1, 2 and 3) are possibly involved in cell wall biosynthesis, and v) extracellular matrix protein is considered to be an adhesion protein. In addition, eight deduced proteins were predicted to localize in the plasma membrane and six in the cell wall. We also identified proteins involved in the synthesis, attachment and cleaving of the GPI anchor in the *P. brasiliensis* transcriptome.

Key words: *Paracoccidioides brasiliensis*, GPI-anchored proteins, Plasma membrane, Cell wall

INTRODUCTION

Cell surface membrane proteins constitute an important class of biomolecules in living cells, as they are at the interface with the surrounding environment. Most eukaryote membrane proteins are post-translationally modified, and a subset of them is modified by the attachment of a glycosylphosphatidylinositol (GPI) moiety at the C-terminal end of the protein (Ferguson et al., 1988). Although fungal and mammalian cells contain the same mechanism by which they attach carbohydrates to nascent proteins, mammalian GPI anchors tether proteins to cell membranes, whereas in fungal cells GPI anchors are also used to covalently link proteins to cell wall glucans (Varki et al., 1999).

GPI-modified proteins are widely found in lower and higher eukaryotes (Eisenhaber et al., 2001). The primary sequence of GPI proteins share a general pattern, with N-terminal signal peptides and C-terminal features that mediate GPI anchor addition at an amino acid residue designated the omega (ω)-site (Hamada et al., 1998b). GPI anchor addition occurs in the endoplasmic reticulum (ER), following proteolytic cleavage of the C-terminal propeptide (Orlean, 1997). In addition to these signal sequences, the GPI proteins present a serine-threonine-rich sequence that provides sites for glycosylation. Moreover, the cellular localization of GPI-anchored proteins seems to be at least partly determined by basic or hydrophobic residues in the ω-region (Caro et al., 1997; Vossen et al., 1997; Hamada et al., 1998b, 1999).

The core structure of the GPI anchor consists of a single phospholipid spanning the membrane and a head group consisting of a phosphodiester-linked inositol, to which a glucosamine is linked, a linear chain of three mannose sugars linked to glucosamine and an ethanolamine phosphate (EtNP) linked to the terminal mannose. Composition differences in the lipid portion and side chain substitutions in the tetrasaccharide backbone of the conserved headgroup promote variants in the structure of the GPI anchor. One of the most prominent aspects of GPI anchor diversity is glycan substitution of the conserved mannose residues (McConville and Ferguson, 1993).

The biosynthesis of the GPI moiety occurs in the ER, and the complete GPI anchor is fully assembled prior to attachment to the protein. A series of sequential enzymatic steps adds the various GPI components. GPI proteins enter the ER where the GPI anchor is covalently added to the ω-site by a transamidase complex of at least five proteins (Fraering et al., 2001;

Hong et al., 2003). The GPI-anchored proteins are transported from the ER to the Golgi apparatus in distinct vesicles from the non-GPI-anchored proteins (Muniz et al., 2001). A Rab GTPase is specifically required for GPI protein trafficking. Also, the tethering factors Vso1 and Sec34/35p are necessary for the sorting of GPI-anchored proteins upon ER exit (Morsomme and Riezman, 2002).

Most available evidence suggests that there are two terminal fates for GPI proteins. They can reside at the plasma membrane (GPI-anchored plasma membrane proteins) or reside at the cell wall (Lu et al., 1994). Caro et al. (1997) proposed, based on *in silico* analysis of GPI-anchored proteins of *Saccharomyces cerevisiae*, that a signal of two basic amino acids in the four residues upstream to the ω-site acts to retain the protein at the plasma membrane. Hamada et al. (1998b, 1999) suggested that in the absence of this retention signal, hydrophobic amino acids at positions 2, 4, and 5, upstream to the ω-site act positively to localize the protein to the cell wall.

The intact GPI anchor confers an amphiphilic character to the protein, which by the action of phospholipases (PLs) cleaving the ester bond of the phosphatidylinositol (PI), render the protein hydrophilic. In this way, a proposed role for the GPI anchor and their solubilizing PLs is that it may be an alternative to proteolysis for the regulated release of proteins from membranes (Ehlers and Riordan, 1991). The location of GPI proteins makes them ideal candidates for such function.

Several studies have now established that GPI-anchored proteins are a large class of functionally diverse proteins. They can be enzymes, surface antigens, adhesion molecules, or surface receptors (Chatterjee and Mayor, 2001; Hoyer, 2001; Sundstrom, 2002). GPI-anchored proteins reported in various microbial pathogens have been shown to be immunogenic and are suggested to be important virulence factors (Hung et al., 2002; McGwire et al., 2002). In addition, GPI-bound proteins can display enzymatic properties, playing an active role in cell wall biosynthesis (Hartland et al., 1996; Mouyna et al., 2000). In fungi, synthesis of GPI anchors is essential for viability, since their cell wall mannoproteins require a GPI anchor so that they can be covalently incorporated into the cell wall (Leidich et al., 1994).

Yeast has been extensively used to study the GPI-anchoring system, and it is now well understood (Ash et al., 1995; van der Vaart et al., 1995). However, in contrast to the case for *S. cerevisiae*, little is known about the structure and biosynthesis of the GPI anchor in filamentous fungi. *Aspergillus fumigatus* presents about nine GPI-anchored protein homologs to the yeast counterparts (Bruneau et al., 2001). Fontaine et al. (2003) characterized four GPI-anchored proteins from a membrane preparation of *A. fumigatus*. In contrast to yeast, only ceramide was found in the GPI anchor structure of *A. fumigatus*. The glycan moiety is mainly a linear pentomannose structure, linked to a glucosamine residue.

The thermal dimorphic fungus *Paracoccidioides brasiliensis* causes paracoccidioidomycosis, the leading endemic deep mycosis in Latin America. The disease may develop as different forms, ranging from benign and localized to severe and disseminated forms (Franco et al., 1993). Fungal conidia start the infection, which undergo conversion to the yeast parasitic phase in human lungs (McEwen et al., 1987). The morphological switch from mycelia to yeasts is the most important biological feature that enables *P. brasiliensis* to colonize, invade and survive in host tissues during infection (San-Blas et al., 2002). Previous reports described that *P. brasiliensis* makes use of GPI as a means of membrane anchorage of surface proteins (Heise et al., 1995). The addition of complete GPI anchors is required for morphogenesis, virulence and

for host-fungus interactions (Richard et al., 2002; Sundstrom, 2002; Delgado et al., 2003). These reasons can be invoked to account for the importance of GPI-anchored proteins in *P. brasiliensis*.

An efficient method for retrieving novel GPI proteins is a genome sequence-based approach. Computational methods provide a useful starting point for genome-wide screening of potential GPIs in a variety of organisms. *Saccharomyces cerevisiae* DNA sequencing and Von Heijine algorithm studies identified 58 potential GPI-anchored proteins (Caro et al., 1997). Recently, *P. brasiliensis* transcriptome information (https://www.biomol.unb.br/Pb) have been obtained and released in public databases. The availability of this transcriptome gives us a new strategy for identifying genes that are likely GPI proteins. We report 20 putative GPI-anchored predicted proteins in the *P. brasiliensis* transcriptome.

MATERIAL AND METHODS

Sequence and motif similarity is the most commonly used method for assigning a putative function to newly discovered genes. The identification of putative GPI-anchored proteins was based on the following criteria: i) the presence of an N-terminal signal peptide for secretion; ii) a hydrophobic tail, and iii) the GPI-attachment site.

Two GPI-anchored prediction tools, big-PI fungal predictor (http://mendel.imp.univie.ac.at/gpi/fungi/gpi_fungi.html) (Eisenhaber et al., 2004) and DGPI (http://129.194.185.165/dgpi/index_en.html) were used to screen the *P. brasiliensis* GPI-anchored proteins. The presence of a signal sequence for import into ER was confirmed by using SignalP version 3.0 (http://www.cbs.dtu.dk/services/SignalP/) (Nielsen et al., 1997; Bendtsen et al., 2004). The presence of hydrophobic regions was analyzed with DAS (http://www.sbc.su.se/~miklos/DAS/) (Cserzo et al., 1997) and PSORT II (http://www.psort.org/) (Horton and Nakai, 1997). PSORT II was also used for protein localization predictions. BLAST searches were performed at NCBI (http://www.ncbi.nlm.nih.gov/BLAST/) (Altschul et al., 1997) and Pfam (http://www.sanger.ac.uk/software/pfam/index.shtml) (Bateman et al., 2002). A phylogenetic tree was constructed by multiple sequence alignments by using the Clustal X program, version 1.8 and the neighbor joining method (Thompson et al., 1997). Robustness of branches was estimated using 100-bootstrapped replicates. The amino acid sequences were visualized using the TreeView software.

RESULTS AND DISCUSSION

Putative GPI-anchored proteins of *P. brasiliensis*

Several studies have now established that GPI-anchored proteins are a large class of functionally diverse proteins. The predicted GPI-anchored proteins of *P. brasiliensis* could be enzymes, surface antigens, or adhesion molecules, and they have a structural role in the cell wall biogenesis (Table 1). For instance, α-amylase, proline-rich antigen/antigen 2 (PRA/Ag2), Cu-Zn superoxide dismutase (Cu-Zn SOD), GPI-anchored cell wall, ECM33, Crh-like, DFG5-like, PLB, extracellular matriz protein (EMP), aspartic proteinase precursor, expression library immunization antigen 1 (ELI Ag1) and β-1,3-glucanosyltransferase (Gels 1, 2 and 3) proteins were found. Their predicted functions were obtained by comparison to the homologs for which a role has been defined.

Table 1. Functional diversity of GPI-anchored proteins of *Paracoccidioides brasiliensis*.

Putative function	Product	References
Enzymes	β-1,3-glucanosyltransferases (Gel1, 2, 3)	Mouyna et al., 2000
	α-amylase	Nagamine et al., 2003
	Aspartic proteinase	Komano et al., 1999
	Cu-Zn superoxide dismutase	Martchenko et al., 2004
	DFG5-like	Kitagaki et al., 2002
	Phospholipase B	Mukherjee et al., 2001; Noverr et al., 2003
	ECM33	Lussier et al., 1997; Ross-MacDonald et al., 1999
Structural role	Crh-like	Rodrigues-Pena et al., 2000
	GPI-anchored cell wall protein	Moukadiri et al., 1997
Surface antigens	Expression library immunization antigen 1	Ivey et al., 2003
	Proline-rich antigen	Zhu et al., 1996, 1997; Peng et al., 2002
	β-1,3-glucanosyltransferase 1	Delgado et al., 2003
Adhesion molecules	Extracellular matrix protein	Ahn et al., 2004
Unknown function	Hypothetical protein PbAEST 2445	
	Hypothetical protein PbAEST 61	
	Hypothetical protein PbAEST 2429	
	Hypothetical protein PbAEST 4050	
	Hypothetical protein PbAEST 3834	
	Hypothetical protein PbAEST 3516	

In our 20 predicted GPI-anchored proteins nine in our list are supposed to have enzymatic activity. The α-amylase enzyme is located on the cell wall of fungi, and it plays a crucial role in the fermentation process in yeast (Yabuki and Fukui, 1970; Nagamine et al., 2003). Aspartic proteinase could act in the processing of cell wall precursors or precursors of enzymes involved in cell wall synthesis or remodeling (Komano et al., 1999). Eukaryotic Cu- and Zn-containing superoxide dismutase 1 (SOD1) is a key superoxide scavenging enzyme that is largely localized in the cytosol but is also found in the intermembrane space of mitochondria and in other organelles (Weisiger and Fridovich, 1973; Chang et al., 1988; Keller et al., 1991; Okado-Matsumoto and Fridovich, 2001; Sturtz et al., 2001).

Some of the newly identified proteins, ECM33 and DFG5-like, have been reported to be involved in cell wall biogenesis (Lussier et al., 1997; Ross-MacDonald et al., 1999; Kitagaki et al., 2002) and cell growth at high temperature (Terashima et al., 2003). In addition, the Gel family is also required for proper cell wall assembly and morphogenesis due to their activity elongating β-1,3-glucans of human fungal pathogens (Mouyna et al., 2000). Two proteins have been reported to have a structural role: Crh-like, which has a putative glycosidase domain and could be involved in the development of cell wall architecture (Rodriguez-Pena et al., 2000), and GPI-anchored cell wall protein, which has a structural role in association with the glucan network, since both have the same localization (Moukadiri et al., 1997).

All known GPI-anchored proteins share a number of common features, including the predominantly hydrophobic region in the C-terminus, which most likely functions as a recogni-

tion signal for a transamidase system, the absence of transmembrane domains in the mature molecule and the presence of a cleavable N-terminal secretion signal for translocation into the ER. Based on the algorithms described above, we detected 20 predicted GPI-anchored proteins in the *P. brasiliensis* transcriptome (Table 2). In mammalian cells, over 100 cell surface proteins are putative GPI-anchored proteins (Low, 1989; Kinoshita et al., 1995). Fifty-eight potential GPI-anchored proteins were identified in the *S. cerevisiae* genome (Caro et al., 1997).

Among the identified GPI proteins, 16 presented the N-terminal signal peptides (Table 2). Among the 20 *P. brasiliensis* GPI-anchored proteins we were able to detect C-terminal regions in 11 predicted proteins (Table 2). Several residues of S and T, potential sites for O-glycosylation, were detected, even in the partial sequences (Table 2). GPI proteins usually have a high percentage of S and T residues, the side-chains of which are potential sites for O-glycosylation (Klis et al., 2002). The S/T content in the putative *P. brasiliensis* GPI proteins varies from 9 to 28%, with an average of 20%, which is similar to predicted GPI-anchored proteins of *Neurospora crassa* (21%) and slightly lower than in *S. cerevisiae* (25%), *Candida albicans* (28%) and *Schizosaccharomyces pombe* (29%) (de Groot et al., 2003).

Putative cellular localization of the predicted GPI-anchored proteins of *P. brasiliensis*

Although most GPI-anchored proteins in yeast and other fungi localize to the cell wall, some are believed to reside at the plasma membrane. Evidence indicates that the amino acids immediately upstream to the ω-site serve as the signal determining protein localization. Two kinds of signals have been proposed for GPI-anchored protein cellular localization: i) dibasic residues (K and/or R) in a short ω-minus region are favored in proteins that are predominantly localized in the plasma membrane (Caro et al., 1997; Vossen et al., 1997) and ii) the specific amino acid residues V, I or L 4 or 5 amino acids upstream of the GPI-attachment site (the ω-site) and Y or N at the ω-2 site have been shown to act as a positive signal for cell wall localization (Hamada et al., 1998b, 1999).

In order to predict the cellular localization of putative GPI-anchored proteins of the *P. brasiliensis* transcriptome, we analyzed the corresponding amino acids in the ω-minus region and also examined the results of *k*-NN prediction (PSORT II server) (data not shown). We also compared those analyses to data from other organisms. Accordingly, among the 20 GPI-anchored proteins, 11 sequences which presented at least the 42 last amino acids in the C-terminal region were selected to study their putative localization (Table 2). The three proteins of the Gel family, the ECM33 protein and the hypothetical protein PbAEST 2429 presented basic motifs upstream to the predicted ω-site, as detected by the big-PI fungal predictor (Eisenhaber et al., 2004). These results were compatible with the *k*-NN prediction (Horton and Nakai, 1997) and with the literature description of plasma membrane localization (Vai et al., 1991; Hamada et al., 1998a; Terashima et al., 2003). However, no basic amino acid was found in the ω-minus region for the Cu-Zn SOD and DFG5-like proteins. In both, plasma membrane localization prevails, as described by Karpinska et al. (2001), Kitagaki et al. (2002) and Spreghini et al. (2003).

The PRA/Ag2 and GPI-anchored cell wall proteins were predicted as putatively anchored in the cell wall on the basis of descriptions from *Coccidioides immitis* (Zhu et al., 1996) and *S. cerevisiae* (Moukadiri et al., 1997; Hamada et al., 1998a), respectively. Two hypothetical proteins, PbEST 2445 and PbEST 61, were predicted as cell wall proteins, only by the PSORT II analysis.

Table 2. Putative GPI-anchored proteins of the *Paracoccidioides brasiliensis* transcriptome.

Product/PbAEST/ GenBank accession number	% Amino acid identity by BLASTp analysis[1]	N- and C-terminus sequences[2,3]	Predicted cellular localization[4]	S/T content (%)[3,5]
Glucanosyltransferase Gel3p/PbAEST 1370/ AY324033	*Aspergillus nidulans* 311/543 (57%)	N- MKFASVLAGAALAGTAFA ADLDPIVIKGSK C- GPAGTS**K**GAA[S]VGAVPAVDFGMVRVGAGVVAGVIAGMSILLL	**PM** (Vai et al., 1991; Hamada et al., 1998a)	12
β-1,3-glucanosyltransferase Gel2p/PbAEST 2375/ AY340235	*Aspergillus fumigatus* 156/272 (57%)	N- MTLLRSFTVLFALVASTVHA VTPISIEGSQ C- GEKTSGAPGAVKEK**KK**[G]AASTLSTSNALSLLAAVVGLTLLMV	**PM** (Vai et al., 1991; Hamada et al., 1998a)	13 (IC) 13 (IC)
β-1,3-glucanosyltransferase 1, Gel1/AY380566*	*Aspergillus fumigatus* 42/76 (55%)	N- MKAIAASALSAAVLA SSALTGEASIIKSRT C- KAGQLFAL**R**TQ[S]AAAGLEPPKILSAFLYVPLLLERLRSLAFH	**PM** (Vai et al., 1991; Hamada et al., 1998a)	14 (IC) 12 (IC)
ECM33/PbAEST 4500	*Aspergillus nidulans* 61/127 (48%)	N- IC C- TGTGTNSGPG**K**P**K**PS[G]AAMGPLSPPSGMTMLALAGGVLGFAL	**PM** (Hamada et al., 1998a; Terashima et al., 2003)	14 (IC)
Hypothetical protein/ PbAEST 2429	*Aspergillus nidulans* 84/202 (41%)	N- MFVFSVLLTVSVLASLSSS QGLDPNNIPLQ C- NPGMAP**K**GG**R**[N]GAERGLVLEIGQVYGVGILVAVFKAGFSMVV	**PM**	19
Cu-Zn superoxide dismutase/ PbAEST 50	*Magnaporthe grisea* 71/159 (44%)	N- MKPTFSILACSLGFALRATA QVMMEAVTTE C- ASINGTAVPTP[S]ASQRPSQGPANRVGAFGLGVMLAGVAAMIW	**PM** (Karpinska et al., 2001)	15
DFG5-like/ AY307855*	*Magnaporthe grisea* 171/403 (42%)	N- MKSQLWAVLATVVSLGPWATVA LDGSDLDS C- VTQLPTGKSQGDE[S]QAEILEEHRLLELHQILHLPAWPRRLWI	**PM** (Kitagaki et al., 2002; Spreghini et al., 2003)	13
Aspartic proteinase precursor/PbAEST 5557	*Magnaporthe grisea* 84/147 (57%)	IC	**PM** (Ash et al., 1995; Hamada et al., 1998a)	22 (IC)
Phospholipase B/ PbAEST 3306	*Aspergillus nidulans* 121/199 (60%)	IC	**PM** (Hamada et al., 1998a)	15 (IC)
Proline-rich antigen /antigen 2 (Ag2)/ PbAEST 5497	*Coccidioides immitis* 112/194 (57%)	N- MQFSHALIALVAASLANA QLPNIPPCALSC C- SKPVPTSTPTTSRPAEFP[G]AGSNLNANIGGVAAALLAVAAYL	**CW** (Zhu et al., 1996; Peng et al., 2002)	22

Continued on next page

Table 2. Continued.

Product/PbAEST/ GenBank accession number	% Amino acid identity by BLASTp analysis[1]	N- and C-terminus sequences[2,3]	Predicted cellular localization[4]	S/T content (%)[3,5]
GPI-anchored cell wall protein/ PbAEST 440/ AY495673	*Aspergillus nidulans* 31/87 (35%)	N- MLAAKSIFVVALLALFNIVFA IPPGCLISA C- GSSTTSGGSGASPTGSGAGYVHKVDSMAVTAIVAFVGFVSAL	**CW** (Moukadiri et al., 1997; Hamada et al., 1998a)	22
Hypothetical protein/ PbAEST 2445	*Aspergillus nidulans* 38/97 (39%)	N- MRLSMAVLPSLLGLVAAQGLNG LPECAKSC C- CNTTQSSSTPTTSPTPVPSQNAAAKIGVGAGLVLVMAVWGMF	CW	21
Hypothetical protein/ PbAEST 61	*Aspergillus nidulans* 40/86 (46%)	N- MKFFTLMALAGLFASAAA LPQENPATTTTT C- PPNGTSTGNFQTTPSGGAGVINVQLGSFAAGIVGLLMAAVVL	CW	27
α-amylase/ PbAEST 5676	*Aspergillus nidulans* 120/189 (63%)	N- MLRLFILCYLAGLALA ADTVDWKSRSIYQV C- IC	**CW** (Nagamine et al., 2003)	9 (IC)
Crh-like protein/ PbAEST 5441	*Magnaporthe grisea* 73/204 (35%)	N- MKVSSGSMASLALVLFSGSALVGA QTFTEC C- IC	**CW** (Hamada et al., 1998a)	16 (IC)
Expression library immunization antigen 1 /PbAEST 2838	*Coccidioides immitis* 91/158 (57%)	N- MRFQTTLLPLTGLLTLTSA HFDLLQPPSRG C- IC		21 (IC)
Extracellular matrix protein/PbAEST 1208	*Aspergillus nidulans* 45/116 (38%)	N- MHLVKALVASALLVATAVA QGISFTSFPDN C- IC		28 (IC)
Hypothetical protein/ PbAEST 4050	*Magnaporthe grisea* 63/154 (40%)	N- MKSIFSTIALIATAIA ETIDVKVGENGLTI C- IC		18 (IC)
Hypothetical protein/ PbAEST 3834	*Aspergillus nidulans* 69/133 (51%)	N- MRLRHVALFSLSLLSSSLCLARG HQDPGPS C- IC		28 (IC)
Hypothetical protein/ PbAEST 3516	*Aspergillus nidulans* 66/137 (48%)	IC		27 (IC)

[1]Comparison by BLASTp to the nr database (GenBank).
[2]The predicted signal peptide cleavage sites indicated with a space between the first 30 amino acids. The best ω-sites (fungal big-PI predicted) of each sequence are boxed and the hydrophobic regions are double underlined within the last 42 amino acids. The amino acid residue V at the ω-5 site is underlined. The basic amino acid residues K and R in the short ω-minus region are marked in bold.
[3]IC indicates incomplete cDNA.
[4]Predicted localization in plasma membrane (PM) and cell wall (CW).
[5]Percentage of S plus T amino acids along each open reading frame.
*Not detected in the *P. brasiliensis* transcriptome: obtained by PCR of total DNA.

GPI-anchored proteins putatively associated with the fungus host interaction

Recent studies suggest that the GPI proteins are instrumental in fungal adhesion, recognition by host receptors, and may play a role in cell wall expansion. GPI-anchored proteins are leading vaccine candidates that are thought to be of major importance for infection (Smythe et al., 1988; Delgado et al., 2003). Table 3 shows some *P. brasiliensis* proteins that could be involved in host interaction and virulence. Two of the newly identified proteins, PRA/Ag2 and ELI Ag1, have been reported to be surface antigens. PRA/Ag2 is a component of a glycopeptide, which is probably the main T-cell-reactive component of *C. immitis* cell walls (Zhu et al., 1996). Also, the recombinant PRA/Ag2 protein is reactive with sera from patients with active coccidioidomycosis (Zhu et al., 1997). This protein is suggested to have an endoglucanase activity and to be important for spherule cell-wall morphogenesis during the infection process by *C. immitis* (Zhu et al., 1996). It is located in the fungal cell wall (Galgiani et al., 1992), most probable attached to the cell wall matrix (Peng et al., 2002). The expression of this protein can be considered phase specific since it is up-regulated during the spherule phase in *C. immitis* (Galgiane et al., 1992; Peng et al., 1999). ELI Ag1 is the first protective *C. immitis* antigen that has been identified by expression library immunization, inducing a strong level of protection in BALB/c mice. The mechanism by which this antigen protects mice against a lethal challenge with *C. immitis* arthroconidia is not yet known (Ivey et al., 2003).

We identified EST homologs to the EMP of *Magnaporthe grisea*. Although the function of EMP1 remains unclear at the biochemical level, it is suggested that it has a role in sensing a surface signal and/or transmitting a signal into the cell to promote conidial adhesion and appressorium formation in *M. grisea* (Ahn et al., 2004).

Among the identified enzymes, PLB, Cu-Zn SOD and Gel1 are reported as necessary for the virulence of fungal pathogens. It has been postulated that PLs assist in the penetration of phospholipid-rich host barriers, such as membranes and lung surfactant (Cox et al., 2001). Supporting evidence for this role has been shown by deletion of the *PLB1* gene in *C. albicans*, which results in a significant reduction in the ability of the pathogen to traverse the stomach mucosa and disseminate hematogenously to the liver (Mukherjee et al., 2001). Furthermore, PLB1 of *Cryptococcus neoformans* may act as a virulence factor, by enhancing the ability to survive the macrophage antifungal defenses, possibly by facilitating fungal eicosanoid production during cryptococcal infection (Noverr et al., 2003).

The main function of SOD is to scavenge O_2^- radicals generated in various physiological process, thus preventing the oxidation of biological molecules (Liochev and Fridovich, 1994; Fridovich, 1995). SOD can be classified according to metal co-factor(s) bound to them. Cu-Zn SOD has copper and zinc as metal co-factors (Martchenko et al., 2004). *Candida albicans* Sod1 was shown to protect cells against extracellular superoxide radicals produced by macrophages, and it was reported to be important for the virulence of *C. albicans* in a mouse model (Hwang et al., 2002).

It was found that mice immunized with the recombinant Gel1 of *Coccidioides posadasii* and infected against a lethal challenge of this pathogen had a significant reduction in fungal burden and increased survival compared to nonimmune mice. The mature Gel1 was immunolocalized to the surface of endospores, and the highest level of the Gel1 mRNA was detected during the endosporulation stage of the parasitic cycle (Delgado et al., 2003). Furthermore, it was found that two homologous genes in *C. albicans* are pH-regulated and required for viru-

Table 3. Putative role of GPI-anchored proteins in *Paracoccidioides brasiliensis*.

Product	Functional grouping	Putative role in *P. brasiliensis*	Reported relation to host interaction in the pathogens
Proline-rich antigen/ antigen 2/ immunoreactive protein precursor		Surface antigen	Major immunoreactive component of *Coccidioides immitis* mycelium- and spherule-phase cell walls (Zhu et al., 1996, 1997)
Expression library immunization antigen 1		Surface antigen	Induced a strong level of protection in BALB/c mice in *C. immitis* (Ivey et al., 2003)
Extracellular matrix protein		Adhesion molecule	Putative role in conidial adhesion and appressorium formation in *Magnaporthe grisea* (Ahn et al., 2004)
Phospholipase B (PLB)	Phospholipase	Virulence factor	*Cryptococcus neoformans* PLB1 may act as a virulence factor by enhancing the ability to survive macrophage antifungal defense (Noverr et al., 2003)
Cu-Zn superoxide dismutase	Cu-Zn superoxide dismutase	Virulence factor	Scavenging oxygen radicals: hypha-induced Sod5p is instrumental in virulence (Martchenko et al., 2004)
β-1,3-glucanosyltransferase 1 (Gel1)	β-1,3-Glucanosyl transferase, Gas/Phr/Epd CAZy family 72*	Virulence factor	Expressed in high levels during the endosporulation stage of the parasitic cycle and infection of host lung tissue of *C. posadasii* (Delgado et al., 2003)

*CAZy, carbohydrate-active enzyme classification according to Coutinho and Henrissat, 1999.

lence. These genes nominally include *PHR1*, a gene expressed maximally at pH 5.5 to 8.0, which encodes a protein promoting systemic infection of mice, and a *PHR2* gene, the expression pattern of which is the inverse and is involved in pathogenesis in a mouse model of vaginal infection (Saporito-Irwin et al., 1995; Muhlschlegel and Fonzi, 1997; De Bernardis et al., 1998).

Phosphatidylinositol-glycan proteins and transamidases

The biosynthesis of GPI occurs on the membrane of the ER by the sequential addition of sugar residues to PI by the action of glycosyltransferases (Stevens, 1995). The common core structure of GPI consists of inositol phospholipid, GlcN, three mannoses and EtNP (Ferguson and Williams, 1988).

Genes encoding the enzymes in GPI biosynthesis have been identified by cloning, sequencing and by using the techniques of knock out and rescue. In mammals, around 20 genes participate in this pathway and have been identified as phosphatidylinositol-glycan (PIG) gene products (Ferguson, 1999; Kinoshita and Inoue, 2000; McConville and Menon, 2000). The glycosyltransferase complex composed by the proteins PIG-A, PIG-C, PIG-H, GPI1, PIG-P, and DPM2 (dolichol-phosphate-mannose 2) catalyzes the first step in the GPI synthesis (Watanabe et al., 2000). PIG-A encodes a subunit of GPI-N-acetylglucosamine transferase (Mayor and Riezman, 2004).

Table 4 summarizes the PIGs found in the *P. brasiliensis* transcriptome. PIG-H and GPI1 encoding ESTs were not detected in the *P. brasiliensis* transcriptome. Studies on *S. cerevisiae* had shown that the Gpi12 homolog of PIG-L participates in the second step of GPI synthesis (Watanabe et al., 1999) and the mannosylation reactions are mediated by PIG-M (GPI-α-1-4 mannosyltransferase) and PIG-B (GPI-α-1-2 mannosyltransferase) (Kinoshita and Inoue, 2000). Only PIG-L was found in the *P. brasiliensis* transcriptome (Table 4). The EtNP transfer to the first and third mannose residues is mediated by PIG-N and PIG-F and PIG-O, respectively. The first two ESTs had not been detected in our analysis.

Attachment of the GPI to the protein involves cleavage of the lumenally located preprotein at a hydrophobic stretch, followed by the attachment of the cleaved sequence to the fully assembled GPI via a transamidase reaction (Udenfriend and Kodukula, 1995). Components of the transamidase complex have been identified in yeast and other organisms (Hamburger et al., 1995; Ohishi et al., 2000). Humans and *S. cerevisiae* GPI transaminidases are well conserved, containing five homologous components (Hong et al., 2003). Five human components, GAA1 (glycosylphosphatidylinositol anchor attachment 1), GPI8, PIG-S, PIG-T, and PIG-U are homologous to yeast Gaa1p, Gpi8p, Gpi17p, Gpi16p, and Cdc91p, respectively (Fraering et al., 2001; Ohishi et al., 2001). Several lines of evidence indicate that GPI8/Gpi8p are the catalytic components responsible for the cleavage of the GPI-attachment signal sequences (Benghezal et al., 1996; Meyer et al., 2000; Ohishi et al., 2000; Spurway et al., 2001; Vidugiriene et al., 2001). All of those encoding transamidase ESTs were detected in our analysis of the *P. brasiliensis* transcriptome, with the exception of ESTs encoding PIG-U (Table 4).

Phylogenetic relationships of *Pb*PIGs were generated with PIGs available on the Pfam database. The PIGs were well resolved into clades corresponding respectively to PIG-C, PIG-DPM2, PIG-P, PIG-M, and PIG-L (Figure 1). Consequently, we suggest conservation of PIG sequences during evolution.

GPI solubilizing phospholipases

The intact GPI anchor confers an amphiphilic character to the proteins, which by the action of PLs cleaving the ester bond of the PI, render the protein hydrophilic (Stambuk and Cardoso de Almeida, 1996). Thus, a proposed role for the GPI anchor and their solubilizing PLs is that it may be an alternative to proteolysis for the regulated release of proteins from membranes (Ehlers and Riordan, 1991).

The term "phospholipases" refers to a heterogeneous group of enzymes that are able to hydrolyze one or more ester linkages in glycerophospholipids (Cox et al., 2001). The action of PLs can result in the destabilization of membranes, cell lysis and release of lipid second messengers (Schmiel and Miller, 1999; Ghannoum, 2000). Although all PLs target phospholipids as

Table 4. *Paracoccidioides brasiliensis* phosphatidylinositol-glycan (PIG) biosynthesis and transamidases.

Protein/PbAEST	Amino acid length[1]	% Amino acid identity by BLASTp analysis[2]	Putative function in organisms
PIGs			
PIG-A/PbAEST 4859	141 - IC	*Aspergillus nidulans* 115/145 (79%)	Participates in catalysis of the first step in GPI anchor synthesis: transferring GlcNac (N-acetylglucosamine) from UDP-GlcNac to PI to form GPI. Probably provides the catalytic center (Kostova et al., 2000)
PIG-C/PbAEST 2893	186 - IC	*Aspergillus fumigatus* 133/183 (72%)	Participates in catalysis of the first step in GPI anchor synthesis: transfer of GlcNac from UDP-GlcNac to PI to form GPI (Delorenzi et al., 2002)
PIG-P/PbAEST 2368	342 - IC	*Aspergillus nidulans* 213/276 (77%)	Participates in catalysis of the first step in GPI anchor synthesis: transfer of GlcNac from UDP-GlcNac to PI to form GPI (Watanabe et al., 2000)
DPM2/PbAEST 3119	85	*Gibberella zeae* 53/75 (70%)	Implicated in first step of the GPI anchor biosynthesis (Watanabe et al., 2000) and is required for assisting the transfer of mannose units from dolichol phosphate by the catalytic DPM1 (dolichol-phosphate-mannose 1) (Maeda et al., 1998)
PIG-L (Gpi12)/PbAEST 3269	190 - IC	*Aspergillus nidulans* 124/195 (63%)	Catalyzes the second reaction in GPI anchor synthesis: deacetylation of GlcN-PI (glucosamine-phosphatidylinositol) (Watanabe et al., 1999)
PIG-O/PbAEST 4120	164	*Aspergillus nidulans* 109/187 (58%)	Involved in transferring EtNP (ethanolamine phosphate) to the third mannose of the GPI (Hong et al., 2000)
PIG-M	ND[3]		Mannosyltransferase that transfers the first mannose to GPI (Maeda et al., 2001)
PIG-F	ND[3]		Involved in the addition of EtNP to Man3 (Hong et al., 2000)
PIG-B	ND[3]		Involved in transferring the third mannose (Takahashi et al., 1996)
PIG-N	ND[3]		Involved in transferring EtNP to the first mannose of the GPI (Hong et al., 1999a)
PIG-H (Gpi15)	ND[3]		Participates in the catalysis of the first step in anchor synthesis: transfer of GlcNac from UDP-GlcNac to PI to form GPI (Watanabe et al., 2000)

Continued on next page

Table 4. Continued.

Protein/PbAEST	Amino acid length[1]	% Amino acid identity by BLASTp analysis[2]	Putative function in organisms
GPI1	ND[3]		Necessary for the stable formation of GPI-GnT (GPI-GlcNAc transferase) and probably required for the efficient association of PIG-C with a complex of PIG-A and PIG-H (Hong et al., 1999b)
TRANSAMIDASES			
GAA1/PbAEST 3403	231 - IC	*Aspergillus nidulans* 182/231 (78%)	Required for a terminal step of GPI anchor attachment (Hamburger et al., 1995)
GPI8 (PIG-K)/PbAEST 3107	179 - IC	*Aspergillus nidulans* 152/179 (84%)	Is intimately involved in the recognition of GPI precursor proteins (Meyer et al., 2002) and several lines of evidence indicate that GPI8 is responsible for the cleavage of the ω-site (Benghezal et al., 1996; Meyer et al., 2000; Ohishi et al., 2000; Spurway et al., 2001; Vidugiriene et al., 2001)
PIG-S (Gpi17)/PbAEST 3267 and	157 - IC	*Aspergillus nidulans* 112/157 (71%)	Forms a protein complex with GAA1 and GPI8 (Ohishi et al., 2001)
PbAEST 3811	149 - IC	*Aspergillus nidulans* 95/149 (63%)	
PIG-T (Gpi16)/PbAEST 5428	139 - IC	*Aspergillus nidulans* 89/142 (62%)	PIG-T and PIG-S form a protein complex with GAA1 and GPI8, and PIG-T maintains the GPI transamidase complex by stabilizing the expression of GAA1 and GPI8 (Ohishi et al., 2001)
PIG-U	ND[3]		It is suggested that Pig-U is involved in an event preceding the cleavage of the ω-site in the precursor protein (Hong et al., 2003)

[1]IC: incomplete cDNA.
[2]Comparison by BLASTp to the nr database (GenBank).
[3]ND: not detected.

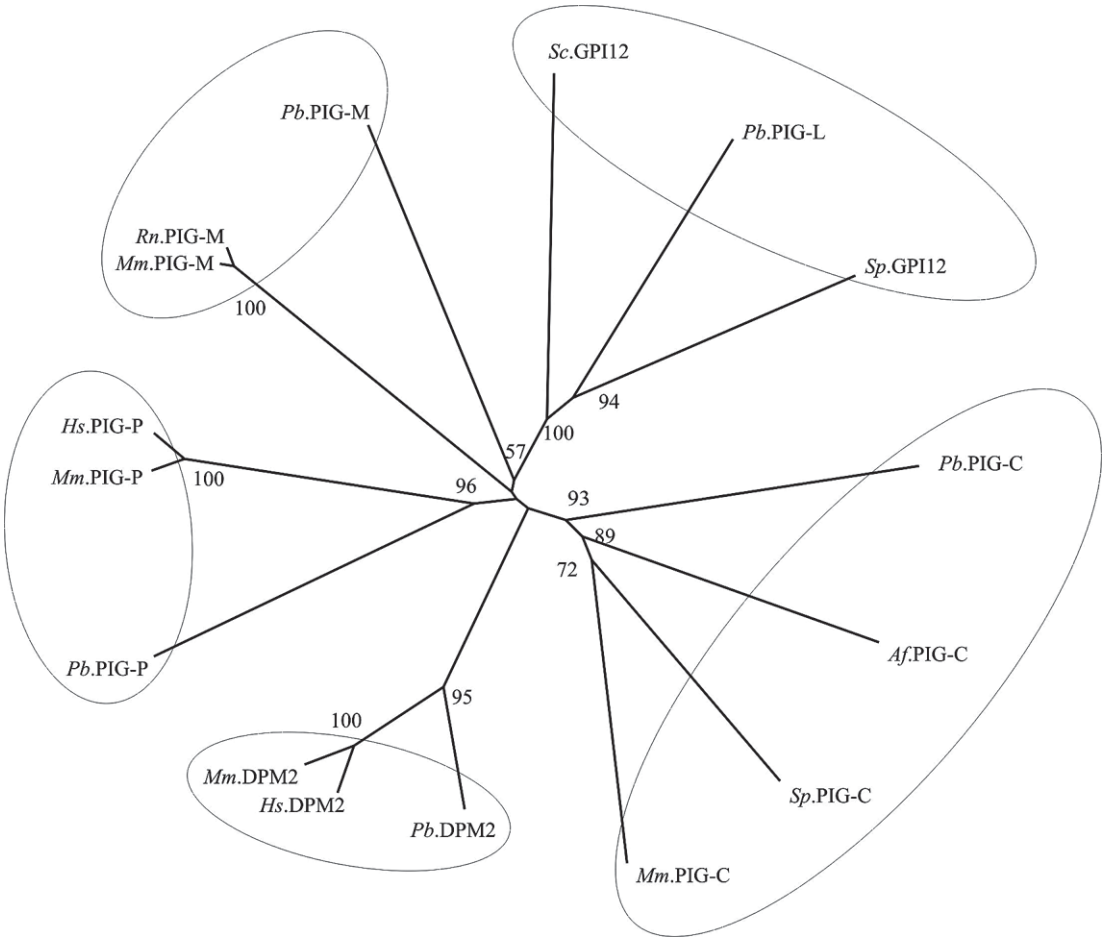

Figure 1. Phylogenetic tree of phosphatidylinositol-glycans (PIG). Sequences were aligned using the CLUSTAL X program. Sequences were taken from the Pfam database. *Pb*, *Paracoccidioides brasiliensis*, *Pb*PIG-P, *Pb*DPM2, *Pb*PIG-C, *Pb*PIG-L, and *Pb*PIG-M; *Rn*, *Rattus norvegicus*, *Rn*PIG-M (GenBank accession No. NP077058); *Hs*, *Homo sapiens*, *Hs*DPM2 (GenBank accession No. NP003854) and *Hs*PIG-P (GenBank accession No. P57054); *Sp*, *Schizosaccharomyces pombe*, *Sp*PIG-C (GenBank accession No. NP588096) and *Sp*GPI12 (GenBank accession No. CAC21467); *Af*, *Aspergillus fumigatus*, *Af*PIG-C (GenBank accession No. AAS68361); *Mm*, *Mus musculus*, *Mm*DPM2 (GenBank Accession No. NP034203), *Mm*PIG-M (GenBank accession No. NP080510), *Mm*PIG-P (GenBank accession No. NP062416), and *Mm*PIG-C (GenBank accession No. NP080354); *Sc*, *Saccharomyces cerevisiae*, *Sc*GPI12 (GenBank accession No. NP014008). DPM2 = dolichol-phosphate-mannose 2; GPI = glycosylphosphatidylinositol.

substrates, each enzyme has the ability to cleave a specific ester bond (Cox et al., 2001).

Several mammalian PL activities that seem to be capable of removing the GPI anchors from proteins have been reported (Low and Saltiel, 1988). In *P. brasiliensis*, Heise et al. (1995) reported the detection of a potent PLC capable of selectively hydrolyzing the GPI anchor, with the consequent release of proteins. The search for cDNAs homologous to PLs in the *P. brasiliensis* transcriptome revealed two open reading frames with high sequence homology to PI-PLC and PLD of *A. nidulans* and *A. oryzae*, respectively (Table 5). This finding suggests that PI-PLC and PLD could be capable of hydrolyzing the GPI anchor in *P. brasiliensis*. The GPI-specific PLC, which is another type of phospholipase C capable of cleaving the GPI anchor, was not found in the *P. brasiliensis* transcriptome.

Table 5. *Paracoccidioides brasiliensis* phospholipases capable of cleavage the ω-site.

Product/PbAEST	Amino acid length[1]	% Amino acid identity by BLASTp analysis[2]	Putative functional
Phosphatidylinositol-specific phospholipase C/ PbAEST 2355	170 - IC	*Aspergillus nidulans* 88/174 (50%)	Hydrolyzes the phosphodiester bond in the phospholipid backbone to yield phosphatidic acid (Timpe et al., 2003)
Phospholipase D/ PbAEST 3496	173 - IC	*Aspergillus oryzae* 109/182 (59%)	Hydrolyzes the phosphodiester bond in the phospholipid backbone to yield 1,2-diacylglycerol (Timpe et al., 2003)

[1]Incomplete cDNAs are indicated by IC.
[2]Comparison by BLASTp to the nr database (GenBank).

CONCLUDING REMARKS

The cell wall is a plastic and dynamic structure that is constantly changing in response to environmental signals and to different stages of the fungal cell cycle. GPI anchoring is a eukaryotic mechanism for attaching proteins to the cell surface. In fungi, GPI proteins are known to be either covalently incorporated into the cell wall network or to remain attached to the plasma membrane. The GPI-anchored proteins localized in the cell wall may determine surface hydrophobicity and antigenicity, and they are reported from various microbial pathogens as immunogenic and adhesion molecules; they have also been suggested to be important virulence factors. On the other hand, the GPI proteins localized in the plasma membrane are known to play a role in cell wall biosynthesis and remodeling.

This is the first analysis of *P. brasiliensis* GPI-anchored proteins in the fungus transcriptome. Many of the identified proteins can be broadly categorized as being involved in cell wall remodeling, in host-fungus interaction, providing some insight into the purposes of GPI-anchoring.

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