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The incorporation of mannoproteins in the cell wall of *S. cerevisiae* and filamentous *Ascomycetes*

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Abstract

In yeast, glucanase extractable cell wall proteins are anchored to the plasma membrane at an intermediate stage in their biogenesis via a glycosylphosphatidylinositol (GPI) moiety before they become anchored to the wall glucan via a β1,6-glucan linkage. The mechanism of the membrane processing step of cell wall proteins is not known.

Here, we report that *Ascomycete* filamentous fungi involved in food spoilage such as *Aspergillus*, *Paecilomyces* and *Penicillium*, also contain GPI membrane-anchored proteins some of which are processed by an endogenous phospholipase C activity. Furthermore, similar to the situation in yeast, their cell walls contain mannoproteins which are linked to the glucan backbone through a β1,6-glucan linkage. Interestingly, one mould which contains a significant amount of non covalently linked β1,6-glucosylated cell wall proteins, is much more sensitive towards β1,3-glucanases and membrane perturbing peptides than the others.

Introduction

The cell wall of the yeast *Saccharomyces cerevisiae* is a dynamic structure that plays a role in mating and flocculation, is a site of numerous enzymes (Klis et al., 1994) and also controls the passage of macromolecules. The components of the *S. cerevisiae* cell wall are well characterised. It consists mostly (>95%) of equal amounts of glucan and mannoproteins. A small amount (1-2%) of the wall consists of chitin, which is present mostly in bud scars and close to the cell envelope (Klis, 1994; Cid et al., 1995). Chitin is however an essential component of the *S. cerevisiae* cell wall as deletion of all three genes responsible for chitin synthesis is lethal (Bulawa, 1993; Shaw et al., 1991). Glucan consists of two forms: a linear β1,3-glucan, and a branched glucan containing β1,6 and β1,3 linkages. The glucan layer forms the backbone of the cell wall and is linked to both chitin and mannoproteins. The mannoproteins, which reside mainly in the very outer layer of the yeast cell wall, give this structure its active properties (see above) and have an important role in controlling the porosity of the cell wall (De Nobel et al., 1989, 1990; Brzobohaty & Kovar, 1986).

The mannoprotein layer itself has been extensively studied in recent years. One area in particular that has been focused on is the processing which occurs in the transit of the glucanase-extractable mannoproteins from inside the cell to the cell wall itself. As with all glycosylated proteins of eukaryotes, the addition of sugar residues is initiated in the endoplasmic reticulum (ER) followed by modification in the Golgi apparatus (see e.g. Lu et al., 1994). It is now known that after these processing steps mannoproteins which are to be covalently linked with the wall sugar polymers remain associated for some time with the plasma membrane via a glycosylphosphatidylinositol (GPI) anchor (Lu et al., 1994; see also Figure 1). Subsequently, processing of the GPI-anchor takes place. The part of the anchor that remains associated with the wall proteins is linked to β1,6-glucan which is in itself linked to β1,3-glucan (Kapteyn et al., 1996). The precise part of the GPI

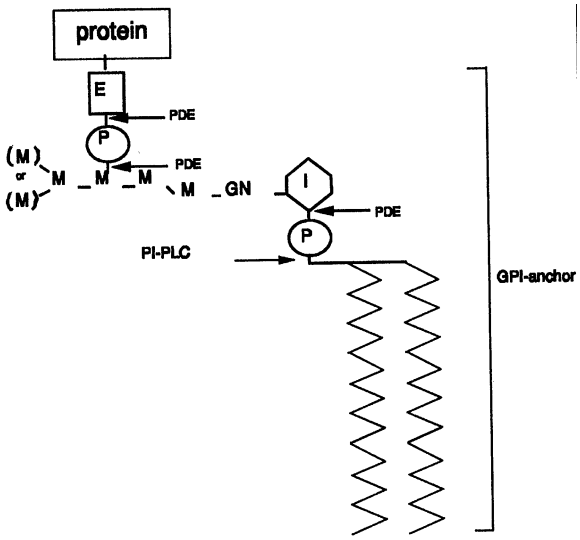

Figure 1. Typical structure of a *S. cerevisiae* protein attached to the plasma membrane via a glycosyl-phosphatidylinositiol (GPI) anchor structure. E=ethanolamine, P=phosphate, M=mannose, I=*myo*-inositol, GN=glucosamine, PI-PLC=phosphatidylinositol-phospholipas e-C, PDE=phosphodiesterase. Cleavage sites of the PI-PLC and PDE are also shown. The major epitope of the anti-CRD antiserum is exposed upon PI-PLC cleavage. (see also Zamze et al., 1988).

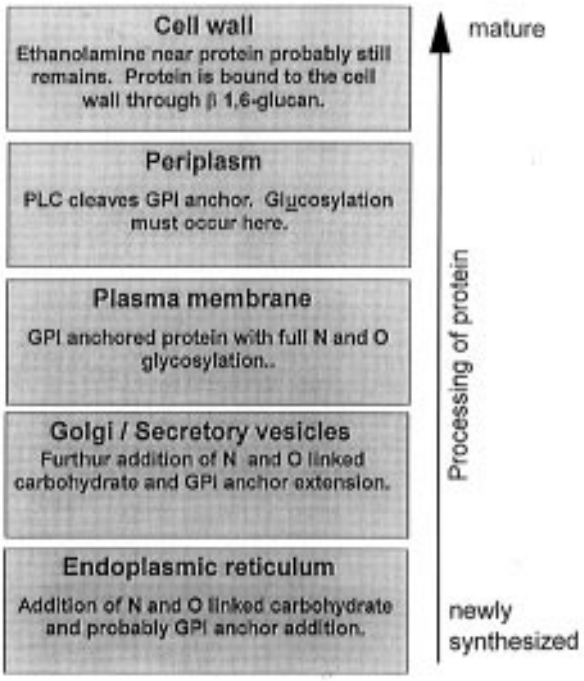

Figure 2. Proposed processing events of glucanase extractable cell wall mannoproteins in *S. cerevisiae*. Processing in the endoplasmic reticulum (ER) and the Golgi apparatus occurs in the same way as for secretory proteins. Addition of β1,6-glucose units must occur after release from the membrane (Lu et al., 1994).

anchor structure that remains in the mature protein is still matter of debate. Müller et al., (1996) suggest that both ethanolamine and *myo*-inositol are removed in a two step process involving phospholipase C (PLC) activity and a proteolytic step. Kapteyn et al., (1996) believe that the ethanolamine remains, which is consistent with the fact that proteins can be removed from the cell wall by aqueous HF treatment (which cleaves phosphodiester bonds). Similar results have been obtained with the fungal pathogen *Candida albicans* (Kapteyn et al., 1994, 1995a,b). Figure 2 summarizes the proposed processing events of the glucanase extractable yeast wall mannoproteins.

Cell walls of filamentous fungi generally contain more chitin than walls of yeast, although interspecies variation in the actual amount occurs. Furthermore, α1,3-linked glucan is often found (Griffin, 1994). Proteins may contain galactose in addition to mannose on their *N*- and *O*- glycan chain (Griffin, 1994; Nakajima & Ichishima, 1994). To date very little study has been done on the biogenesis of cell wall proteins of filamentous fungi. GPI anchored proteins have been demonstrated in the slime mould *Dictyostelium discoidium* (Reymond et al., 1995). In the plant pathogen *Fusarium oxysporum* β1,6-glucan linked wall proteins have been identified (Schoffelmeer et al., 1996). We have selected three filamentous fungi, *Penicillium roqueforti*, *Paecilomyces variotii* and *Aspergillus niger*, which are all representative of species that cause food spoilage (Samson et al., 1995). Here we show that, similar to *Saccharomyces cerevisiae*, their cell walls contain GPI-anchored proteins which are linked with β1,6-glucan to the β1,3-glucan backbone. We also provide evidence that these wall proteins are processed at the plasma membrane by a phosphatidylinositol-specific phospholipase C.

Materials and methods

Strains and growth conditions

Yeast strains used: *Saccharomyces cerevisiae* SU51 (*ura*3, *his*4, *leu2*) and*S. cerevisiae mnn*9 (*ura*3, *his*3, *leu*2, *mnn*9), only capable of core *N*-glycosylation (Ballou, 1990). The filamentous fungi used were *Aspergillus niger* van Tiegham (CBS 109.30), *Pae-*

cilomyces variotii Banier (CBS 338.51) and *Penicillium roqueforti* Thom (CBS 221.30). Additionally, all experiments were performed at least once with similar food-product isolates. For wall and membrane analysis, log phase yeast cultures were prepared by repeated subculturing in fresh Malt Extract Broth (MEB, Difco). The other fungi were grown overnight in MEB. Conidia were taken from -80 °C spore stocks (inoculation level 10^6 CFU/ml). For the generation of protoplasts, minimal medium (0.17% yeast nitrogen base (Difco), 0.5% $(NH_4)_2SO_4$ and 2% glucose) was used. The original culture of cells was carried out in a 200-ml volume whilst the regeneration of protoplasts was in 5-ml volumes. Cultures were shaken at 200 rpm, while protoplast regeneration was performed at 85 rpm.

Protoplast formation and regeneration

Mycelia grown in minimal media were harvested through Miracloth (Calbiochem). After removal of excess liquid the cell mass was transferred to a solution of 1M sorbitol in 10mm Tris-HCl (pH 7.4) containing 25mg. ml^{-1} of Novozym 234 (Novo Nordisk). This was done at a ratio of 20ml.g^{-1} of cells. The mycelia were then incubated at 30 °C for 2 hours at 85 rpm. Protoplasts were recovered by filtration through Miracloth followed by 2 rounds of centrifugation at 1,000 g. Protoplasts, washed once in 1M sorbitol, were then allowed to regenerate in 5 ml minimal medium stabilised with 1M sorbitol, both in the presence and absence of 25μg. ml^{-1} tunicamycin (Sigma). Periodically, protoplast stability was tested by plating onto malt agar, with and without 1M sorbitol. Supernatants were collected and concentrated by precipitation (see below). Proteins recovered from the 5-ml incubations were resuspended in 200μ1 polyacrylamide gelelectrophoresis (PAGE) loading buffer and subsequently analysed by SDS-PAGE and Western blot.

Preparation of cell walls, membranes and wall protein extraction

Yeast strains were harvested by centrifugation at 2500*g* for 10 minutes and washed once in 10mM Tris-HCl (pH 7.8) plus 1mM PMSF. Filamentous fungi were harvested via filtration through a 0.2μm cellulose/mixed ester filter membrane (Schleicher & Schuell GmbH) and washed with the same buffer. The filamentous fungi were then ground lightly under liquid nitrogen using a mortar and pestle. Cells were completely broken by shaking with glass beads of 450-625μm diameter. Subsequently, cell walls and cell membranes were isolated as described by Schreuder et al., (1993). Procedures for treating cell walls with SDS and Mollusc laminarinase (Sigma) were essentially as in Schreuder et al., (1993). The first SDS extraction was in a 2:1 volume to weight ratio of SDS extraction buffer to cell walls. The second SDS extraction was carried out with 10ml of extraction buffer, regardless of the cell wall weight. A range of laminarinase concentrations were used (see results). Mock treatments with no laminarinase were also included. For treatment with pure β1,3-glucanase, Quantazyme *ylg* (Quantum Biotechnologies Inc.) was used as in Kapteyn et al., (1995b).

Phosphatidylinositol-Phospholipase C (PI-PLC) treatment of cell membranes

Membranes (from 100ml cultures) were resuspended in 200μ1 of 100mM NaCl in 50mM Tris-HCl (pH 7.5). The samples were subsequently split into two portions. One portion was treated for 1 hour at 37 °C with 0.1U of *Bacillus cereus* PI-PLC (Boehringer Mannheim Biochemica). After centrifugation at 14,000 rpm in a micro-centrifuge, the supernatants were retained. The other portion was treated in exactly the same manner, except that no PI-PLC was included.

Protein analysis

Proteins were precipitated with methanol as described by Wessel & Flügge (1984). This method was used both for concentration of proteins from protoplast experiments and buffer exchange for the various enzymatic treatments.

Phosphodiesterase I (PDE I, bovine intestinal mucosa, Sigma) treatment of proteins was carried out as in Müller et al. (1996), using 0.25U of the enzyme in 50 μ1 of protein solution. For HF treatment, proteins were precipitated and then treated with 50% aqueous HF for 72 hours as described by Ferguson (1992).

Finally, proteins were digested with endoglycosidase-H (Endo-H, Boehringer Mannheim GmbH) for 72 hours at 37 °C using 4mU of Endo-H per 20 μ1 of sample in 100mM Na-acetate (pH 5.0).

SDS PAGE and immunoblotting

SDS PAGE was carried out with 4–20% gradient Laemmli gels (Laemmli, 1970) using Bio-Rad Mini-Protean II equipment, and Silver staining was done as described by De Nobel et al., (1989). Immunoblot-

ting was performed according to Towbin et al. (1979) using polyvinylidene-difluoride membranes (Immobilon P, Millipore). After blotting overnight at 30V constant at 4 °C, the membranes were blocked with 5% skimmed milk powder in phosphate buffered saline (PBS, 1% NaCl, 0.225% $Na_2H_2PO_4.2H_2O$ and 0.03% NaH_2PO_4). Subsequently, the filters were incubated with either a 1 in 2000 dilution of rabbit anti-β 1,6-glucan (gift of Roy Montijn) or a 1 in 4000 dilution of rabbit anti-CRD (Zamze et al., 1988, Oxford Glyco Systems). As secondary antibody we used horse radish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Sigma, 1:2000 dilution). The blotting membrane was incubated with both the primary and the secondary antibodies for two hours in 3% bovine serum albumin containing PBS. Detection was carried out using chemiluminescence and Amersham ECL Western blotting reagents according to the manufacturers instructions. X-ray film was from Fuji. All experiments were performed at least in duplicate.

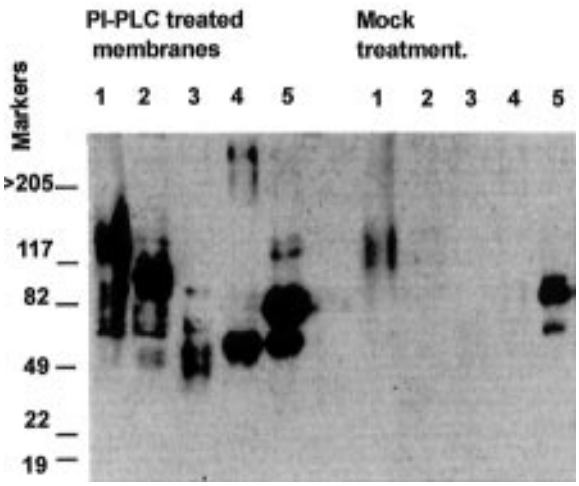

Figure 3. Membrane-bound GPI proteins in filamentous fungi. Protein bands located with anti-CRD on an immunoblot of a 4–20% SDS-PAGE gel of membrane proteins. The left hand side of the blot shows the proteins cleaved by the phospholipase enzyme and the right hand side the control treatment with no phospholipase treatment. The middle lane is loaded with the PI-PLC at the concentration used (no bands visible). Lane 1 =*Saccharomyces cerevisiae* wild type, 2 =*S. cerevisiae mnn*9, 3 = *Penicillium roqueforti*, 4 = *Paecilomyces variotii* and 5 = *Aspergillus niger*.

Results

Cell membrane experiments

Isolated cell membranes of the fungal strains were treated with phospholipase C, which cleaves the phosphodiester bond nearest to the membrane, leaving a cyclic phosphate on the *myo*-inositol. This epitope is recognized by the anti-CRD antibody (Figure 1). The results of an analysis of proteins liberated from the membranes by PI-PLC treatment followed by a reaction with the anti-CRD antiserum are shown in Figure 3. All of the strains tested contained proteins which could be liberated from membranes by phospholipase C. For wild-type *S. cerevisiae* and *A. niger*, some proteins could be visualised on the Western blot of the mock treatments suggesting the involvement of an endogenous PLC activity in processing GPI-anchored proteins. A similar blot, developed using the anti β1,6-glucan antiserum gave no reaction (data not shown). In conclusion, GPI-anchored proteins are present in membranes of filamentous fungi.

Protoplast regeneration

Kapteyn et al., (1995a) used regenerating protoplasts to show that a class of cell wall proteins is secreted in the early stages of wall regeneration, due to the absence of glucan to which they are attached normally. Similar experiments were carried out with *P. variotii* and *A. niger* to test which proteins are secreted and thus are candidate cell wall proteins. This was done with and without the addition of 25μg. ml^{-1} tunicamycin, which is an inhibitor of *N*-linked glycosylation. A typical result is shown in Figure 4 for *P. variotii*. Bands corresponding to high molecular weight proteins and a distinct species of $\approx$60 kDa were present in both the PI-PLC treated membrane fraction and in the regeneration medium of protoplasts. The latter contained additionally a band of $\approx$30 kDa. There is a clear time dependence in the concentration of these proteins in the incubations without tunicamycin indicating that they are actively secreted. While the secretion of the proteins seems to be enhanced by tunicamycin, neither the 60 kDa nor the 30 kDa band are altered in molecular weight by the presence of the drug.

In order to explain these observations we favour the thesis that at least some *P. variotii* GPI anchored membrane proteins are processed by a PI-PLC and subsequently secreted. Their processing does not depend on N-glycosylation. We presume that these proteins may be incorporated in the mycelial wall (see also Discussion).

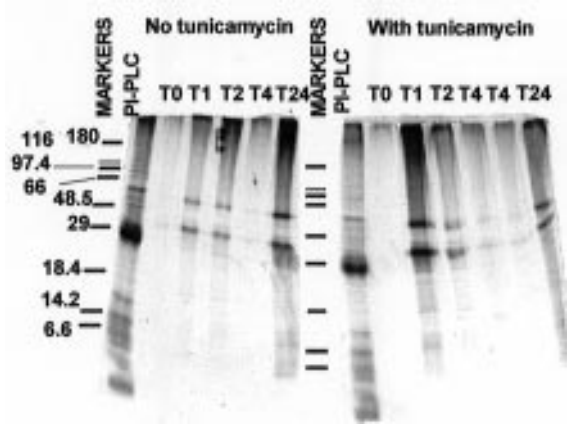

Figure 4. Protein profile of the medium of regenerating protoplasts of *Paecilomyces variotii*. Proteins were recovered from the supernatants of regenerating *P. variotii* protoplasts between 0 hours (T0) and 24 hours (T24), with and without the addition of 25μg/ml tunicamycin and separated on 4–20% gradient gels. PI-PLC cleaved membrane proteins have also been loaded. The gels were silver stained. The T4 samples contained less protein due to a partial loss of the samples.

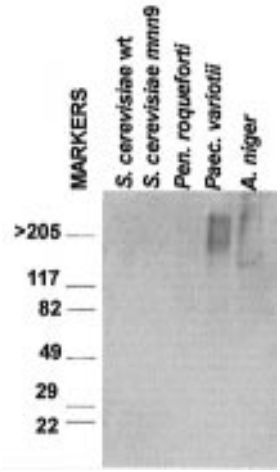

Figure 5. β-Glucosylated SDS-extractable cell wall proteins. Hot SDS / β-mercaptoethanol-extracted cell wall proteins were separated on a 4–20% SDS PAGE gel and then immunoblotted with an anti-β1,6-glucan antiserum.

Interestingly, Western analysis indicated that, when significant amounts of secreted proteins are present in the culture media, a CRD epitope can be detected on these proteins irrespective of the presence of tunicamycin (not shown). This is in accordance with the proposed involvement of a PLC activity in wall protein processing. At all time-points the intactness of the protoplasts was microscopically verified.

For *A. niger*, essentially similar results were obtained. Neither supernatants from *P. variotii* nor from *A. niger* contained proteins which could be detected with the anti-β1,6-glucan antibody (data not shown).

Analysis of cell wall mannoproteins

The cell walls were examined for the presence of mannoproteins using extraction with Mollusc laminarinase, which has primarily β1,3-glucanase activity (90%) apart from β1,6-glucanase, α-mannosidase and proteinase activity. Before laminarinase treatment, non-covalently bound proteins and proteins bound only through disulphide bonds were removed by hot SDS / β-mercaptoethanol extraction. Subsequently, the first SDS extracts of all strains were immunoblotted and the presence of any β1,6-glucan epitope was investigated. *P. variotii* and to a lower extent *A. niger* SDS extracts appear to contain proteins $>$ 150 kDa with β1,6-glucan attached to them (Figure 5). Laminarinase extraction of walls was performed with a range of concentrations. Figure 6 shows the results of the Western blot analysis. With the exception of *P. variotti*, extracts of all strains tested contained proteins which reacted with anti-β 1,6 glucan sera. Thus these strains contained proteins linked to the β1,3-glucan cell wall via a β1,6-glucose structure. The absence of covalently β1,6-glucan linked wall proteins is in accordance with the fact that *P. variotii* is very sensitive to β1,3-glucanase treatment. Wild type *S. cerevisiae* reacted less strongly than the *mnn*9 mutant. This could be due to less efficient transfer during blotting of the wild type proteins caused by heavy glycosylation (which decreases transfer efficiency). The diffuse appearance of most of the bands is caused by their heterogeneous sugar content. The positive control is not heavily glycosylated and gives a distinct band of 67kDa (Van der Vaart et al., 1996). Anti-β1,6-glucan did not bind to laminarinase itself (fig 6a) and this enzyme cocktail therefore contains none of the β1,6-glucan epitope.

The laminarinase extracts were pooled, separated by SDS PAGE, transferred to a membrane for Western blot analysis and probed with an anti-CRD antiserum (Figure 7). All of the samples tested contained proteins which reacted with the anti-CRD antiserum. For *P. roqueforti*, proteins extracted with different concentrations of laminarinase reacted with the anti-CRD antiserum proportionally to the amount of enzyme used (not shown). As proof that a phosphodiester bond is involved, a digest of *S. cerevisiae* cell wall proteins

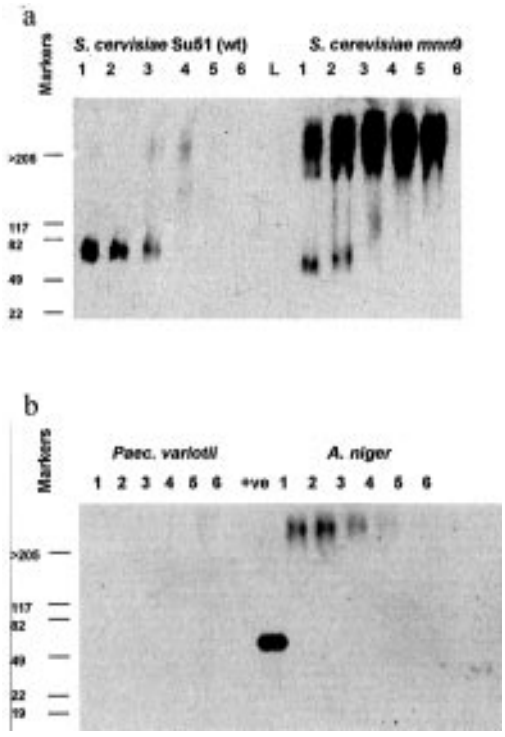

Figure 6. Anti β1,6-glucan reactive cell wall proteins. Cell wall proteins were extracted with varying concentrations of laminarinase, separated on 4–20% gradient gels , transferred to membranes and probed with an anti β1,6-glucan antiserum. (a) Laminarinase digests for the yeasts, and (b) for *P. variotii* and *A. niger*. Laminarinase concentrations used were as follows; 1 = 0.25mU mg^{-1}of cell wall (in 2μ1 buffer), 2 = 0.125mU, 3 = 0.05mU, 4 = 0.025mU, 5 = 0.0125mU and 6 = no laminarinase (mock digestion). A positive control (+ve)$*$ is shown in Figure 6b and laminarinase, at the highest concentration used, (L) in Figure 6a. $*$The positive control is a fusion protein of *S. cerevisiae* cell wall protein 2 and α-galactosidase (Van Der Vaart et al., 1996).

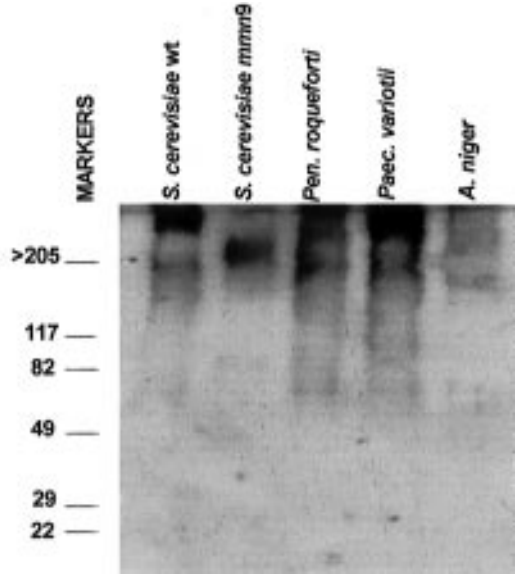

Figure 7. Anti-CRD reactive cell wall proteins. Pooled laminarinase digests of cell walls were separated on a 4–20% gradient gel and immunoblotted. Proteins were located with an anti-CRD antiserum.

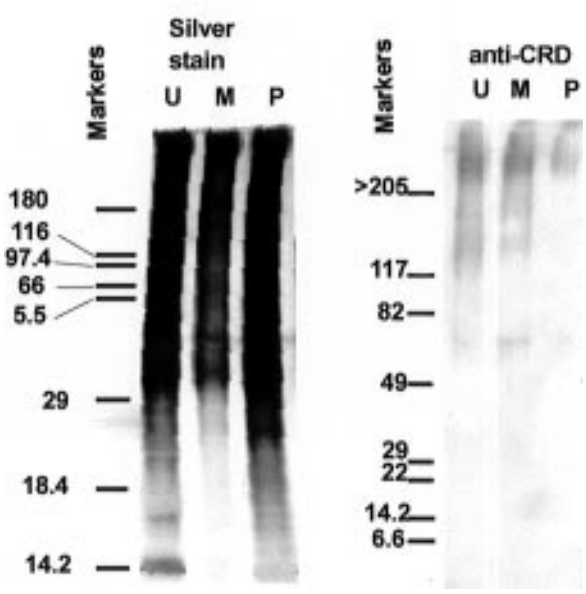

Figure 8. Phosphodiesterase treatment of CRD positive yeast wall proteins. Pooled laminarinase extracts of cell walls from a *S. cerevisiae* wild type strain were separated on a 4–20% SDS PAGE gel, silver stained or immunoblotted and then developed with anti-CRD antibodies. U = untreated samples, m = mock treated and P = phosphodiesterase I treated samples.

was carried out with phophodiesterase I (PDE I), which cuts various phosphate bonds in the GPI anchor (see Figure 1). Figure 8 shows that with the anti-CRD antiserum, bands of $>$205, $\approx$150 and 60 kDa were detected. Also with anti β1,6-glucan antisera similar proteins bands were recognized (not shown). The amount of these proteins which can be visualised is less in the lanes for the PDE I treated proteins. In summary, these data point to a class of proteins which (a) can not be removed from the cell wall by hot SDS / β-mercaptoethanol treatment, (b) have been cleaved by a phospholipase C enzyme and (c) react to the anti-β 1,6-glucan and/or anti-CRD antisera. Laminarinase does not react with the anti-CRD antibody (not shown).

In addition to laminarinase, Quantazym *ylg* a pure β1,3-glucanase, was also used to extract cell walls. The proteins released by the activity of this enzyme were also subjected to Western analysis with both the anti-CRD and anti β1,6-glucan sera. The results are

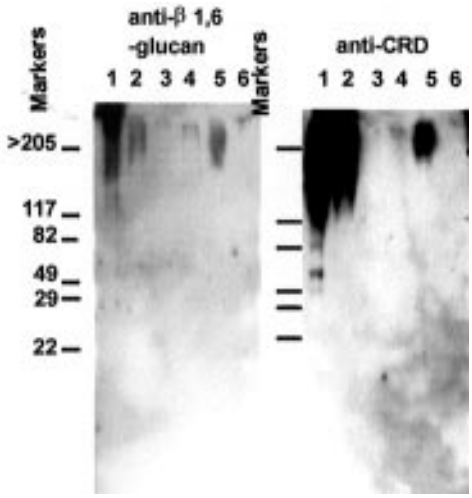

Figure 9. Quantazym*ylg* digests of fungal walls. *S. cerevisiae* wild type (1), *S. cerevisiae mnn*9 (2), *P. roqueforti* (3), *P. variotii* (4) and *A. niger* (5) cell walls were extracted with 0.6 U.mg^{-1} Quantazym *ygl*, separated on a 4–20% gradient gel, immunoblotted and developed with anti-β1,6-glucan and anti-CRD antisera. Positions of molecular weight markers are also shown (kDa). Lane 6 shows a mock digest of *S. cerevisiae* wild type.

shown in Figure 9. Quantazym *ylg* released cell wall proteins from isolated walls of both yeast strains and *A. niger*, which reacted with both anti-β1,6-glucan and anti-CRD antisera. In contrast to the experiment described in Figure 6, here also for wild-type yeast high molecular weight proteins were observed on the blot and smearing of the other samples was equal or less.

In order to analyse the nature of the linkage between the cell wall proteins and the β1,6-glucose with which they are attached to the wall, the Quantazym *ylg* released cell wall proteins were treated with Endo-H, which removes N-linked sugars. Upon endo-H digestion β1,6-glucan antiserum still recognized the wall proteins both in *S. cerevisiae* and in *A. niger*. There was, however, a shift in molecular weight of the proteins indicating that these proteins contain *N*-linked sugars (not shown).

Discussion

Using either laminarinase or pure β1,3-glucanase, it was possible to liberate proteins from the cell walls of all of the fungi analysed. These proteins were usually of high molecular weight and migrated as diffuse smears, indicating a high degree of glycosylation. In samples of *S. cerevisiae* wild-type and mnn9 mutants, and in *A. niger*, the level of smearing was lower or equal in the proteins released with the recombinant β1,3-glucanase Quantazym *ylg*, than in proteins released with laminarinase which is a mixture of β1,3- and β1,6-glucanase. This suggests that heterogeneity in β1,3-glucan is mainly responsible for the smearing of the proteins. The poor release of wall proteins of *P. roqueforti* by Quantazym *ygl* compared to laminarinase could indicate an impaired accessibility of the β1,3-glucan due to extensive β1,6-glucan (see e.g. Kapteyn et al., 1996). In *Paecilomyces variotii* and to some extent *A. niger*, some β1,6-glucosylated proteins could also be released by SDS / β-mercaptoethanol extraction. These proteins may represent cell wall proteins which have been freed by endogenous glucanase activity, or proteins which have become β1,6 glucosylated, but not yet covelently cross-linked into the wall β1,3-glucan. Indeed, *P. variotii* is more sensitive to β1,3-glucanases than the other fungi (see also chapter 3 in Montijn, 1996).

Interestingly, the results suggest that a PI-PLC activity is involved in targeting of fungal wall proteins (Figures 3, 4, 7, 8). This means that there is the potential for a similar cell wall protein processing system existing across a broad range of fungi. Indeed, proteins from glucanase cell wall digestions could be visualised with anti-CRD in all fungal extracts tested. Previous work on α-agglutinin (Lu et al., 1994, 1996) and a cell wall cAMP binding protein (Muller et al., 1996) has suggested that *myo*-inositol is removed from the cell wall proteins prior to cell wall attachment. Results presented here however, show that this observation cannot be generalised. In the laminarinase digests of *Penicillium roqueforti*, the intensity of the anti-CRD reaction is proportional to the level of the enzyme cocktail used. It is thus likely that these proteins were liberated from the cell wall by laminarinase activity, rather than being artifacts of the cell fractionation procedure (e.g. cell membrane proteins). Van der Vaart et al., (1996), reported that after removal of loosely associated membrane proteins by Na-deoxycholate treatment, the remaining membrane bound GPI-anchored proteins would not react with the anti-CRD antibody until treated with PI-PLC. This proves the absolute requirement for PLC processing of the GPI-anchor for a positive reaction with the anti-CRD antiserum to occur. In the current paper, cleavage of phosphodiester bonds in the *S. cerevisiae* proteins with PDE I resulted in a decrease in epitope detection. It is therefore concluded that the detection by the anti-CRD antisera of the

enzyme-liberated cell wall proteins, is caused by the presence of the *myo*-inositol with a cyclic phosphate attached, rather than non-specific binding. The conversion of cell membrane forms of most GPI anchored proteins to cell wall bound forms thus seems to require the activity of a phospholipase C type enzyme. A phospholipase C, Plc1p, has been discovered in *S. cerevisiae*, but protoplasts of a temperature sensitive knockout mutant did not secrete less cell wall proteins at the non-permissive temperature (J. M. Van der Vaart, personal communication). A PI-PLC enzyme involved in cell wall protein processing therefore still remains to be identified. Finally, a possibility is that the proteins detected by the CRD antiserum represent intermediate processing products. This hypothesis will be evaluated using radioactive pulse-chase experiments with ^{35}S-methionine and ^{3}H-inositol. *N*-glycosylation is not responsible for the linkage of the cell wall proteins to glucan. This was already known for *S. cerevisiae*, but not for filamentous fungi. Removal of *N*-linked oligosaccharides with endo-H does not result in removal of the β1,6-glucan epitope in *A. niger*. Recent experiments by Van der Vaart et al. (in press) also rule out *O*-glycosylation mediated cell wall linkage of proteins in bakers yeast. In summary, cell wall mannoproteins in major filamentous *Ascomycete* fungi are processed at the plasma membrane, and linked to the cell wall using similar mechanisms to those found in yeast. The scheme outlined in Figure 2 seems therefore to be generally applicable. The processing steps constitute optimal targets for novel antifungal systems (Schoffelmeer et al., 1996).

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