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Proteome analysis of *Aspergillus fumigatus* identifies glycosylphosphatidylinositol-anchored proteins associated to the cell wall biosynthesis

Previous studies in *Aspergillus fumigatus* (Mouyna I., Fontaine T., Vai M., Monod M., Fonzi W. A., Diaquin M., Popolo L., Hartland R. P., Latgé J.-P., *J. Biol. Chem.* 2000, 275, 14882–14889) have shown that a glucanoyltransferase playing an important role in fungal cell wall biosynthesis is glycosylphosphatidylinositol (GPI) anchored to the membrane. To identify other GPI-anchored proteins putatively involved in cell wall biogenesis, a proteomic analysis has been undertaken in *A. fumigatus* and the protein data were matched with the yeast genomic data. GPI-anchored proteins of *A. fumigatus* were released from membrane preparation by an endogenous GPI-phospholipase C, purified by liquid chromatography and separated by two-dimensional electrophoresis. They were characterized by their peptide mass fingerprint through matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF)-mass spectrometry and by internal amino acid sequencing. Nine GPI-anchored proteins were identified in *A. fumigatus*. Five of them were homologs of putatively GPI-anchored yeast proteins (Csa1p, Crh1p, Crh2p, Ecm33p, Gas1p) of unknown function but shown by gene disruption analysis to play a role in cell wall morphogenesis. In addition, a comparative study performed with chitin synthase and glucanoyl transferase mutants of *A. fumigatus* showed that a modification of the growth phenotype seen in these mutants was associated to an alteration of the pattern of GPI-anchored proteins. These results suggest that GPI-anchored proteins identified in this study are involved in *A. fumigatus* cell wall organization.

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1 Introduction

Glycosylphosphatidylinositol (GPI) proteins anchored to the plasma membrane are known to play a role in fungal morphogenesis and cell wall organization. Two types of functions have been assigned to these proteins depending on their localization in the cell wall. First, numerous reports have shown that GPI-mannoproteins are bound covalently to cell wall polysaccharides in yeast [1–4]. These mannoproteins are linked to the cell wall β -1,6-glucan through a remnant of their GPI-anchor encompassing the ethanolamine-phosphate moiety and several mannose residues [5, 6]. These proteins have a biological

function such as filamentation, mating, flocculation, or adhesion to the external matrix which requires their localization at the cell surface [7–9]. Their covalent association with the polysaccharides is a way for the proteins to reach and stay at the cell surface. The role of these proteins in maintaining the cell wall shape remains debatable since deletion mutants grow equally well as the parental strain [4]. Second, GPI-bound proteins can display enzymatic properties playing an active role in cell wall biosynthesis. A family of β -1,3-glucanoyltransferases encoded by GPI-proteins has been identified in *Aspergillus fumigatus*, *Saccharomyces cerevisiae*, *Candida albicans*, and *Candida maltosa* [10–17]. Gene disruption experiments have shown that this enzymatic activity, that is responsible for the elongation of the cell wall β -1,3-glucans, was essential for mould and yeast morphogenesis.

With the exception of the β -1,3-glucanoyltransferase mentioned above, other enzymes involved in the remodeling of the neosynthesized polysaccharides are unknown. The location in the periplasmic space of GPI-proteins make them ideal candidates for such function and

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Abbreviations: CRD, cross reacting determinant; GPI, glycosylphosphatidylinositol; OG, octylglycoside; Q, quadrupole; PCMPSA, *p*-chloromercuriphenylsulfonic acid; PLC, phospholipase C; SB3-10: *N*-decyl-*N,N*-dimethyl-3-ammonio-1-propane-sulfonate

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prompted us to identify as many GPI-anchored proteins as possible in our model organism, *A. fumigatus*, using a selective proteomics approach. Membrane-bound proteins are not usually amenable to 2-D electrophoresis [18, 19]. To identify membrane-bound GPI-proteins, we have taken advantage of the fact that upon cleavage by a GPI-specific phospholipase C (GPI-PLC), GPI-anchored proteins are released in a soluble form and the terminal anchor is transformed into the so-called cross-reacting determinant (CRD), an inositol 1,2-cyclic phosphate containing structure [20]. Antibodies reacting specifically with this structure have been used to trace the presence of released GPI-anchored proteins. In this report, we show the identification of nine different GPI-anchored proteins in *A. fumigatus* and compare their sequence to the genomic databases in view to identify GPI-anchored proteins common to yeast and filamentous fungi which could have a role in cell wall morphogenesis.

2 Materials and methods

2.1 Strains and fungal cultures

CBS 144-89 and LAR 2965B2 strains are clinical isolates of *A. fumigatus*. The construction of cell wall mutants Δ CHSE/ Δ CHSG and Δ GEL1/ Δ GEL2 will be reported elsewhere (Mellado *et al.*, manuscript in preparation; Mouyna *et al.*, manuscript in preparation). Flasks containing 50 mL of Sabouraud liquid medium (2% glucose, 1% mycopeptone; Biokar, Beauvais, France) were inoculated and shaken for 3 days at 200 rpm at 25°C. These precultures were then inoculated into a 2 L fermenter (LSL Biolaftite SA, Saint-Germain en Laye, France) containing 1.2 L Sabouraud liquid medium. Cultures were performed at 25°C for 24 h with an agitation of 500 rpm and an aeration of 0.5 L air per min. The mycelium was then collected by paper filtration under vacuum and extensively washed with water before use.

2.2 Preparation of the anti-CRD polyclonal antibodies

Hy/Cr rabbits (Charles River France) were immunized by subcutaneous injection of 1 mg of sVSG protein (provided by M.A. Ferguson; [21]) in Freund's complete adjuvant. One boost injection was done after 2 weeks with 0.5 mg sVSG in Freund's incomplete adjuvant. Bleeding of the rabbit was done 3 weeks after the last immunization and anti-CRD antibodies were purified by affinity chromatography on a sVSG column prepared by coupling of the protein on CNBr activated Sepharose CL-4B (Amersham Pharmacia Biotech, Uppsala, Sweden) according to the

specifications of the manufacturer. The purified antibodies fractions were assayed by Western blotting and immunodetection as described below.

2.3 Preparation of *A. fumigatus* membranes and purification of CRD-positive proteins

2.3.1 Preparation of *A. fumigatus* membranes

Mycelium was resuspended (0.3 g fresh weight mL⁻¹) in a 200 mM Tris-HCl buffer, pH 8, containing 50 mM EDTA, 1 mM PMSF, and disrupted for 3 min in a CO₂-cooled MSK homogenizer (B. Braun Biotech, Bailly, France) with 1 mm-diameter glass beads. Cell walls were removed by centrifugation at 4000 × *g* for 15 min at 4°C. The supernatant was centrifuged at 100 000 × *g* for 1 h at 4°C. The membrane pellet was homogenized in a 50 mM Tris-HCl buffer, pH 8.5, containing 5 mM EDTA, 0.5 mM PMSF, 1 mM DTT using a glass-glass potter and immediately frozen in liquid nitrogen and stored at -80°C until used. When required, 5 mM *p*-chloromercuriphenylsulfonic acid (PCMPSA) was added to buffers to prevent GPI-PLC driven cleavage of GPI-anchor during the extraction procedure.

2.3.2 Solubilization of CRD-positive proteins

Membranes were thawed and β -*n*-octylglucopyranoside and PMSF were added to reach a final concentration of 1% and 0.5 mM respectively. A 30 min–1 h incubation at room temperature released GPI-anchored proteins from the membrane homogenate. The preparation was ultracentrifuged at 100 000 × *g* for 1 h at 4°C and the supernatant was used for CRD-positive protein purification.

2.3.3 CRD-positive protein purification

Solubilized proteins from the previous step were diluted fivefold with a buffer containing 25 mM Na₂HPO₄/25 mM NaH₂PO₄ pH 7, 5 mM EDTA (buffer B) with 0.5 M (NH₂)SO₄ added to it (buffer A). This solution was loaded onto a previously equilibrated (buffer A) 25 mL Octyl Sepharose column (Sigma, St. Louis, MO, USA) at a flow rate of 0.5 mL min⁻¹ and optical density at 280 nm was recorded. Fractions were collected as soon as proteins begun to exit from the column (flow-through fraction). After a wash with buffer A followed by a wash with buffer B until optical density returned to the baseline, bound material was eluted by means of a 30 min linear gradient at 0.5 mL min⁻¹ from buffer B to 50% ethylene glycol in the same buffer. Eluted proteins were collected and saved for further treatment (ethylene glycol eluate). The flow-

through fraction, containing most of the CRD-positive proteins, was dialyzed extensively against water (four dialysis bath changes) and freeze-dried using a Heto Drywinner 6-55 freeze dryer (Fisher Bioblock Scientific, Ulkirch, France). Since the ethylene glycol eluate contained low amount of proteins, material was accumulated from four identical experiments and processed in an identical manner as compared to the flow-through fraction.

2.4 Electrophoresis separation

For SDS-PAGE (1-D) separation, protein samples were solubilized in reducing sample buffer (62.5 mM Tris-HCl, pH 6.8, 3% SDS, 5% β -mercaptoethanol, 10% glycerol) and heated for 5 min at 100°C. Proteins were separated on a 8% acrylamide Tris-glycine polyacrylamide gel from Novex (Invitrogen, Leek, The Netherlands). For 2-D electrophoresis, freeze-dried purified proteins were solubilized in 1 mL of sample buffer containing 6 M urea (Merck, Darmstadt, Germany), 2 M thiourea (BioRad, Richmond, CA, USA), 4 mM tributylphosphine (Acros Organic, Geel, Belgium), 2% CHAPS, 2% SB 3–10, 0.5% Immobiline pH 4–6.5 (Pharmacia, Uppsala, Sweden), and a trace of bromophenol blue. Any insoluble material was removed by centrifugation at $10\,000 \times g$. This sample was used to rehydrate $180 \times 3 \times 0.5$ mm Immobiline dry strips pH 3–10 NL or pH 4–7 (IPG strips; Amersham Pharmacia Biotech) in an IPGPhor system (Amersham Pharmacia Biotech) for 10 h at 50 V. Isoelectrofocusing separation was carried out using the following programming steps: gradient from 50 to 500 V in 1 h, gradient from 500 to 1000 V in 1 h, gradient from 1000 to 3000 V in 1 h, hold at 8000 V to achieve a total migration of 100 kVh. IPG-strips were then equilibrated in 50 mM Tris-HCl, pH 8.3, 6 M urea, 2 M thiourea, 30% glycerol, 2% SDS (equilibration buffer) containing 1% DTT for 10 min followed by equilibration buffer containing 2.5% iodoacetamide and a trace of bromophenol blue for 10 min. Equilibrated IPG-strips were loaded on the top of Excel-Gel 12–14% acrylamide (Amersham Pharmacia Biotech) and the second dimension was done according to the specifications of the manufacturer. 2-D gels were either stained with 0.2% Coomassie blue or transferred to nitrocellulose for Western blotting.

2.5 Western blotting

After 1-D electrophoresis separation, proteins were transferred to ECL nitrocellulose (NC) membranes (Amersham Pharmacia Biotech) using the Novex X Cell II blot module (Invitrogen) in the conditions recommended by the manufacturer. Transfer of 2-D gels was performed in a Nova Blot system (Amersham Pharmacia Biotech) at a constant

current of 0.8 mA cm^{-2} of membrane for 2 h. The NC membrane was then incubated for 2 h at room temperature or overnight at 4°C in a blocking solution containing 5% non-fat dry milk (Life Technologies, Paisley, Scotland) and 0.05% Tween 20 in phosphate-buffered saline (PBS; Life Technologies). After two washes with 0.05% Tween 20 in PBS to remove proteins in excess, the membrane was then incubated for 1 h at room temperature in a solution containing a 1:75 dilution in PBS, 0.05% Tween 20 of an anti-CRD purified antiserum (see above). The NC membrane was washed three times in PBS, 0.05% Tween 20 (10 min each) and incubated for 1 h at room temperature in a solution containing a 1:2500 dilution in PBS, 0.05% Tween 20 of a donkey anti-rabbit IgG-horseradish peroxidase conjugate (Amersham Pharmacia Biotech). Bound antibodies were detected using the enhanced chemiluminescence (ECL) reagents and autoradiography on ECL-Hyperfilm (Amersham Pharmacia Biotech). Pre-stained proteins were used for calibration (Full range Rainbow; Amersham Pharmacia Biotech).

2.6 Protein identification and searches in sequence databases

Coomassie blue stained gels images were acquired using a Personal Densitometer SI (Amersham Pharmacia Biotech). Molecular weight and isoelectric point of protein spots were determined through calculation by comparison with a molecular weight standard (standard MARK 12; Novex, San Diego, CA, USA) loaded on one edge of each gel, and the theoretical pH gradient shape of IPG strips, respectively, using the Melanie3 2D electrophoresis imaging software (GeneBio).

2.6.1 In-gel digestion of protein spots

Coomassie blue stained protein spots which were CRD-positive, were excised from the gels and cut in approximately 1 mm^2 pieces. In-gel digestion by bovine trypsin (Roche Diagnostics, Indianapolis, IN, USA) was done in a Digest Pro workstation (Abimed, Langenfeld, Germany). Gel slices were washed for 10 min with acetonitrile followed by a 10 min rehydration by 50 mM NH_4HCO_3 . This step was repeated twice. Proteins were then reduced at 37°C for 30 min, washed with acetonitrile for 10 min (room temperature), and alkylated for 15 min using 10 mM dithiotreitol and 50 mM iodoacetamide, respectively. After three 10-min acetonitrile washes, the temperature was set at 37°C and gel slices were dried under a nitrogen flux. Gel pieces were then rehydrated by a $25 \text{ }\mu\text{g/mL}$ solution of bovine trypsin in 0.01% TFA (10 min) followed by 50 mM NH_4HCO_3 (15 min). Excess of protease was removed and 25 mM NH_4HCO_3 was added to cover the

gel pieces. Digestion was allowed to proceed for 6 h. Peptides were then extracted by two acetonitrile incubations (15 min), followed by three extractions by 10% formic acid (10 min) and acetonitrile (15 min) at room temperature. Peptides extracts were pooled and dried under vacuum in a speed-vac centrifuge (Savant Instruments, Holbrook, NY, USA). For Edman sequencing of internal peptides, in-gel digestion by Lys-C (Roche Diagnostics), separation of peptides by microbore reversed-phase HPLC and *N*-terminal sequencing of peptides was performed as previously described [22].

2.6.2 Desalting of samples and mass spectrometry analysis of tryptic digests

Peptides were solubilized by 10 μ L of 2% formic acid and loaded on a previously equilibrated (50% acetonitrile followed by 2% formic acid) ZipTip C18 pipette tips (Millipore, Bedford, MA, USA). Tips were washed with 2% formic acid and peptides were eluted using 10 μ L of a solution containing 60% acetonitrile, 2% formic acid in water. 0.5 μ L of sample was mixed with 0.5 μ L of MALDI matrix (saturated solution of dihydroxybenzoic acid) on the MALDI-TOF target and allowed to dry. MALDI-TOF-MS spectra were acquired and recorded with a Voyager DE-STR mass spectrometer (PerSeptive Biosystems, Framingham, MA, USA) using an accelerating voltage of 21 kV in the reflector mode. When necessary, residual peptide samples were used to obtain amino acid sequences information. For nano-ES-MS/MS, samples were infused using the nanoelectrospray source kit (Protona A/S, Odense, Denmark) for the Finnigan TSQ 7000 (ThermoQuest, Austin, TX, USA). Samples were resuspended in 5 μ L of water, 50% methanol, 0.5% formic acid and sprayed at 0.6–0.7 kV. Under these conditions, MS/MS was done on the doubly charged protonated molecules using argon (2.5 millitorr) as collision gas at 20–38 eV. Scan accumulation was done until sufficient signal was obtained. Quadrupole (Q)-TOF-MS/MS analysis was done in a similar way using a Q-TOF2 system (Micromass, Manchester, England) except that doubly, triply or quadruply charged ions were obtained. Transformation of multicharged-spectra into monocharged spectra was done with the Maxent 3.0 program (Micromass) and interpretation of data was done using the Pepseq program [23] included in the Biolynx software (Micromass). MALDI-TOF-MS and MS/MS data were matched against the NCBI nr and the Genome Therapeutics Corporation Pathogenome databases using the MS-Fit and MS-Seq and/or MS-Edman programs (assembled into the Protein Prospector package. Baker, P. R. and Clauser, K. R. <http://prospector.ucsf.edu>), respectively, over an intranet connection. Searches were done over the

full molecular weight and isoelectric point range in order to have the best chance to identify the proteins. Concerning MS-Fit, mass accuracy was set at ± 50 ppm for most MALDI-TOF analysis (internal calibration was done with trypsin autolysis generated ions). Possible post-translational and chemical modifications such as oxidation of methionine, *N*-terminal acetylation, *N*-terminal pyroglutamic acid, carbamidomethylation of cysteine, and modification of cysteine by acrylamide, were taken into consideration for the queries. Protein sequences homologies were determined using the Wisconsin analysis Package version 10.1 from the Genetics Computer Group (Madison, WI, USA) through the SeqWeb Version 1.2 interface.

3 Results

3.1 Purification of CRD-positive proteins and separation by 2-D electrophoresis

Previous studies have shown that membrane preparations of *A. fumigatus* contained GPI-anchored proteins that can be labeled specifically with anti-CRD antibodies after cleavage by exogenous GPI-phospholipase C [11]. Incubation of *A. fumigatus* membrane preparations for 1 h at room temperature in a Tris-EDTA-PMSF-DTT buffer resulted in the release of CRD-positive protein bands (Fig. 1). The number and amount of proteins labeled by the anti-CRD antibodies was highly increased after incubation of the membrane preparations for 30 to 60 min with 1% octylglucoside (OG) (Fig. 1). This increase was due to the activation of an endogenous GPI-PLC of *A. fumigatus* since: (i) known inhibitors of phosphatidylinositol specific (PI)-PLC such as PCMPSA (5 mM) or HgCl_2 (1 mM) blocked the release of these CRD-positive proteins and (ii) similar Western blot patterns were obtained when a crude membrane preparation of *A. fumigatus* was incubated with bacterial commercial PI-PLC from *Bacillus thuringiensis* (data not shown). The optimal concentration of OG was 1% although 0.5% was also effective in increasing the signal compared to the untreated membranes. GPI-PLC activation was not detergent-specific since other nonionic detergents such as Triton X-100 at a concentration of 0.1% stimulated also the release of CRD-positive proteins, although less effectively (data not shown). SDS-PAGE 1-D gels showed that major CRD-positive signals were obtained for proteins with apparent molecular masses between 114 and 40 kDa. Since the soluble fraction of the OG-treated membrane preparation contained also numerous CRD-negative proteins, purification of CRD-positive proteins was carried out. Hydrophobic interaction liquid chromatography using Octyl-Sepharose was the most efficient procedure to separate GPI-

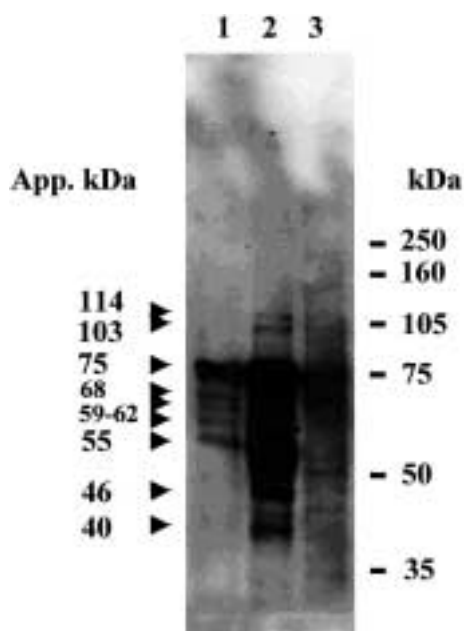


Figure 1. Presence of GPI-anchored proteins and of a GPI-phospholipase C activity in membrane preparations of *A. fumigatus*. 16 μ L of a microsomal membrane preparation were incubated at room temperature for 1 h in the conditions described below. The mixture was centrifuged and 5 μ L of supernatant (36 μ g protein) was diluted twice with 2 $\times$ reducing SDS sample buffer, boiled for 5 min, and loaded on a 8% Tris-glycine polyacrylamide gel. Proteins were transferred to nitrocellulose and immunodetection was done with the affinity-purified anti-CRD antiserum as described in the experimental procedures. Following ECL detection, the film was exposed for 10 min. Lane 1, membranes alone; 2, membranes in the presence of 1% *n*-OG; 3, membranes in the presence of 1% *n*-OG and 5 mM PCMPA. Molecular weights are indicated on the right and arrow heads indicate the position of the major signals. Calculated molecular masses of these proteins are noted on the left as apparent kDa (App. kDa).

anchored proteins from the bulk of membrane proteins. The majority of CRD-positive proteins was not retained on the column or was eluted during the low-ionic-strength wash, and only two of them, with molecular masses of 103 and 114 kDa, were eluted during an ethylene glycol gradient.

Further separation of GPI-proteins that did not bind to Octyl-Sepharose, was achieved by 2-D electrophoresis. In early experiments, separations by 2-D electrophoresis were done using nonlinear pH 3–10 gradient for the first dimension, and premade 12–14% acrylamide gradient gels for the second dimension. All CRD-positive proteins were located in the acidic separation part of the gel (data not shown). Therefore, subsequent separations were

done in pH 4–7 18 cm IPG strips for the first dimension. An example of pH 4–7 separation is shown in Fig. 2. Nineteen CRD-positive spots (Fig. 2A) could be matched with Coomassie blue stained protein spots on the original gels (Fig. 2B). The pattern, in terms of size separation, was similar to the one previously obtained in 1-D SDS-PAGE (see Fig. 1 for comparison). However, the 40 and 45 kDa signals observed in 1-D SDS-PAGE were missing suggesting that some protein degradation or cleavage of the GPI-anchor occurred during the purification steps. IEF separation shows a number of horizontally aligned spots indicating that more than a single polypeptide was present at a given molecular weight, some of them being likely isoforms of the same protein. The latter was particularly true for the 75 kDa signal, which was made of eight regularly spaced protein spots.

3.2 Identification of the Octyl-Sepharose unbound CRD-positive proteins

Identification of the different GPI-anchored proteins separated by 2-D electrophoresis (Fig. 2) is shown in Table 1. The same CRD-positive protein pattern was seen when two different wild-type mutants (CBS 144-89 and LAR 2965B2) were analyzed, showing that the proteins identified were representative of the *A. fumigatus* species and not of a peculiar strain (data not shown).

3.2.1 Spots 1–5

These five spots matched with the most abundant CRD-positive protein that had a molecular mass of 75 kDa. The major polypeptide (spot 5) was subjected to Lys-C endoprotease digestion and *N*-terminal Edman sequencing of the purified peptides. Two of the peptides yielded the sequences (K)HNPLVLYDSVTK and (K)TGYVNYDVDT. When matched to the NCBI nr database, similarities with the *Penicillium chrysogenum* (accession P37274; [24]) and *Aspergillus niger* (accession P34724; [25]) phosphate-repressible acid phosphatase gene product PhoAp were found. Five protein spots among the eight migrating at the same molecular mass were analyzed by nano-ES-MS/MS. All the spots (spots 1–5) corresponded to the *A. fumigatus* PhoA protein, suggesting that all these eight polypeptides were isoforms of the same protein (Table 1).

3.2.2 Spots 6, 7

Two proteins migrating at a slightly higher molecular mass than PhoAp (78 kDa) with isoelectric points of 4.43 and 4.39 (Table 1) were unambiguously matched with an already known *A. fumigatus* allergen named rAsp f 9 ([26];

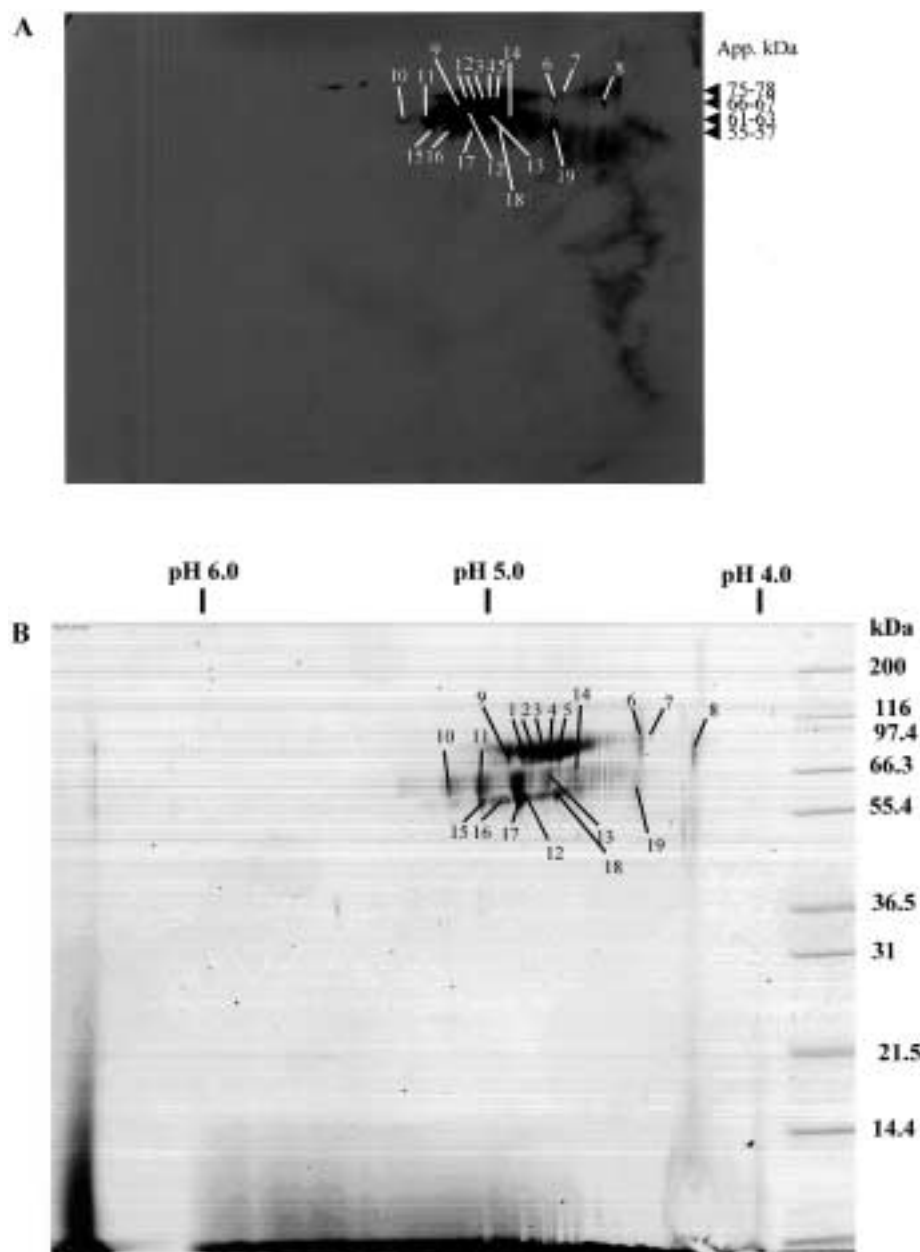


Figure 2. 2-D electrophoresis separation of proteins present in the flow-through fraction of the Octyl-Sepharose column. Proteins were separated in pH 4–7 IPG-strips for the first dimension and in acrylamide 12–14% gradient gels for the second dimension as described in the experimental procedures. (A) Separated proteins were transferred to nitrocellulose and immunodetection was done with anti-CRD antiserum and autoradiography after revelation with a horseradish peroxidase (HRP)-conjugate. Following ECL detection, the film was exposed for 1 min. Arrow heads on the right indicate the correspondence of the CRD-positive signals found also on 1-D separation together with the calculated apparent molecular masses (App. kDa). (B) Coomassie blue staining of a gel done in the same conditions than in (A). Protein spots that were visible on both images are labeled from 1 to 19 and indicated with an arrow. Molecular masses are indicated on the right.

GenBank accession no. AJ223327). This protein displayed 46% identity with the *Saccharomyces cerevisiae* CRH1/YGR189C gene product, a putative GPI cell wall protein [27, 28]. The calculated isoelectric point of Crh1p (4.56) was similar to the one calculated from spot 6 migration. In contrast, the 53 kDa molecular mass calculated upon the sequence of Crh1p was much lower than the one obtained with its ortholog in *A. fumigatus*. The increase in molecular mass between the observed *A. fumigatus* homolog and the calculated molecular mass of the *S. cerevisiae* Crh1p may result from the presence of *N*- and *O*-glycans attached to this protein. Indeed

Crh1p contains three potential *N*-glycosylation sites as well as a Ser- and Thr-rich C-terminal region, a typical feature of highly *O*-glycosylated proteins.

3.2.3 Spot 8

Only two amino acid sequences were obtained from peptides derived from spot 8 migrating between 67 and 75 kDa. One sequence was unambiguously matched with a pathogenome incomplete entry that showed 32 and 35% identity at the nucleotide level with a *Cocci-*

Table 1. Identification of the CRD-positive proteins separated by 2-D electrophoresis

Spot ^{a)} no.	Exp. ^{a)} <i>M_r</i>	Exp. pI ^{a)}	MS-Fit output ^{b)}		Calc. <i>M_r</i> ^{c)}	Calc. pI ^{c)}	MS/MS ^{d)}		MS-Seq/MS-Edman results ^{e)}	
			Nb pep. matched/ total	Seq. cover.			Sequence tag	Method	Name	
1	75 000	4.87					YSPV ...	ES-MS/MS	PA7501739816: homologies with <i>P. chrysogenum</i> / <i>A. niger</i> PhoA	
2	75 000	4.84					KHNPLV(LYD)SVTK	ES-MS/MS	PA7501739816	
3	75 000	4.80					(SP)VSNEGVA(FNR)	ES-MS/MS	PA7501739816	
							TGYVNYDVD ...	ES-MS/MS	PA7501739816	
4	75 000	4.76					TGYVNYDVDTTNCR	ES-MS/MS	PA7501739816	
5	75 000	4.73					KHNPLVLYDSVTK	ES-MS/MS	PA7501739816	
6	78 000	4.43	13/45	35%	32 318	4.63	LGSWAGGD(PSN) ...	ES-MS/MS	NCBI 2979890 rAspF9 <i>A. fumigatus</i>	
							FPQTPMR	ES-MS/MS	NCBI 2979890	
							DPQNTGI/LI/LAQSQQFK	Q-TOF-MS	Not found	
					52 758	4.56	() SSVSSD	Q-TOF-MS	NCBI6321628 Crh1p of <i>S. cerevisiae</i>	
7	78 000	4.39	8/12	23%	32 318	4.63	(VP)VG(PQ)G(AEFTV)AK	ES-MS/MS	PA7501803123: rAspF9 <i>A. fumigatus</i>	
							TLTY ...	ES-MS/MS	PA7501803131: rAspF9 <i>A. fumigatus</i>	
							LGSWAGGD(PSN)(PK)	ES-MS/MS	PA7501803131	
							I/LV VASSAPK	ES-MS/MS	Not found	
8	67 000	4.23					NTGI/LI/LAQV ...	Q-TOF-MS	Not found	
							CLCSQANYGYGIR	ES-MS/MS	PA7501939934: homologies with Csa1p <i>C. albicans</i>	
9	66 000	4.8–4.9	10/17	21%	52 305	4.64	LSFVMoxNQYYK	Q-TOF-MS	PA7501832353 Epd1 homolog	
							(AD)PAW ...	Q-TOF-MS	PA7501832353	
							(AD)PAWNTDLYKR	Q-TOF-MS	PA7501832353	
							(SS)LLVGATDDDD ...	Q-TOF-MS	PA7501832353	
10	61 000	5.14	Identical fingerprint than spot 11				NDELSSIDL ...	ES-MS/MS	PA7501829984: Ecm33p homolog	
11	61 000	5.03					WTGAVDF ...	ES-MS/MS	PA7501829984	
							WTGAVDFSGK	ES-MS/MS	PA7501829984	
12	61 000	4.89					DELSSIDDL ...	ES-MS/MS	PA7501829984	
							QYMPQT ...	ES-MS/MS	PA7501914965: Utr2p homolog	
							QYMoxYPQTSPR	ES-MS/MS	PA7501914965	
13	62 000	4.77					SDWVY ...	ES-MS/MS	PA7501914974: Utr2p homolog	
							QYMoxY(PQT)PSR	ES-MS/MS	PA7501914965	
							(QY)MY ...	ES-MS/MS	PA7501914965	
14	63 000	4.67					QYMY ...	ES-MS/MS	PA7501914965	
15	57 000	5.02	10/10	20%	48 000	4.94		nd	NCBI 3608406: Gel1p	
16	57 000	4.94	Identical fingerprint than spot 14					nd		
17	57 000	4.88	17/27	36%	48 000	4.94	FKELG ...	ES-MS/MS	NCBI 3608406	
							GPGFAGP(GSQD)R	ES-MS/MS	NCBI 3608406	

Table 1. Continued

Spot ^{a)} no.	Exp. ^{a)} <i>M_r</i>	Exp. <i>pI</i> ^{a)}	MS-Fit output ^{b)}		Calc. <i>M_r</i> ^{c)}	Calc. <i>pI</i> ^{c)}	MS/MS ^{d)}		MS-Seq/MS-Edman results ^{e)}	
			Nb pep. matched/ total	Seq. cover.			Sequence tag	Method	Name	
18	57 000	4.74	8/10	15%	48 000	4.94	ELPD ...	ES-MS/MS	NCBI 3608406	
							TSG ...	ES-MS/MS	NCBI 3608406	
							GNA ...	ES-MS/MS	NCBI 3608406	
							GPGFAGPGSQDR	ES-MS/MS	NCBI 3608406	
19	55 000	4.45					TQVSLSSGSF ...	ES-MS/MS	PA7501727705: Unknown protein	
							LOY(PT)SR	ES-MS/MS	PA7501727705	

- a) Experimental molecular weight (Exp. *M_r*) and isoelectric point (Exp. *pI*) as determined by the protein spots migration in 2-D electrophoresis
- b) Results given by the MS-Fit program when queried with the peptide mass fingerprint of the indicated protein. The number of peptides, among the total used for the query, that matched with the identified protein (Nb pep. Matched) is indicated as well as the sequence coverage (Seq. cover.) of this protein with the matching peptides
- c) Calculated molecular weight (Calc. *M_r*) and isoelectric point (Calc. *pI*) based on the amino acid sequence of the identified protein
- d) Indicates the MS/MS method used for obtaining amino acid sequence information from the protein as well as the obtained sequence tags. Residues between brackets correspond to nonfragmented ions found in MS/MS spectra. Mox indicates an oxidized methionine, I/L indicates the presence of either a isoleucine or a leucine (isomers) in sequence tags that could not be matched with a sequence present in databases. Although Gln and Lys cannot be discriminated in MS (isobar amino acids), Gln (Q) was chosen when found within a sequence
- nd, not done
- e) The pathogenome (PA) or the NCBI identifier is indicated as well as the name of the protein if known in *A. fumigatus* or the name noted in the sequence annotation. Annotations in pathogenome sequences, were generated by a Blast search of the public databases with the pathogenome entries.

dioides immitis immunoreactive cell wall protein named ag2 and the mycelial surface antigen Csa1p of *C. albicans* ([29]; GenBank accession no. AF080221), respectively. The deduced amino acid sequence of Csa1p suggested that the C-terminus contains a core of hydrophobic residues that could function as a GPI-anchoring determinant.

3.2.4 Spot 9

As can be seen in Fig. 2A, a CRD-positive train of spots was seen just below the PhoAp signal. This signal accounted for the 68 kDa response obtained in 1-D gels. This protein was identified by Q-TOF-MS/MS as an homolog of Epd1p of *Candida maltosa* (Table 1), a known GPI-anchored membrane protein homologous of the Gelps of *A. fumigatus* [14]. The sequences obtained from this protein were however different from the ones of Gel1p, Gel2p and Gel3p, the three members of the GEL family identified in *A. fumigatus* [12]. This result indicated the presence of a new member of this gene family that we have named Gel4p.

3.2.5 Spots 10–11

The major CRD-positive signal at 61 kDa in Fig. 2A was identified as isoforms of an homolog of the *S. cerevisiae* putative GPI-anchored membrane protein Ecm33p [30]. *ECM33/YBR078W* was found during a genetic screen aimed at identifying genes involved in *S. cerevisiae* cell surface biosynthesis and architecture [30]. ECM33 mutant had indeed a disturbed cell wall biosynthesis as shown by increased sensitivity to Calcofluor white and zymolyase [31].

3.2.6 Spots 12–14

The three other CRD-positive polypeptides also detected at 61 kDa were isoforms of the putative GPI-cell wall protein Utr2p, although spot 12 consisted in a mixture of Utr2p and Ecm33p homologs. *UTR2/CRH2/YELO40W* belongs to the same gene family than *CRH1* [27]. Interestingly, *CRH1/CRH2* double null mutant was more sensitive to Congo red and Calcofluor white than either single mutant and parental wild type strain and had an increased amount of alkali-soluble glucan [32].

3.2.7 Spots 15–18

The CRD-positive response corresponding to protein spots 15, 16, 17, and 18 in Fig. 2B were due to the presence of the GPI-anchored glucanoyltransferase Gel1p previously identified in *A. fumigatus*. Six sequence tags obtained from these different spots matched without any mismatch with this protein. In addition, 36% coverage of the Gel1p sequence was obtained with the peptide mass fingerprint. Gel1p migrates at 57 kDa and isoforms were present between *pI* 4.74 and 5.02. These values were in agreement with those calculated from the sequence data (48 kDa, *pI* 4.78) since it was previously shown that Gel1p was *N*-glycosylated [10, 11]. The identification of this known membrane GPI-anchored protein in our 2-D gels confirmed also the specificity of the immunolabelling with anti-CRD antibodies.

3.2.8 Spot 19

Amino acid sequences were obtained from the 55 kDa protein but could not be assigned to known proteins although the two sequences obtained from spot 19 could be matched unambiguously with a Pathogenome database entry (see Table 1).

3.3 Identification of the Octyl-Sepharose bound CRD-positive proteins

Ethylene glycol eluates from the Octyl-Sepharose column contained two CRD-positive proteins with apparent molecular masses of 114 and 103 kDa (Fig. 1). Proteins were subjected to 1-D SDS-PAGE and Coomassie blue staining. The amount of the 103 kDa molecule was too low even after four enrichment steps to allow its identification. Two sequence tags obtained from the 114 kDa protein (IQDFDAAGYIDR and ALMNGAGAIK) matched with a Pathogenome entry (PA7501726476) that shared strong homologies with phospholipases from various organisms. Particularly, 76 and 43% identity in a 110 amino acids overlap was found with a phospholipase B from *Penicillium chrysogenum* (accession GenBank X60348; [33]) and Plb3p encoded by the YOL011W gene from *S. cerevisiae* (accession GenBank Z74753; Hughes and Pohl, 1996 unpublished), respectively. Interestingly, the C-terminal region of Plb3p contains a motif for a putative GPI anchor attachment [34].

3.4 Comparative analysis of GPI-proteins of *A. fumigatus* wild-type and cell wall mutants

Since it was recently shown that at least some members of the GPI-anchored protein family had a functional role in cell wall biosynthesis [11], it was of interest to investigate the population of CRD-positive proteins present in the membrane of cell wall mutants. The two mutants selected were a double chitin synthase ($\Delta CHSE/\Delta CHSG$) and a double glucanoyltransferase ($\Delta GEL1/\Delta GEL2$) mutants which are the only *A. fumigatus* cell wall mutants which displayed to date the most reduced growth phenotype (Mellado *et al.*, manuscript in preparation; Mouyna *et al.*, manuscript in preparation). The comparison of the patterns of GPI-anchored proteins showed a marked difference between the mutants and the wild-type parental strain (Fig. 3) although the total amount of proteins used for the purification were similar for the mutant and wild-type strains (verified by Coomassie staining in 1-D SDS PAGE, not shown). No additional CRD-positive proteins were detected in both mutants. In contrast, various CRD-positive spots were missing in the membrane preparations of the two mutants. In the $\Delta CHSE/\Delta CHSG$ mutant, the signal corresponding to PhoAp disappeared almost entirely and the signal corresponding to spots 8 and 19 decreased significantly. The other CRD-positive spots remained unchanged.

Changes were the most pronounced in the $\Delta GEL1/\Delta GEL2$ double mutant. A global decrease in the CRD signal was apparent in this mutant since the time exposure of the X-ray film had to be highly increased ($\times 7$) to be able to identify CRD-positive proteins when a similar amount of starting material was used for the purification. In spite of this long time exposure, almost all the CRD-positive signals disappeared. The strongest signal was seen for Ecm33p and spot 19. The PhoAp signal was decreased but was comparatively more abundant than in the case of the $\Delta CHSE/\Delta CHSG$ mutant. The absence of the Gel1p signal was in agreement with the disruption of *GEL1*. These results showed that cell wall perturbations consecutive to the disruption of the chitin synthase and glucanoyltransferase genes were associated to a modification of the expression or anchoring of GPI-anchored proteins in *A. fumigatus*.

4 Discussion

Interest in the study of GPI-bound proteins comes from recent studies showing that GPI-proteins play a role in cell wall biogenesis. Enzymes involved in cell wall bio-

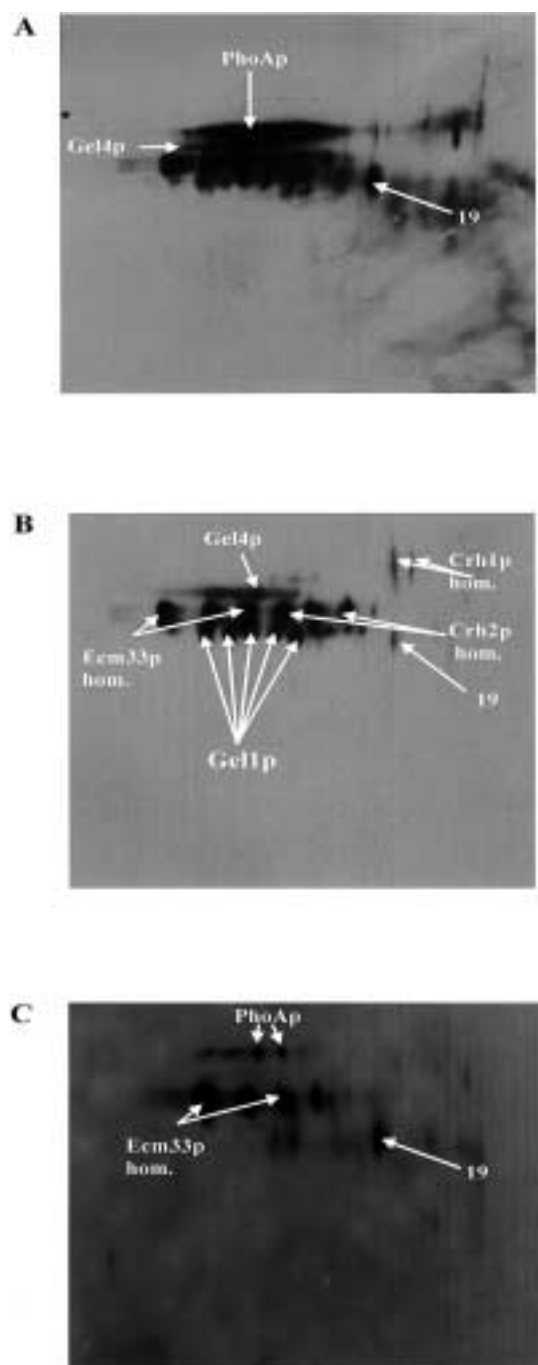


Figure 3. 2-D electrophoresis separation patterns comparison of *A. fumigatus* wild-type and mutants extracts. Separation conditions are identical to the ones in Fig. 2A. (A) Wild-type, (B) Δ CHSE/ Δ CHSG mutant, (C) Δ GEL1/ Δ GEL2 mutant. Figures are close views of area of gels containing the protein spots. Film exposure was 1 min for images shown in (A) and (B) and 7 min for the one shown in (C). Spots indicated by an arrow were individually digested by trypsin and analyzed by MS/MS. Ecm33p hom., Crh1p hom., Crh2p hom. stands for homologs of the *S. cerevisiae* corresponding proteins.

synthesis common to both filamentous and yeast pathogens such as *Aspergillus* and *Candida* are ideal targets for the development of new antifungals [35]. Comparing GPI- proteins of *A. fumigatus* to other yeast GPI-proteins has indeed brought a new insight in the understanding of the role of these GPI-proteins in fungal cell wall biosynthesis. In *A. fumigatus*, nine GPI-anchored proteins were identified. Six of these proteins have a putative enzymatic function as shown by comparison with the databases, two β 1-3-glucanosyltransferases (Gel1p and Gel4p), one phosphatase, one phospholipase (Plb3p) and β 1-3 or β 1-3/1-4 glucanases (Crh1p/Crh2p). In contrast, no homolog of the GPI-CWPs putatively covalently bound to *S. cerevisiae* cell wall polysaccharides was identified in *A. fumigatus*. This result is in agreement with an unpublished biochemical study from our laboratory suggesting that no proteins are covalently linked to β 1-3 glucan and chitin in *A. fumigatus* (Bernard *et al.*, manuscript in preparation).

Three members of the Gelp (Gel1-3) family of *A. fumigatus* were already cloned and sequenced [12]. In our membrane preparations, we have identified two members of the Gelp family. One is the already mentioned Gel1p whereas the second one is a new member of the Gelp family, called Gel4p. If the absence of Gel3p in the 2-D pattern was in agreement with its lack of expression under normal growth conditions, it was astonishing to see that Gel2p was not detected in our 2-D gels whereas the gene was transcribed under the growth conditions used in this study [12]. This result indicated that either Gel2p was processed and destroyed through proteolytic cleavage or present in a too low amount to be identified. This result indicated that not all the expressed proteins were identified by our 2-D electrophoresis approach and confirmed the interest to complement a proteome approach by a transcriptome that showed that *GEL2* was indeed transcribed.

Three additional GPI-anchored proteins identified in *A. fumigatus* have been previously described as putatively GPI-anchored and involved in cell wall maintenance in the budding yeast. Ecm33p has been found in genetic screens aimed at identifying genes involved in cell wall biosynthesis (De Groot *et al.*, submitted) [30] and in a genome-wide analysis of disruption phenotypes [31] in *S. cerevisiae*. *ECM33* null mutants displayed Calcofluor white, K1 killer toxin, zymolyase and hygromycin B hypersensitivity as well as a reduced mannose/glucose ratio [30]. Crh1p and Crh2p, although not found in the above-mentioned screens, are considered to be potential glycosidases involved at different stages of the yeast life cycle [32]. In addition, *CRH1* transcription was found to be regulated through the cell integrity

signaling pathway [36] and increased following the disruption of *FKS1*, the putative catalytic subunit of β 1-3 glucan synthase [37].

Three GPI-anchored proteins identified in *A. fumigatus* were not found in *S. cerevisiae*. No homolog was found in databases for the polypeptide of spot 19. Spot 8 is homologous to a cystein-rich hydrophobin Csa1p of *C. albicans* which is preferentially expressed during the mycelial growth phase [29] and is restricted to the sites of cell surface elongation. The major GPI-anchored protein present in *A. fumigatus* membrane fraction is a homolog of the acid-phosphatase PhoAp from *Penicillium chrysogenum* and *A. niger* [24]. An acid-phosphatase, Pho610, has been described to be cell wall associated in the yeast *Kluyveromyces marxianus* [38]. A 46% amino acids identity was found between Pho610 and the phosphate repressible acid-phosphatase PhoA from *P. chrysogenum*. In addition, Pho610 was found to be GPI-anchored. This suggests a similarity between the *A. fumigatus* PhoAp found in this study and the *Kluyveromyces* enzyme.

This proteome study in *A. fumigatus* suggests that GPI-anchored proteins common to both yeast and filamentous fungi such as CRHs and ECM33 could play a similar enzymatic role in the construction of the fungal cell wall of both yeast and moulds since (i) membrane enzymes involved in cell wall biosynthesis such as *FKS*, *CHS*, *AGS*, *GEL*, have a very conserved function among yeast and moulds, and (ii) the structure of the polysaccharidic core of filamentous fungi and yeasts is very conserved [2, 39]. Disruption of the genes encoding these GPI-proteins in *A. fumigatus* and production of recombinant proteins are presently undertaken to investigate the enzymatic role of these GPI-anchored proteins in the organization of the cell wall of *A. fumigatus*.

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