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Characterisation of CwpA, a putative glycosylphosphatidylinositol-anchored cell wall mannoprotein in the filamentous fungus *Aspergillus niger*

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Abstract

Glycosylphosphatidylinositol (GPI)-anchored proteins in fungi are found at the cell surface, either as plasma membrane proteins (GPI-PMPs) or attached by a remnant of the GPI-anchor to the cell wall (GPI-CWPs). GPI-CWPs can be extracted from the cell wall by treatment with hydrofluoric acid (HF), which cleaves the phosphodiester bond that is present in the remnant of the GPI-anchor. The filamentous fungus *Aspergillus niger* contains at least seven HF-extractable cell wall mannoproteins. One gene encoding an HF-extractable cell wall mannoprotein, *cwpA*, was cloned and further characterised. The protein sequence of CwpA indicated the presence of two hydrophobic signal sequences both at the N-terminus and C-terminus of the protein, for entering the ER and the addition of a GPI-anchor, respectively. A CwpA-specific antiserum was raised and in combination with fractionation experiments, we show that this protein was abundantly present as an HF-extractable protein in the cell wall of *A. niger*.

Keywords: Cell wall integrity; GPI-anchoring; Calcofluor White; Hypersensitive; HF-extractions; ω-site; Glucan

1. Introduction

The cell wall of a fungus is an intriguing component of the fungal cell. It not only determines the shape of the cell, it also protects the cell from the harsh external environment and is the site at which the fungus physically interacts with its environment. Electron microscopic images of fungal cell walls have revealed a layered structure. The outer, electron dense layer is composed of cell wall mannoproteins (Hazen and Hazen, 1992; Zlotnik et al., 1984). The inner, electron transparent layer is composed mainly of glucan (β-1,3- and β-1,6-glucans) and chitin which acts as a scaffold for exposing cell wall mannoproteins (Kollar et al., 1997; Zlotnik et al., 1984). The cell walls of filamentous fungi consist of the same basic components as the cell walls of yeasts (mannoproteins, β-glucans, and chitin), but generally contain more chitin (10–15% chitin instead of 1–2% chitin in the yeast cell wall) and can contain additional polymers such as α-1,3-glucan or α-1,3-α-1,4 glucan (Fontaine et al., 2000; Schoffelmeer et al., 1999). The composition and structure of the cell wall are not static but are modified in response to changes in the environment (Klis et al., 2002). The surface properties of fungi are primarily determined by the presence of cell wall mannoproteins (reviewed by De Groot et al., 2005). Cell wall mannoproteins are grouped in different classes based on their extractability and

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proposed linkage: (i) SDS-extractable cell wall mannoproteins which are bound to the cell wall by hydrogen bonds, (ii) β-mercaptoethanol/DTT-extractable cell wall mannoproteins which are attached covalently to the cell wall by disulphide bonds (Cappellaro et al., 1994, 1998) or (iii) cell wall mannoproteins that are covalently linked to the glucan part of the cell wall. Two different classes of glucan-linked cell wall mannoproteins have been described for *Saccharomyces cerevisiae*. The first class of glucan-linked cell wall mannoproteins consists of members that belong to the protein with internal repeats (PIR)-family (Toh-e et al., 1993). These proteins can be liberated from the cell wall after mild-alkali treatment (Mrsa et al., 1997). The repeats consist of a glutamine-rich consensus sequence (Q-I/V-X-D-G-Q-I/V/P-Q) and the number of repeats varies between the different PIR proteins (Toh-e et al., 1993). Pir4p/Ccw5p contains only a single PIR motif and it has been shown that this sequence is required for the covalent linkage of Pir4p/Ccw5p to the cell wall (Castillo et al., 2003). PIR proteins are most likely linked to the β-1,3-glucan part of the cell wall. However, the nature of this linkage has not yet been established (Castillo et al., 2003; Mrsa and Tanner, 1999). The second class of glucan-linked cell wall mannoproteins is attached to the cell wall through glycosylphosphatidylinositol (GPI) linkages (Kapteyn et al., 1995, 1996; Lu et al., 1994; Montijn et al., 1994). GPI-anchored cell wall proteins (GPI-CWPs) contain a hydrophobic sequence at their C-terminus that acts as a GPI-anchoring signal. GPI-anchor addition takes place in the endoplasmic reticulum (ER) where the hydrophobic domain is replaced by the pre-assembled GPI-anchor (Orlean, 1997). After transport through the secretory pathway and arrival at the plasma membrane the GPI-anchor is processed and attached to β-1,6-glucan (Kollar et al., 1997; Montijn et al., 1994). GPI-CWPs are further characterised by the presence of a hydrophobic N-terminal signal sequence for import into the ER, and are often heavily O-glycosylated. GPI-CWPs can be removed from the cell wall by enzymatic and chemical treatments. Both β-1,3- and β-1,6-glucanases liberate GPI-CWPs from the cell wall (Kapteyn et al., 1996). In addition, GPI-CWPs can be extracted from the cell wall by treatment with hydrofluoric acid (HF), which cleaves the phosphodiester bonds in the GPI-anchor (de Groot et al., 2004; Kapteyn et al., 1996).

Cell–cell or cell–surface interactions of the fungal cell are often determined by the presence of GPI-CWPs. In *S. cerevisiae*, flocculation and agglutination properties are mediated via the flocculins and agglutinins, respectively, and these proteins are exposed and bound to the cell surface of the cell wall through their GPI-anchor (Bony et al., 1997; Lu et al., 1994; Teunissen and Steensma, 1995). ALS proteins in *Candida albicans* and Epa1p in *Candida glabrata* are GPI-anchored cell wall proteins that are important for mediating the adhesion properties of the *Candida* cell to human epithelial cells (Cormack et al., 1999; Frieman et al., 2002; Hoyer, 2001; Klis et al., 2001; Sundstrom, 2002). In addition to a few examples of cell wall proteins with a clear biologically relevant function, numerous GPI-CWPs have been identified whose function is not yet clear (Klis et al., 2002). In general, they are considered to be cell wall proteins with a structural role. Interestingly, the transcription of genes encoding GPI-CWPs is highly regulated in response to internal and external factors. Progression through the cell cycle is accompanied by a regulated expression of several genes encoding GPI-CWPs (Caro et al., 1998; Smits et al., 1999; Spellman et al., 1998). Expression of GPI-CWPs encoding genes is also highly regulated in response to different forms of environmental stress. Cell wall stress inducing conditions (Jung and Levin, 1999; Ram et al., 1998a; Terashima et al., 2000), high osmolarity (Gasch et al., 2000; Posas et al., 2000; Rep et al., 2000), extreme pH (Kapteyn et al., 2001; Lamb et al., 2001), anaerobic growth conditions (Abramova et al., 2001), and entering the stationary growth phase (Puig and Perez-Ortin, 2000; Shimoi et al., 1998), all have been shown to increase the expression level of a number of GPI-CWP encoding genes. The induced expression of certain GPI-CWPs (like Cwp1p) is not limited to a specific growth condition indicating that their expression might be under the control of different transcriptional activating networks. The genes encoding members of the PIR protein family are also induced by a wide range of environmental stimuli (Jung and Levin, 1999; Lagorce et al., 2003; Toh-e et al., 1993).

GPI-CWPs have been identified and characterised most extensively in *S. cerevisiae* and *Candida* ssp., but also recently in *Yarrowia lipolytica* (Jaafar and Zueco, 2004). For filamentous fungi, the presence of a GPI-CWP (Fem1p) has only been reported in the cell wall of the plant-pathogenic fungus *Fusarium oxysporum* (Schoffelmeer et al., 1999, 2001). In this paper, we have cloned a gene encoding an HF-extractable cell wall mannoprotein and named the gene *cwpA*. As expected for an HF-extractable cell wall mannoprotein, the protein contains a putative GPI-anchor addition signal and demonstrates further conservation of the presence of GPI-linked cell wall mannoproteins in filamentous fungi.

2. Materials and methods

2.1. Strains, culture conditions, and transformations

The *Aspergillus niger* strains used in this study are N402 (a *cspA1* derivative of ATCC9029, Bos et al., 1988) and the *pyrG* negative derivative of N402, AB4.1 (van Hartingsveldt et al., 1987). Strains were grown in *Aspergillus* minimal medium (MM) (Bennett and Lasure, 1991) or *Aspergillus* complete medium (CM) which

contains the same compounds as minimal medium with the addition of 10 g L^{-1} yeast extract and 5 g L^{-1} casamino acids. When required the growth medium was supplemented with 10 mM uridine (Serva). Transformation of *A. niger* was carried out as described by Punt and van den Hondel (1992) using lysing enzymes (L1412, Sigma) for the formation of protoplasts. Conidiospores were obtained by harvesting spores from a CM-plate after 4–6 days of growth at 30 °C, using a 0.9% NaCl solution. The bacterial strain used for transformation and amplification of recombinant DNA was *Escherichia coli* XL1-Blue or DH5α. Bacterial transformations were performed according to the heat shock protocol as described by Inoue et al. (1990).

2.2. Molecular biological techniques

Small-scale chromosomal DNA isolations for PCR screening of transformants were performed using a FastPrep FP120 (Bio101). *A. niger* spores were transferred to FastPrep tubes containing 1 ml of CM and 0.3 g acid washed glass beads (Ø 400–600 μm, Sigma). After growth for 16 h at 37 °C, the mycelium was spun down and the medium was removed. Subsequently, 500 μl cold extraction solution (2:2:1 mixed, TNS, 40 mM tri-isonaphthalene sulphonic acid, PAS, 0.70 M *p*-aminosalicyclic acid, and RNB, 1.0 M Tris–HCl, pH 8.5, 1.25 M NaCl, and 0.25 M EDTA) and 500 μl phenol–chloroform–isoamyl alcohol (25:24:1 v/v %) were added. Vials were closed carefully and vigorously shaken two times for 30 s at speed 6.0 using a FastPrep120 grinder (Bio101) and cooled 5 min on ice between runs. When larger quantities of chromosomal DNA were required, as for Southern analysis, genomic DNA was isolated as described by Kolar et al. (1988). RNA was extracted from mycelium flash-frozen in liquid nitrogen using TRIzol reagent (Invitrogen). Both Southern and Northern blot analyses were carried out as described by Sambrook et al. (1989). Electrophoresis of RNA was performed as described (Damveld et al., 2005). [α-^{32}P]dCTP-labelled probes were synthesised using Rediprime II DNA labelling System (Amersham Pharmacia Biotech) according to the instructions of the manufacturer. PCR was performed on a PTC-100 Programmable Thermal Controller (MJ Research) using Super Taq (HT Biotechnology) or when required Expand High Fidelity PCR system (Roche). Primers were obtained from Isogen and are listed in Table 1. For ligation, the Rapid DNA Ligation Kit (Boehringer–Mannheim) was used. Sequencing was carried out with a Perkin-Elmer ABI PRISM 310 sequencer using the ABI prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). Restriction enzymes were obtained from Invitrogen and used according to the protocol supplied by the manufacturer.

Table 1
Primers used in this study

Primer name	Sequence 5′–3′[a]
AnCWPP1	taataacatacctaccactc
AnCWPP2	gagttgatctccgagacc
pyrGAO-*Nar*I-F	atggcgccgttgctcggtagctgatta
pyrGAO-*Nar*I-R	atggcgcccgatggataattgtgccg
F1590	gctatcatttcctttgatattgg
F1591	aaattaccggatcaattcgagc
cwpAp9	aacaacaatattcaggcctc
pAO9	aatgtcaattccagcagcg
cwpA-Rec1	ttttttggatccatggccaaccccaccaag
cwpA-Rec2	ttttttctcgagagccacgtccttgta
rodA1dw	ggagcactcctggaagagac
rodA1up	tcaccgctgctgttcttg
brlAdw	gcatgagatcttgggacttgg
brlAup	cagcttctcacccatggaatc

[a] Restriction sites are underlined.

2.3. Isolation of the cwpA gene from A. niger

We identified three cDNAs in the DDBJ/EMBL/GenBank databases with Accession Nos. BE759683, BE758883, and BE759871 (Tsang and Storms, unpublished data) which displayed a significant homology to the mannoproteins from *Penicillium marneffei* Mp1 (*E* value 4e−09) and *Aspergillus fumigatus* Mp1 (1e−12). Using these cDNA sequences, two primers were designed, AnCWPP1for and AnCWPP2rev (Table 1), which in combination with the vector primers F1590 and F1591 (Table 1) were used to amplify two overlapping parts of the *cwpA* gene from an *A. niger* cDNA library in pEMBLyex4 (Veldhuisen et al., 1997). The PCR fragments obtained were 0.4 (F1591 and AnCWPP2rev) and 1.2 kb (AnCWPP1for and F1590) in size and were cloned into pGEM-T Easy (Promega) and sequenced. Southern blot analysis on genomic DNA from *A. niger* showed that the *cwpA* gene was located on a 3 kb *Bgl*II fragment (data not shown). Genomic DNA was digested with *Bgl*II and fragments of approximately 3 kb were isolated from gel to ligate into a *Bam*HI opened pBluescript II SK vector (Stratagene). This *Bgl*II mini-library was transformed to *E. coli* and white colonies were transferred to new LB plates. Using the colony lift protocol from Hybond N+ (Amersham, see suppliers manual), approximately 200 colonies were lysed on Hydond N+ filters and subsequently hybridised with the *cwpA* cDNA fragment as a probe. One hybridising clone was identified and named pCwpA3.0 and sequenced. The *A. niger cwpA* nucleotide sequence data have been submitted to the DDBJ/EMBL/GenBank databases under Accession No. AY704270.

2.4. Construction of the cwpA::AopyrG deletion plasmid

To construct a plasmid for deletion of the *cwpA* gene, the *Aspergillus oryzae pyrG* gene was amplified using

primers pyrGAO-*Nar*I-F and pyrGAO-*Nar*I-R to introduce *Nar*I sites at both sides of the *pyrG* gene using pAO4-13 (de Ruiter-Jacobs et al., 1989) as a template. After amplification, the expected 1.7 kb fragment was cloned in pGEM-T Easy and transformed to *E. coli*. To confirm the functionality of the *pyrG* selection marker, the DNA obtained from small-scale DNA isolations was transformed to *A. niger* strain AB4.1. Transformation of plasmid pGEMT-PyrGAO-*Nar*I#2 resulted in the formation of uridine prototrophic transformants indicating that this plasmid contains a functional *AopyrG* gene. The *AopyrG* gene was isolated from pGEMT-PyrGAO-*Nar*I#2 as a 1.7 kb NarI fragment and cloned into the *Nar*I digested pCwpA3.0. Digestion of pCwpA3.0 with *Nar*I removed a 990 bp fragment, containing 610 bp of the *cwpA* promoter sequence and 380 bp of the *cwpA* coding region, which was then replaced with the 1.7 kb *Nar*I fragment containing the *AopyrG* gene, to give plasmid pΔ*cwpA*. The orientation of the insert was determined by *Eco*RV digestion. Constructs with both orientations were obtained and a plasmid, pΔcwpA#2, with the *AopyrG* gene oriented so that the coding strand was in the opposite direction to that of the *cwpA* that gene was used for transformation to *A. niger*. Before transformation to *A. niger* AB4.1, pΔcwpA#2 was linearised by a *Bgl*I digestion. Transformation of 25 μg linearised plasmid yielded 220 transformants. Transformants were screened by PCR using primers cwpAp9, located outside and downstream of the deletion cassette, and pAO9. Only transformants with a targeted deletion of the *cwpA* gene should result in the amplification of a 0.7 kb PCR fragment. PCR positive transformants were analysed by Southern blot analysis. Genomic DNA of two PCR positive transformants was digested with *Bgl*II and probed with a genomic *cwpA* fragment to confirm single copy, targeted integration of the deletion cassette (see also Fig. 4).

2.5. Growth conditions and developmental probes

To analyse the expression of *cwpA* in shake flask cultures, RNA samples were isolated from 50 ml CM cultures that were inoculated with 1×10^6 spores ml^{-1} and grown for 8, 16, 24, 32, and 64 h at 30 °C using Trizol reagent (Invitrogen). Parallel cultures were used for biomass determination and cell wall isolations. Mycelium was harvested using a sieve with a 20 μm aperture (Endecotts) and stored at −80 °C prior to RNA or cell wall isolation. The biomass of the parallel cultures was determined by harvesting the spores over myracloth filter (Omnilabo) and drying the mycelium at 70 °C.

Aspergillus niger sequences encoding BrlA and RodA proteins that were highly homologous to the *Aspergillus nidulans* orthologs (*E* values of e−159 and 1e−27, respectively) were kindly provided by DSM. The *A. niger brlA* and *rodA* homologues were isolated by PCR from *A. niger* with primers pairs brlAdw-brlAup and rodA1dw-rodA1up (Table 1) and cloned into pGEM-T Easy. The isolated fragments were confirmed by sequence analysis and the partial coding sequences were submitted to the DDBJ/EMBL/GenBank databases under Accession Nos. AY817176 (*brlA*) and AY817177 (*rodA*). The probes used are a 300 bp *Eco*RI fragment containing the 5′ region of the *cwpA* gene, an 1147 bp *Eco*RI fragment from pGEMT-brlA containing the *brlA* gene, a 314 bp *Eco*RI fragment from pGEMT-rodA containing a part of the *rodA* open reading frame, and an 18S ribosomal probe as loading control, isolated as a 2 kb *Bgl*I fragment from pMN1 (Borsuk et al., 1982).

2.6. Cell wall extractions

Cell walls were isolated by grinding frozen mycelium using a pestle and mortar, breakage (>95%) was confirmed by phase-contrast microscopy. Ground mycelium was lyophilised, weighed, and resuspended in 25 μl Tris buffer (0.05 M Tris–HCl, pH 7.8) mg^{-1} dry weight. In all cases, approximately 40 mg dry weight mycelium was used for extractions. The cytosolic fraction was separated from the cell walls and membranes by centrifugation at 13,000 rpm for 10 min at 4 °C. This fraction was stored at −20 °C as the cytosolic protein fraction (cyt). The ground mycelium was extracted again with 25 μl Tris buffer mg^{-1} dry weight and pelleted as described above. To remove residual cytosolic contaminants, membrane proteins, and disulphide-linked cell wall proteins, the pellets were boiled three times in 25 μl SDS-extraction buffer (50 mM Tris–HCl, pH 7.8, 2% w/v SDS, 0.1 M Na-EDTA, and 1.6 μl β-mercaptoethanol) mg^{-1} dry weight. The cell walls were pelleted after extractions as described above, and the supernatants were stored as SDS fractions (SDS1–3) (Montijn et al., 1994). Cell walls were washed six times with water, lyophilised, and weighed. Freeze-dried cell walls were incubated with 10 μl HF-pyridine mg^{-1} dry weight for 3 h at 0 °C (de Groot et al., 2004). After centrifugation, the supernatant containing the HF-extracted proteins was collected (in 100 μl aliquots) and proteins were precipitated by the addition of 9 volumes of 100% methanol buffer (100% v/v methanol, 50 mM Tris–HCl, pH 7.8) and subsequently incubated at 0 °C for 2 h. Precipitated proteins were collected by centrifugation (13,000 rpm, 10 min, at 4 °C). The pellet was washed three times with 90% methanol buffer (90% v/v methanol, 50 mM Tris–HCl, pH 7.8) and lyophilised. The HF-extracted proteins were dissolved in 500 μl of 1× sample buffer. Prior to loading on gel both cytosolic- and SDS-fractions were mixed in a 1:1 ratio with 2× sample buffer.

2.7. Western analysis

Protein samples (10 μl) were loaded on a 4–15% Tris–HCl SDS–PAGE gel (Bio-Rad, Art# 161-1104) and run on a Mini-Protean II Electrophoresis cell (Bio-Rad), according

to the supplier's manual. Each 10 µl protein sample that is loaded on SDS–PAGE corresponds to the amount of protein released by an extraction of 200 µg dry weight mycelium (comparable to 75 µg dry weight cell walls). Proteins were transferred overnight to polyvinylidene difluoride membrane (PVDF, Immobilon-P, Millipore) using a Mini Trans-Blot Electrophoretic Transfer cell (Bio-Rad), according the supplier's manual using 25 mM Tris–HCl, 192 mM glycine, and 20% v/v methanol, pH 8.3, as a blotting buffer. The PVDF membranes were blocked with 3% w/v BSA (Sigma–Aldrich, Buchs, Switzerland) dissolved in PBS with 0.1% v/v Tween 20. For immunological detection of CwpA, an antiserum was raised against recombinant CwpA (see below). Blots were incubated for 2 h with 500-fold diluted CwpA antiserum in PBS containing 3% w/v BSA and 0.1% v/v Tween 20. After incubation with the primary antibody, blots were washed twice in PBS with 0.1% v/v Tween 20 for 15 min. Subsequently, the blots were incubated for 2 h with a 2000-fold dilution of goat-anti-rabbit peroxidase-linked antibody (GARPO, Dako, Denmark) in PBS containing 3% w/v BSA and 0.1% v/v Tween 20. Alternatively, for the detection of mannoproteins the blots were incubated for 2 h with peroxidase-conjugated concanavalin A (conA-PO) which was diluted 1000-fold in PBS containing 2.5 mM $CaCl_2$, 2.5 mM $MnCl_2$, 3% w/v BSA, and 0.1% v/v Tween 20. All blots were washed twice in PBS with 0.1% v/v Tween 20 for 15 min prior to detection of peroxidase activity using an ECL-plus kit (Amersham Pharmacia Biotech, UK).

2.8. Raising antibodies against CwpA

For the generation of a CwpA-specific antiserum, part of CwpA (aa1–168) was expressed as a GST-fusion protein in *E. coli* strain BL21 (DE3) and cloned into pLysS. Two primers (cwpA-Rec1 and cwpA-Rec2) were designed to amplify a part of the CwpA gene using pCwpA3.0 as a template. The construction of plasmids and the expression and purification of GST-CwpA were performed by Fusion Antibodies, Belfast. Polyclonal antisera were obtained by the immunisation of a rabbit with the purified recombinant CwpA using a standard immunisation protocol from Harlan Sera-Lab, Leicestershire, UK. To remove non-specific antibodies, 1 ml of the antiserum was pre-absorbed with acetone powder, obtained from a protein extract of the *cwpA* deletion strain according to standard protocols (Harlow and Lane, 1988).

3. Results

3.1. HF-pyridine extraction of the cell wall of A. niger results in the release of putative GPI-CWPs

Extraction of fungal cells walls with aqueous HF or HF-pyridine has been shown to be a reliable method for the release of GPI-anchored cell wall mannoproteins (de Groot et al., 2004; Kapteyn et al., 1996; Schoffelmeer et al., 2001). HF-pyridine specifically cleaves phosphodiester bonds, through which GPI-CWPs are linked to the β-1,6-glucan component of the cell wall. Extensively SDS/β-mercaptoethanol-extracted cell walls of *A. niger* were incubated with HF-pyridine for 3 h on ice. HF-extracted proteins were detected by Western analysis using the lectin concanavalin A (Fig. 1A). At least seven distinct HF-pyridine extractable proteins could be identified with molecular masses of approximately 180, 130, 120, 75, 65, 55, and 40 kDa (Fig. 1A). In mock experiments, in which HF-pyridine was replaced by water, no significant amount of protein was released (data not shown). To clone any of the HF-pyridine extractable proteins, N-terminal sequencing was performed using the same method that was previously used to obtain the Fem1p N-terminal amino acid sequence (Schoffelmeer et al., 2001). An amino acid sequence of the 40 kDa protein was obtained (W-V-T-E-T-N-G-D-L), but attempts to clone the gene encoding this putative cell wall mannoprotein by PCR using degenerated primers were not successful and therefore an alternative approach was used to clone a gene encoding a cell wall protein from *A. niger*.

3.2. Isolation of the cwpA gene

HF-extractable cell wall mannoproteins obtain a GPI-anchor in the endoplasmic reticulum where the hydrophobic C-terminal part of the precursor protein is replaced by a pre-assembled GPI-anchor. Putative cell wall mannoproteins have been identified in *P. marneffei* Mp1, *A. fumigatus* Mp1, and *F. oxysporum* FEM1p (Cao et al., 1998; Schoffelmeer et al., 2001; Yuen et al., 2001) which contain a GPI-anchor addition signal at their C-terminus. We identified in a collection of about 2000 *A. niger* cDNAs (Tsang and

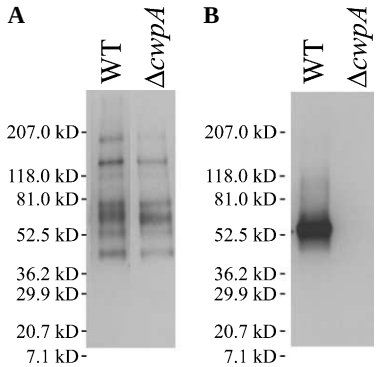

Fig. 1. Western analysis of HF-pyridine extracted proteins from SDS-extracted cell walls of the wild-type (N402) and the *cwpA* deletion strain (*ΔcwpA*). (A) Glycosylated proteins were detected with concanavalin-A-conjugated peroxidase. (B) Proteins detected with a CwpA-specific antibody. In each lane the extracted proteins from 200 µg of dry weight cells were loaded. Molecular weight markers (kDa) are indicated.

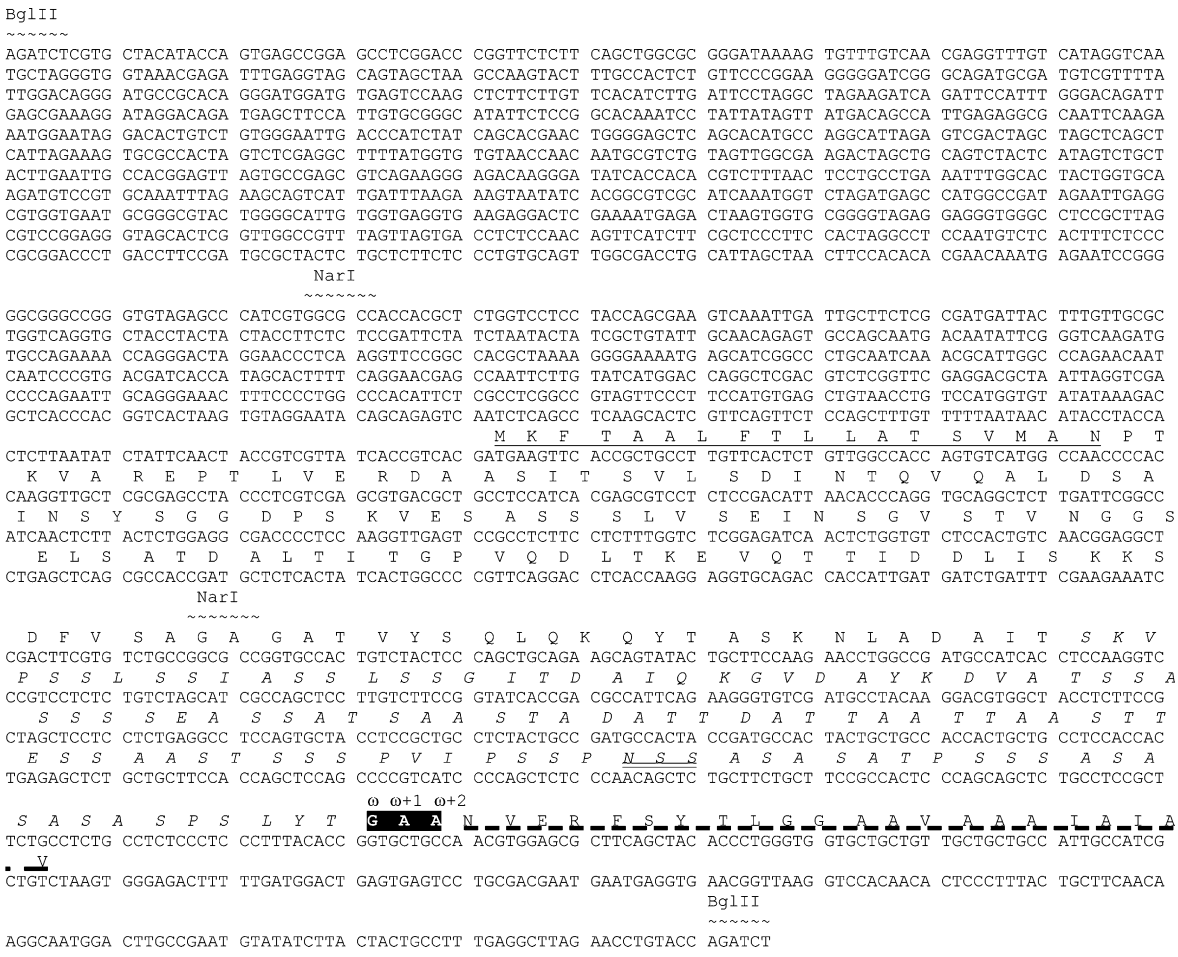

Fig. 2. The sequence of the 2.8 kb genomic *Bgl*II subclone containing the *cwpA* gene. The predicted protein sequence of 288 residues is shown above the open reading frame. Some features shown are characteristic for GPI-anchored cell wall proteins: a 17 amino acid N-terminal cleavable signal sequence (single underlined); a potential N-glycosylation site, at position 238 (double underlined); serine/threonine-rich region (italics) the ω, ω + 1, and ω + 2 sites (black background); and a C-terminal cleavable GPI signal peptide (dashed underlined).

Storms 2000;, unpublished), three almost identical partial clones which displayed a significant homology to the mannoproteins Mp1 from *P. marneffei* (*E* value 4e−09) and Mp1 from *A. fumigatus* (1e−12). Using the available cDNA sequences, we designed two primers to obtain the complete cDNA (see Section 2). Translation of the obtained cDNA fragment (Fig. 2) was shown to encode a protein homologous (56% identity) to the *A. fumigatus* MP1 homologue, and was named CwpA. The cDNA fragment was used to obtain a genomic clone carrying the full-length gene. The plasmid containing the *cwpA* gene was completely sequenced and appeared to contain the full-length *cwpA* gene, flanked by 1.7 kb upstream sequence and 0.2 kb of downstream sequence (Fig. 2).

3.3. CwpA encodes a putative GPI-CWP

The genomic 2766 bp *Bgl*II subclone was analysed and the sequence revealed that the *cwpA* gene has an uninterrupted open reading frame of 864 nucleotides encoding for a 288 residue protein with a predicted molecular weight of 28.2 kDa. Phylogenetic analysis revealed that CwpAp has high homology with several putative cell wall mannoproteins from various filamentous fungi (Fig. 3). The protein sequence of CwpA revealed the presence of both a hydrophobic signal sequence at the N-terminus (residues 1–17) that directs the protein to the endoplasmic reticulum and a C-terminal hydrophobic sequence that meets the criteria required for it to be cleaved off and to be replaced by a GPI-anchor (de Groot et al., 2003; Eisenhaber et al., 2004). The most probable GPI-anchor attachment site (ω-site) in the protein sequence is the glycine at position 264. The two amino acids following the ω-site (ω + 1 and ω + 2 sites) have also been shown to be critical for GPI-anchor attachment, and the alanines at position 265 and 266 (ω + 1 and ω + 2 sites) allow GPI-anchoring. The ω-region (GAA) is conserved in all CWP homologues given in Fig. 3 (data not shown). The ω-region is followed by a rather short spacer of four amino acids and a hydrophobic tail of 18 amino acids (Fig. 2). CwpAp contains one N-glycosylation site (N-X-S/T with X ≠ P) at

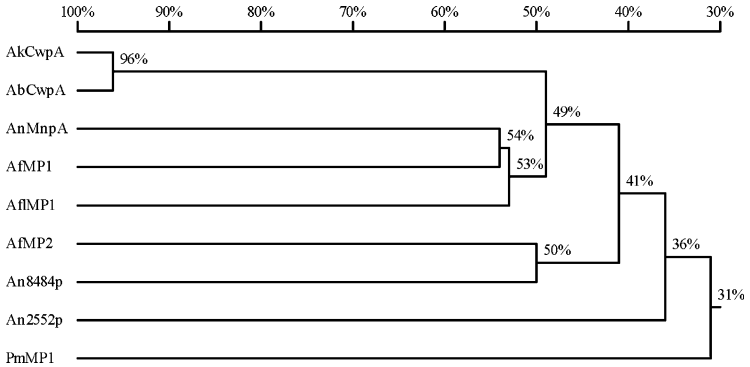

Fig. 3. Phylogenetic tree of putative GPI-CWPs from filamentous fungi. Protein sequences were aligned using DNAMAN (Lynnon BioSoft, v4.0) with standard gap and extension penalties. AkCwpA, AB109764.1 *Aspergillus kawachii* CwpA; AbCwpA, AY704270 *Aspergillus black = Aspergillus niger* CwpA; AnMnpA, AF497720.1 *Aspergillus nidulans* MnpA; AfMP1, AY007312.1 *Aspergillus fumigatus* MP1; AflMP1, AF461762.1 *Aspergillus flavus* MP1; AfMP2, AY460182.1 *Aspergillus fumigatus* MP2; AN8484p, XM_412621.1 *Aspergillus nidulans* AN8484.2p; AN2552p, XM_406689.1 *Aspergillus nidulans* AN2552.2p; PmMP1, AF009957.1 *Penicillium marneffei* MP1. % indicates identical amino acids residues.

residue 238, and many potential O-glycosylation sites (33% of the mature protein consist of serine or threonine residues). The O-glycosylation sites are predominantly located in the C-terminal half of the protein (aa150–263) where 50% (56 of the 114) amino acids are serine or threonine residues.

3.4. Deletion of *cwpA*

To determine whether CwpA is one of the HF-extractable cell wall mannoproteins identified in *A. niger* (Fig. 1), a *cwpA* deletion strain was constructed. The deletion plasmid (*cwpA*::*pyrG*#2) was transformed to the *A. niger pyrG*$^{-}$ strain AB4.1 and uridine prototrophs were selected. Transformants were screened for possible deletion of the *cwpA* gene by PCR (see Section 2). Southern analysis of *Bgl*II digested genomic DNA showed a shift in molecular size from 2.8 kb in the wild-type to the anticipated 3.5 kb hybridising fragment in the *ΔcwpA* strain indicating proper deletion of *cwpA* (Fig. 4).

To investigate whether CwpA is linked to the cell wall by a GPI-anchor, cell wall proteins were isolated by HF-pyridine treatment from the *ΔcwpA* strain and compared to the wild-type HF-extract. In the HF-extract of the *ΔcwpA* mutant, the same set of proteins with identical molecular weights was identified as in the wild-type after detection using with concanavalin-A-conjugated peroxidase (Fig. 1A). To detect CwpA specifically, an antiserum against CwpA was raised. Immunodetection of HF-extracts from the wild-type and the *ΔcwpA* strain with the CwpA antiserum revealed the presence of the CwpA protein, in the HF-pyridine extract of wild-type (N402) cell walls (Fig. 1B). No signal was found in HF-pyridine extracts of cell walls from the *cwpA* deletion strain (*ΔcwpA*), indicating the specificity of the antiserum. The estimated molecular weight based on the Western result of CwpA is around 55 kDa (Figs. 1 and 5). Re-examining the HF-extractable proteins from the wild-type and the *ΔcwpA* strain after detection with the concanavalin-A peroxidase suggests that there is another glycosylated protein at 55 kDa which is detected in *ΔcwpA* (Fig. 1A). The

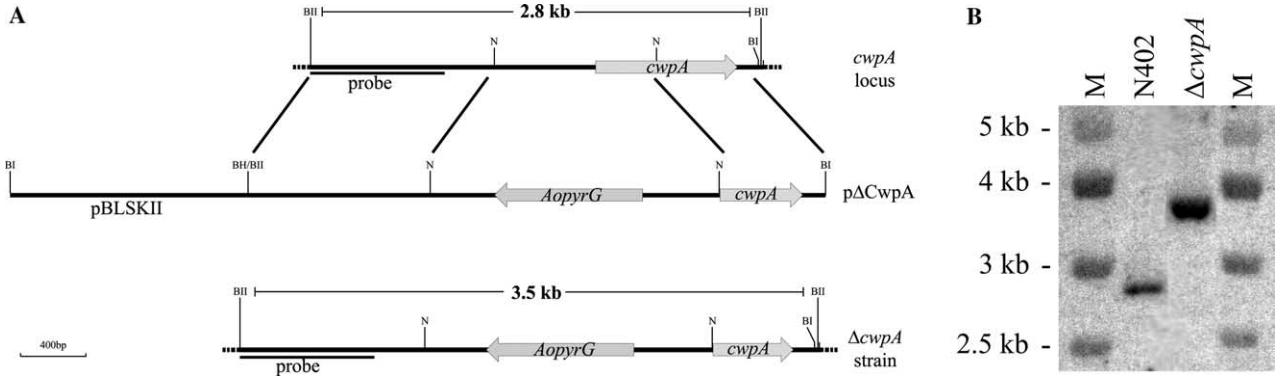

Fig. 4. Deletion of *cwpA*. (A) Schematic representation of the *cwpA* deletion strategy. The wild-type *cwpA* locus, the linear *Bgl*I fragment from the deletion plasmid pΔcwpA#2 used to transform to *A. niger*, and the *cwpA* locus after correct integration of the deletion cassette are shown. Abbreviations: BH, *Bam*HI; BI, *Bgl*I; BII, *Bgl*II; N, *Nar*I. The indicated probe fragment represents a 781 bp *Xba*I fragment from pΔcwpA#2. Regions between diagonal lines are homologous. Scale bar represents 400 bp. (B) Southern analysis of wild-type (N402) and the *cwpA* deletion strain (*ΔcwpA*). Genomic DNA was digested with *Bgl*I and probed with the fragment as indicated in (A). The DNA size markers (M) are shown on both sides of the blot.

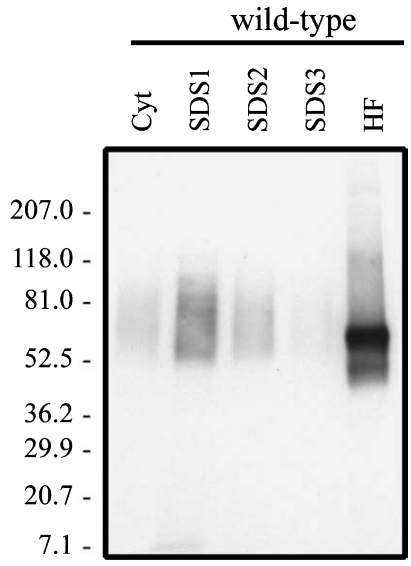

Fig. 5. CwpA is predominantly localised in the cell wall. Cell walls from the wild-type strain (N402) were isolated and extracted with Tris-buffer (Cyt-extract), three times with SDS-buffer (SDS-extracts 1–3) and HF-pyridine (HF-extract). Proteins were detected with a CwpA antiserum. Molecular weight markers (kDa) are indicated on the left side of the blot. In each lane the equivalent of 200 μg of dry weight cells was loaded.

difference in molecular weight between the predicted size of the mature protein (24 kDa) and the observed size (55 kDa) is most likely due to protein glycosylation. In the protein fractions containing predominantly cytosolic proteins (Tris-extract) or predominantly membrane proteins, (SDS-extract) minor amounts of CwpA-reactive material were present (Fig. 5). The proteins that reacted with the CwpA antiserum in the Tris- and SDS-extract were specific, since no reaction with the CwpA antiserum was found in similar extracts from the *ΔcwpA* strain (data not shown). From these results we conclude that CwpA is a HF-extractable cell wall mannoprotein which is most probably anchored to the cell wall through processing of the GPI-anchor and subsequent linkage to the glucan part of the cell wall.

3.5. Phenotypic analysis of cwpA deletion strain shows an increased sensitivity towards CFW

The *cwpA* deletion strain did not show a difference in growth rate or conidiation on CM-plates when compared to wild-type at either 30 or 37 °C. To detect cell wall related phenotypes, the Δ*cwpA* strain was screened for altered sensitivity towards various compounds. Spores of wild-type and the *ΔcwpA* strain were spotted in 10-fold dilutions on CM-plates containing different concentrations of Calcofluor White (CFW), SDS, lysing enzymes (Sigma), caffeine, hydrogen peroxide, KCl, sorbitol or vanadate (Fig. 6 and data not shown). An increase in sensitivity towards CFW was observed when high concentrations (200, 400, or 1000 μg ml^{-1}) were used (Fig. 6). The increased sensitivity could be remedi-

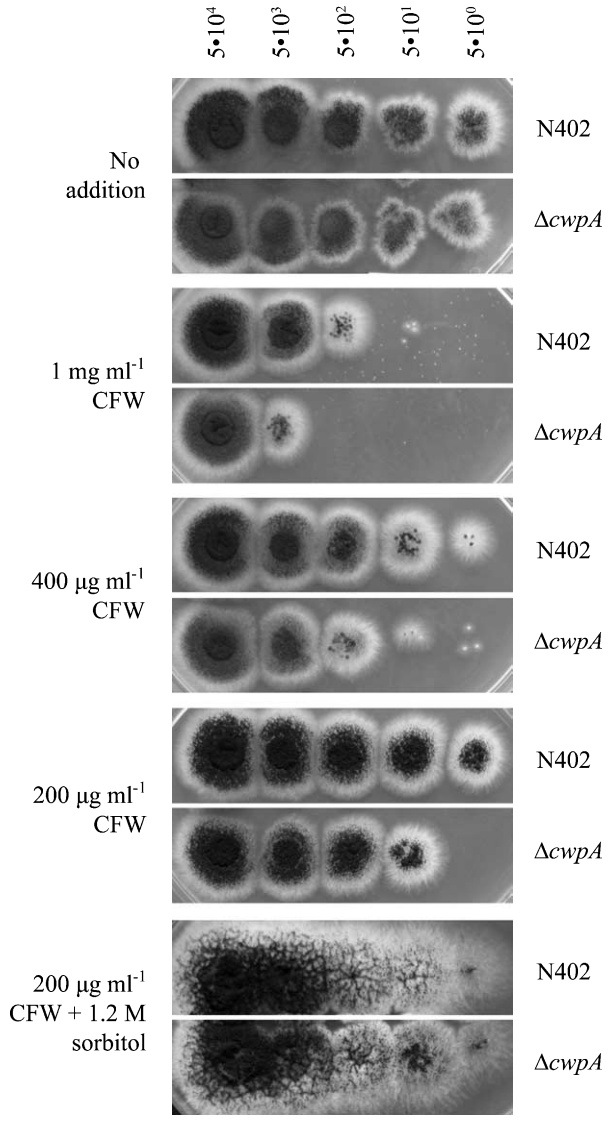

Fig. 6. Phenotype of the *cwpA* deletion strain. Ten-fold serial dilutions of spores of wild-type (N402) and the *cwpA* deletion strain (*ΔcwpA*) were spotted on complete medium plates containing CFW, indicated at the left side of the plates. Pictures were taken after incubation at 37 °C for three days.

ated by the addition of 1.2 M sorbitol. Sensitivity of the wild-type and the *ΔcwpA* mutant towards the other compounds tested did not reveal any difference in growth or sensitivity between the two strains.

3.6. Expression analysis of cwpA

Expression of cell wall proteins in *S. cerevisiae* has been shown to be highly regulated in response to growth conditions and in response to environmental changes (Smits et al., 1999). We examined the expression of *cwpA* under normal growth conditions in shake flask. Spores were inoculated in complete medium and grown at 30 or 37 °C for 8, 16, 24, 32, or 64 h. The expression of *cwpA* seems to peak

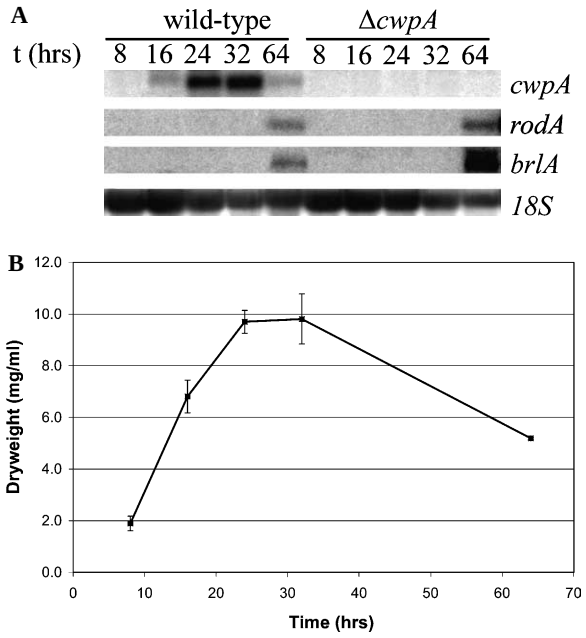

Fig. 7. Analysis of *cwpA* expression. (A) Northern analysis of *cwpA*, *rodA*, and *brlA* expression levels under normal growth at 30 °C in shake flask CM cultures for both wild-type and the *cwpA* deletion strain. Total RNA was isolated after 8, 16, 32, and 64 h. Northerns were probed with an 18S ribosomal probe as a loading control. (B) Growth curve of the wild-type strain grown as described above.

around 24–32 h independent of the growth temperature used (Fig. 7A and data not shown). When the 8- and 32-h time points are compared, a more than 100-fold induction is observed for *cwpA* expression at both temperatures (Fig. 7A and data not shown). As expected, no signal was detected in RNA samples from the *cwpA* deletion strain. The growth curve of these culture conditions showed that maximum biomass level (μg dry weight mycelium ml^{-1} culture) was reached after 24 h, and remained constant for at least 8 h before declining (Fig. 7B). We conclude that the induction of *cwpA* coincides with the later stages of exponential growth and/or with the entrance into stationary phase. At 64 h, the biomass of the culture decreased, which is indicative for cell lysis and is accompanied by a decrease in *cwpA* messenger levels. The induced expression of *cwpA* in the shake flask culture is probably not caused because of conidiation. The expression of conidiation-specific markers (*brlA* and *rodA*) was only observed at the 64 h time point (Fig. 7A). The results suggest that *cwpA* expression is dependent on the growth phase of the fungus and induced during the later phases of growth.

4. Discussion

In this study, we present the identification and characterisation of the *cwpA* gene from *A. niger*. The *cwpA* gene encodes a protein of 228 amino acids which contains a putative signal peptide and a putative GPI-anchor attachment signal. In fungi, GPI-anchored proteins are found at the cell surface, either attached to the plasma membrane (GPI-PMPs) or attached by a remnant of the GPI-anchor to the cell wall and are referred to as GPI-cell wall proteins (GPI-CWPs). Studies in *S. cerevisiae* have shown that GPI-PMPs remain attached predominantly to the plasma membrane, whereas GPI-CWPs become attached to β-1,3-glucan or the chitin part of the cell wall through a flexible β-1,6-glucan moiety (Kapteyn et al., 1995, 1996; Kollar et al., 1997). Upon arrival at the plasma membrane, GPI-CWPs are liberated from the plasma membrane by a still unknown mechanism. Subsequently, a remnant of the GPI-anchor reacts with the β-1,6-glucan part of the cell wall and results in the cross-linking of GPI-CWPs to the cell wall. Whether a GPI-anchored protein is a GPI-PMP or GPI-CWP can be determined by a simple fractionation experiment (Frieman and Cormack, 2003). In their studies, Frieman and Cormack show that GPI-PMPs are present in the SDS-soluble (membrane) fractions, whereas GPI-CWPs are SDS-resistant and found in the cell wall fractions. GPI-CWPs can either be liberated from the cell wall after β-1,3- or β-1,6-glucanase digestion or by HF-treatment. In this study, we have used a similar fractionation method to show that CwpA is a GPI-CWP. CwpA is hardly detectable in the SDS (membrane) fraction, and it is abundantly present in the HF-extract of SDS-extracted cell walls (Fig. 5). To our knowledge, this is the first time that the comparison between SDS- and HF-extractability of a putative cell wall protein has been made for a filamentous fungus. Previously, the most convincing evidence for the presence of GPI-anchored cell wall proteins in filamentous fungi was the report of FEM1p in *F. oxysporum* (Schoffelmeer et al., 2001). However, one could still argue that the amount of FEM1p detected in the HF-extract is only a small part of the total protein present since the amount of FEM1p extracted by SDS has not been reported.

The sorting signal that is determining whether GPI-anchored proteins are GPI-PMPs or GPI-CWP is still under investigation. Based on in silico analysis of GPI-CWPs in *S. cerevisiae*, Caro et al. (1997) predicted that a dibasic motif in the four amino acids upstream of the GPI-anchor attachment site results in the retention of the protein to the plasma membrane. This prediction has been experimentally confirmed by Frieman and Cormack (2003) who have shown that the introduction of a dibasic motif upstream of the ω-site of Cwp2 alters its localisation dramatically from the cell wall to the plasma membrane. In addition, Hamada et al. (1998) predicted that hydrophobic amino acids present at positions ω-2, ω-4, and ω-5 favour the localisation to the cell wall. However, this prediction was not an absolute requirement for cell wall localisation but such amino acids probably increase the efficiency by which a GPI-CWP is incorporated into the cell wall (Frieman and Cormack, 2003). The absence

of a dibasic motif in the amino acids upstream of the predicted ω-site in CwpA is in accordance with the cell wall localisation of CwpA.

Homologues of CwpA have been previously described in *P. marneffei* (Cao et al., 1998), *A. fumigatus* (Chong et al., 2004; Yuen et al., 2001) *A. nidulans* (Jeong et al., 2003), and *Aspergillus flavus* (Woo et al., 2003). All these CwpA homologues are predicted to contain a GPI-anchor attachment signal, and although cell wall localisation has been reported from immuno-EM studies (Cao et al., 1998; Yuen et al., 2001), biochemical evidence that these proteins are covalently bound to the cell wall by their GPI-anchor moiety is still lacking. Based on our observations, we propose that the CwpA homologues will be incorporated into the cell wall in a similar manner to that of the *A. niger* CwpA.

The HF-extractions of extensively SDS-extracted cell walls of *A. niger* revealed that at least seven HF-pyridine extractable proteins could be detected using peroxidase labelled concanavalin-A with CwpA being one of the less abundant proteins. Recently, a rapid and simple method to identify those proteins by LC/MS/MS was successfully used to identify HF-extractable cell wall mannoproteins from *C. albicans* (de Groot et al., 2004), and this method could also be used to identify these proteins in other fungi including *A. niger*.

The protein band corresponding to CwpA in the HF-extract is somewhat smaller and more distinct in size compared to the protein detected in the SDS-extract (Fig. 5). This could be due to the cleavage of additional HF-sensitive linkages which include phosphomannans by HF-pyridine present in N- and O-glycosidic chains as has been shown in the yeast *S. cerevisiae* (Jigami and Odani, 1999).

No obvious function of CwpA could be deduced from its knock out phenotype. The *ΔcwpA* strain did not show an apparent difference in growth and/or conidiation under normal growth conditions. However, the *ΔcwpA* strain showed an increased sensitivity towards Calcofluor White (CFW). CFW is known to interfere with normal cell wall assembly and hypersensitivity towards CFW is indicative for a cell wall integrity defect (Lussier et al., 1997; Ram et al., 1994). At the moment we favour the idea that CwpA has a structural role and that the absence of CwpA leads to alterations in the cell wall which makes the fungus more sensitive to CFW. Sorbitol was shown to fully suppress the CFW-hypersensitive phenotype, which is in agreement with the proposed cell wall integrity defect of the *ΔcwpA* strain. In *S. cerevisiae*, deletion of *cwp1* or *cwp2* or the simultaneous deletion of both genes leads to hypersensitivity towards Congo Red (CR) (van der Vaart et al., 1995). Congo Red binds, like CFW, to chitin fibrils in the cell wall and disturbs proper cell wall assembly.

Certain GPI-anchored cell wall proteins in *S. cerevisiae* have been shown to be specifically induced in response to various cell wall stress inducing conditions (Jung and Levin, 1999; Lagorce et al., 2003; Terashima et al., 2000; Garcia et al., 2004). CFW induced cell wall stress in *A. niger* did not result in an increased expression of *cwpA* (Damveld and Ram, unpublished data). The expression of *cwpA* is most abundant during the late exponential or stationary phase of growth and resembles the expression of Sed1p, a cell wall protein of *S. cerevisiae* involved in lytic enzyme resistance (Shimoi et al., 1998). Deletion of Sed1p had also an effect on the cell wall since Δ*SED1* cells were more sensitive to Zymolyase (β-1,3-glucanase) digestion (Shimoi et al., 1998). To correlate the expression data of *cwpA* with CwpA localisation, a GFP-CwpA fusion was constructed. The GFP was inserted at similar position as has been previously successfully used to analyse the localisation of GFP-CWPs in *S. cerevisiae* (Ram et al., 1998b). However, insertion of GFP did not result in successful transport of GFP-CwpA to the cell surface and it suggests that the GFP-CwpA fusion protein is not transported through the secretory pathway (Damveld, Wierckx and Ram, unpublished results). No further attempts have been undertaken to try to insert GFP at different sites in CwpA protein.

It has been shown for *S. cerevisiae* and *C. albicans* that the GPI-CWPs are linked to β-1,6-glucans through a remnant of the GPI-anchor (Kapteyn et al., 1995; Lu et al., 1994, 1995). An intriguing point of interest, which requires further investigation, is the identification of the exact linkage between the GPI-CWPs and the cell wall in filamentous fungi. The presence of β-1,6-glucosylated cell wall proteins in *A. niger* and *F. oxysporum* has been reported (Brul et al., 1997; Schoffelmeer et al., 1996) which indicates that a similar incorporation mechanism might exist in yeasts and filamentous fungi. However, β-1,6-glucan, as it is in present in *S. cerevisiae*, seems to be absent in the filamentous fungus *A. fumigatus* (Bernard and Latge, 2001) and detailed studies for other fungi are missing. The way in which GPI-CWPs are attached to the cell wall and to which extent a similar incorporation mechanism exists in all filamentous fungi is not known. This, in addition to the identification of enzymes involved in these processes in *A. niger*, is the subject of our current research. The generation of a highly specific antiserum against CwpA will greatly facilitate future studies and will also open the way for a genetic screen to identify mutants disturbed in the incorporation of cell wall mannoproteins in *A. niger*.

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