Disruption of the gene encoding the ChiB1 chitinase of *Aspergillus fumigatus* and characterization of a recombinant gene product

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The gene encoding a major, inducible 45 kDa chitinase of *Aspergillus fumigatus* was cloned and analysis of the deduced amino acid sequence identified a chitinase of the fungal/bacterial class which was designated ChiB1. Recombinant ChiB1, expressed in *Pichia pastoris*, was shown to function by a retaining mechanism of action. That is, the \(\beta\)-conformation of the chitin substrate linkage was preserved in the product in a manner typical of family 18 chitinases. Cleavage patterns with the \(N\)-acetylglucosamine (GlcNAc) oligosaccharide substrates GlcNAc\(_4\), GlcNAc\(_5\) and GlcNAc\(_6\) indicated that the predominant reaction involved hydrolysis of GlcNAc\(_2\) from the non-reducing end of each substrate. Products of transglycosylation were also identified in each incubation. Following disruption of \(chiB1\) by gene replacement, growth and morphology of disruptants and of the wild-type strain were essentially identical. However, during the autolytic phase of batch cultures the level of chitinase activity in culture filtrate from a disruptant was much lower than the activity from the wild-type. The search for chitinases with morphogenetic roles in filamentous fungi should perhaps focus on chitinases of the fungal/plant class although such an investigation will be complicated by the identification of at least 11 putative active site domains for family 18 chitinases in the *A. fumigatus* TIGR database (http://www.tigr.org/).

**INTRODUCTION**

*Aspergillus fumigatus* is the principal aetiological agent of invasive aspergillosis (IA); IA is the most common invasive mould infection worldwide and is associated with high mortality (Denning, 1998; Latgé, 2001). There is an urgent need for improved drugs to treat IA and other human mycoses (Andriole, 2000). Chitin is a major structural component of the cell wall of all fungi pathogenic for humans. However, the polymer is absent from mammals and enzymes of chitin metabolism have therefore been proposed as potential targets for novel antifungal agents.

Chitinases cleave the \(\beta(1\rightarrow4)\)-linkage between \(N\)-acetylglucosamine (GlcNAc) residues in the chitin homopolymer.

**Abbreviations:** GlcNAc, \(N\)-acetylglucosamine; 4MU-(GlcNAc)\(_2\), 4-methylumbelliferyl-\(\beta\)-\(N\)-\(N\)\(_9\)-diacetylchitobioside; 4MU-(GlcNAc)\(_3\), 4-methylumbelliferyl-\(\beta\)-\(N\)-\(N\)\(_9\)\(_0\)-triacetylchitotrioside.

The GenBank accession numbers for the sequences reported in this paper are AY217659 and AY217660.

Genes encoding chitinases have been cloned from a wide range of yeasts and filamentous fungi; all of these enzymes belong to glycohydrolase family 18 (Henrissat, 1999). Within this family two distinct classes of chitinase may be identified based on the similarity of the enzymes to family 18 chitinases from plants or bacteria (Pishko et al., 1995; Takaya et al., 1998a). Chitinases of the plant class have been detected in *Saccharomyces cerevisiae*, *Candida albicans* and several filamentous fungi. Disruption of the CTS1 gene of *S. cerevisiae* led to inability of mother and daughter cells to separate during cell division (Kuranda & Robbins, 1991) while disruption of the *chiA* gene of *Aspergillus nidulans* decreased both hyphal growth rate and the frequency of germination of conidia (Takaya et al., 1998a). These results indicate that family 18 plant class chitinases have roles during growth and morphogenesis in fungi. Chitinases of the bacterial class have been found in filamentous fungi, but not in yeasts. Morphogenetic roles for chitinases of the bacterial class have been proposed (Takaya et al., 1998a; b; Reichard et al., 2000; Kim et al., 2002), but no such role has been demonstrated.
Previously we identified and purified a major, inducible 45 kDa chitinase from A. fumigatus (Escott et al., 1998). Here we report the cloning of the gene encoding the 45 kDa chitinase, ChiB1, which appears most closely related to other chitinases of the bacterial class. The chiB1 gene was overexpressed in the yeast Pichia pastoris and the recombinant enzyme was characterized in detail. The potential of A. fumigatus chitinases of the so-called fungal/bacterial and fungal/plant classes as targets for antifungal drugs is discussed in the light of the results of chiB1 gene disruption studies.

**METHODS**

**Strains, media and growth conditions.** A λ genomic DNA library and a λ Uni-ZAP XR cDNA library were obtained from Stratagene and were constructed using A. fumigatus ATCC 13073. Strain ATCC 13073 was used throughout, except that probes for screening of DNA libraries were prepared using A. fumigatus NCPF 2140 genomic DNA. To obtain conidia, A. fumigatus was grown on Sabouraud’s agar plates for 3 d at 37 °C. Spore suspensions were prepared as described by Escott et al. (1998). For induction of extracellular chitinase by chitin, Vogel’s N medium (Vogel, 1964) containing sucrose (2%, w/v) (50 ml) was inoculated with A. fumigatus conidia (1 x 10⁹ spores ml⁻¹) and incubated at 37 °C at 200 r.p.m. for 20 h. Mycelia were harvested by centrifugation (2000 g for 15 min), washed with Vogel’s N medium (50 ml), resuspended in Vogel’s N medium containing crab shell chitin (1%, w/v), and incubated at 37 °C at 200 r.p.m. for 8 h. Samples (1 ml) were removed and centrifuged (13 000 g for 3 min) and chitinase activity and ChiB1 in the supernatant were detected using zymogram and Western blot procedures, respectively. For induction of extracellular chitinase under conditions of starvation, flasks containing Vogel’s N medium and glucose (1%, w/v) (50 ml) were inoculated with A. fumigatus conidia (1 x 10⁹ spores ml⁻¹) and incubated at 37 °C at 200 r.p.m. At regular intervals, the contents of a flask were filtered under vacuum using Whatman 54 filter paper and the culture filtrate was centrifuged (2000 g for 15 min). Chitinase activity in the supernatant was detected using the zymogram procedure and fluorimetric substrates, and ChiB1 was detected on Western blots.

**Isolation of genomic DNA encoding ChiB1.** Vogel’s N medium (400 ml; Vogel, 1964), supplemented with 2% sucrose, was inoculated to a final concentration of 1 x 10⁹ A. fumigatus spores ml⁻¹, incubated for 24 h at 37 °C at 200 r.p.m. and filtered through Whatman No. 3 paper under pressure to collect the mycelia as a pad on the filter paper. The mycelial pad was washed twice with 0-6 M MgSO₄ and stored at −20 °C. Mycelia (1 g) were ground to a fine powder in liquid nitrogen (approx. 5 ml) and DNA was isolated from 100-200 mg of this material using the Wizard Genomic DNA purification protocol (Promega). A fragment of the gene encoding ChiB1 was isolated from genomic DNA using PCR with a degenerate forward primer, 5'-GTNTAYTTYGNAAYTGGC-3', based on part of the N-terminal sequence (VYYFVNA) of a 45 kDa chitinase isolated previously from the culture filtrate of A. fumigatus NCPF 2140 (Escott et al., 1998), and a degenerate reverse primer, 5'-NGGRTAYTCCARTC-3', based on a conserved sequence (DWEYP) observed in an alignment of published Aphanocladium album (Blaiseau & Lafay, 1992) and Trichoderma harzianum (Hayes et al., 1994) chitinase amino acid sequences. PCR reactions (50 µl) contained buffer (Qiagen), 1.5 mM MgCl₂, 200 µM each dNTP, 2 µM each primer, 100 ng genomic DNA and 1-25 units HotStartTaq polymerase. Samples were heated to 95 °C for 15 min and then cycled 25 times (95 °C, 1 min; 57 °C, 1 min; 72 °C, 1 min). A PCR product of approximately 450 bp was ligated into pGEM-T Easy (Promega) overnight at 4 °C and used to transform competent Escherichia coli JM109 cells (Promega) using standard methods (Sambrook et al., 1989). Forward (5'-CTTCCCCAGGTTCTCCTCG-TC-3') and reverse (5’-CTCACCAACACCGAATCC-3’) primers were designed based on the sequence of the cloned chiB1 fragment and used in a PCR reaction under conditions identical to those described above, except that digoxigenin (Dig)-labelled nucleotides were included in the reaction according to the manufacturer’s instructions (Roche). The Dig-labelled probe was used to screen a λ A. fumigatus ATCC 13073 genomic library (Stratagene) according to the manufacturer’s instructions. Standard methods (Sambrook et al., 1989) were used for cloning, purification and subcloning of appropriate fragments of DNA. Cloned fragments were sequenced by the DNA Sequence Service, University of Durham.

**Isolation of cDNA encoding ChiB1.** A 1 kb fragment of chiB1 was isolated from the λ Uni-ZAP XR cDNA library. The sequence of this fragment confirmed the position of the stop codon of chiB1. For isolation of A. fumigatus cDNA, mycelial pad was ground to a fine powder in liquid nitrogen (see above). Total RNA was extracted from 100 mg ground mycelium using the RNeasy protocol (Qiagen) and polyA⁺ mRNA purified from total RNA using the Oligotex protocol (Qiagen). cDNA was synthesized from 2-5 µg polyA⁺ mRNA using the Clontech Copykit, according to manufacturer’s instructions. Sequencing of a product from a PCR reaction using cDNA, and primers and reaction conditions identical to those described below for expression of ChiB1 in P. pastoris, identified most of the remainder of the cDNA for chiB1. Finally, the 5’ end of chiB1 cDNA was determined using 5’-RACE, according to manufacturer’s instructions (Roche).

**Preparation and purification of recombinant ChiB1.** Forward primer CHIBXF (5’-CATCGGTGTTACGTGAAGTGCGGTGTTATGGTCGTC-3’) and reverse primer CHIBXR (5’-CGAGCTCAATCCTAGATTATCGTTTGCATTGC-3’) were used to amplify a product, with the 5’ region corresponding to sequence encoding the N terminus of native ChiB1 purified from an A. fumigatus culture filtrate (Escott et al., 1998), from cDNA. This product lacks the first 39 aa of the enzyme, including an apparent N-terminal signal peptide which was replaced with the S. cerevisiae a-factor for expression in P. pastoris. PCR reactions (50 µl) contained buffer (Qiagen), 1·5 mM MgCl₂, 200 µM each dNTP, 2 µM each primer, 100 ng cDNA and 1-25 units HotStartTaq polymerase. Samples were heated to 95 °C for 15 min, then cycled 25 times (95 °C, 30 s; 55 °C, 30 s; 72 °C, 1 min) and finally heated to 72 °C for 2 min. The restriction sites, underlined in the primer sequences, SmBI in CHIBXF and XbaI in CHIBXR, were included to facilitate cloning into a modified pPICZαA P. pastoris expression plasmid kindly provided by Dr Lars Hesse (University of Heidelberg, Germany). A single product of approximately 1200 bp was subcloned into the pPICZαA vector and ChiB1 was expressed in the supernatant of P. pastoris X-33, according to manufacturer’s instructions. Supernatant (10 ml) was desalted using a Sephadex G-25 M column (Prepacked P-10; Amersham Biosciences) and elution buffer (0·1 M potassium phosphate buffer, pH 6). Ammonium sulphate was added to a final concentration of 1 M and the sample was subjected to hydrophobic interaction chromatography using a Butyl Sepharose Fast Flow 4 column (20 ml) equilibrated with equilibration buffer (0·1 M potassium phosphate buffer, 1 M ammonium sulphate, pH 6). The sample was applied at a flow rate of 0·1 ml min⁻¹ and the column was eluted with equilibration buffer (100 ml) and elution buffer (100 ml) at a flow rate of 1 ml min⁻¹. Fractions (1 ml) were analysed by SDS-PAGE and those fractions containing the 45 kDa protein were pooled (3 ml), applied to a Sephacryl S-100 (Amersham Biosciences) gel filtration column (100 ml) and eluted with elution buffer. The purity of the eluted proteins was assessed by SDS-PAGE.
and MS. Polyclonal antibodies against purified recombinant ChiB1 were raised in rabbits by Charles River UK Ltd.

**Immunoblot analysis.** Protein blotting onto nitrocellulose was as described by Sambrook et al. (1989) and antibody–antigen conjugates were visualized using a monoclonal anti-rabbit immunoglobulin alkaline phosphatase-conjugate (Sigma–Aldrich) and NBT/BCIP enzyme substrate (Roche).

**Chitinase assays**

**Fluorimetric assays.** Fluorimetric assays with 4-methylumbelliferyl-β,1,2-anhydro-N- neuraminoglycol sulfate [4ωMU-(GlcNAc)2] or 4-methylumbelliferyl-β,1,2-anhydro-N neuraminoglycol sulfate [4ωMU-(GlcNAc)3] as substrates were used to measure chitinolytic activity in A. fumigatus culture filtrates, essentially as described by McCreath & Gooday (1992). All assays were performed in triplicate.

**Anomeric form of products.** The anomeric form of the reaction products was determined by HPLC using a TSK Amide 80 column (Koga et al., 1998; Fukamizo et al., 2001) and the cleavage pattern was assessed from the anomer formation (Koga et al., 1998). Briefly, the enzyme reactions contained 4·8 mM (GlcNAc)6 and 50 mM sodium acetate, pH 5, and were incubated at 30 °C. For HPLC, the elution solvent was 70% acetonitrile and the flow rate was 0·7 ml min⁻¹. Oligosaccharides were detected by UV absorption at 220 nm.

**Reaction time-course.** Products of the hydrolysis of each of (GlcNAc)₆, (GlcNAc)₅ and (GlcNAc)₄ by recombinant ChiB1 were analysed and quantified by gel filtration HPLC using a TSK-GEL G2000PW column (Tosoh) essentially as described by Fukamizo et al. (2001). Briefly, enzyme reactions contained 4·8 mM (GlcNAc)₆ substrate, 0·4 μM enzyme and 50 mM sodium acetate, pH 5, and were incubated at 40 °C. For HPLC, the elution solvent was distilled water and the flow rate was 0·3 ml min⁻¹. Oligosaccharides were detected by UV absorption at 220 nm. The amounts of saccharides produced were determined by computer-aided integration of individual peaks using a standard curve obtained from authentic saccharide solutions.

**Zymogram procedure.** Chitinase activity was detected in polyacrylamide gels containing chitin as described by Escott et al. (1998).

**Construction of gene replacement vector (pΔchiB1) for disruption of the chiB1 gene.** Plasmid pΔchiB1 was designed as a gene replacement vector for the removal of a 484 bp fragment of the chiB1 gene containing the putative active site (Robertus & Monzingo, 1999). The vector was constructed using pAN7.1 (Punt et al., 1987), a fragment of chiB1 incorporating the 5' end of the gene (from 1251 bp upstream of the start codon to 110 bp into the translated sequence) and a fragment of chiB1 incorporating the 3' end of the gene (from 594 bp downstream of the start codon to 70 bp downstream of the stop codon) (Fig. 1a–c). Plasmid pΔchiB1 was used to transform E. coli JM109 and identified in transformants.

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**Fig. 1.** Disruption of chiB1. (a) Part of plasmid pΔchiB1 constructed using pAN7.1 (Punt et al., 1987). The sections labelled 5’ and 3’ correspond to regions located immediately upstream or downstream of the chiB1 gene, respectively (see Methods). (b, c) Restriction map of the 3663 bp BamHI fragment before (b) and after (c) gene replacement using plasmid pΔchiB1. (d) Southern hybridization of genomic DNA with restriction enzyme digests of ΔchiB1 transformants (lanes 1–3) or the parental, wild-type strain (lane W). The size of each band in (d) correlates with the predicted size from (b) and (c), providing evidence for homologous integration.
following restriction digestion with *SstI* and *SapI*, and agarose gel electrophoresis. Construction of the disruption cassette was confirmed by DNA sequencing. The disruption plasmid was excised using *EcoP* and *HpaI*, and used to transform *A. fumigatus* ATCC 13073 essentially as described by Paris (1994). Transformants were subcultured onto MMS agar (Paris, 1994) containing hygromycin (200 μg ml⁻¹) twice and spore suspensions were prepared as described above.

**Electrospray MS.** A recombinant ChiB1 preparation was analysed using a Q-ToF (Micromass) mass spectrometer equipped with nano-electrospray ionization. The preparation was dissolved in 1:1 (v/v) aqueous methanol containing 1% (w/v) formic acid at a concentration of approximately 20 pmol protein ml⁻¹. Positive ionization was used with a capillary voltage of 900 V and a sampling cone voltage of 40 V. Nitrogen was employed as the drying gas. The micro-channel plate detector was set at 2700 V. Data were acquired over the appropriate m/z range and spectra were processed using MassLynx software. The spectra were calibrated with a separate introduction of horse heart myoglobin (16951-49 Da) and a mass accuracy of ±0.01% was expected.

**RESULTS**

**Cloning and heterologous expression of chiB1**

A positive clone isolated from the *A. fumigatus* ATCC 13073 genomic library was sequenced and found to contain a predicted coding region of approximately 1.5 kb. Sequencing of a cDNA clone corresponding to the entire sequence of an *A. nidulans* chitinase, ChiB (GenBank accession no. AY217659). The *A. fumigatus* gene was named *chiB1* because interrogation of the TIGR database (http://www.tigr.org/) indicates that a further six closely related sequences are present in the *A. fumigatus* genome (data not shown). The deduced sequence for ChiB1 also exhibits a high level of identity (61-5%) with the deduced sequence of an *A. nidulans* chitinase, ChiB (GenBank accession no. D87063). The *A. fumigatus* gene was named *chiB1* because interrogation of the TIGR database (http://www.tigr.org/) indicates that a further six closely related genes are present in the *A. fumigatus* genome (data not shown). The deduced sequence for ChiB1 also exhibits a high level of identity (61-5%) with the deduced sequence of the *Coccidioides immitis* fungal/bacterial chitinase CTs1 (Pishko et al., 1995). However, *A. fumigatus* ChiB1 exhibits only 14% identity with the deduced sequence of ChiA1, an *A. fumigatus* chitinase of the fungal/plant class (GenBank accession no. AY217659). The genomic and deduced amino acid sequences of the *A. fumigatus* chiB1 gene and ChiB1 protein were deposited in GenBank (accession no. AY217660).

Recombinant ChiB1 was purified from the culture filtrate of *P. pastoris* following heterologous expression of a truncated form of the *chiB1* gene. SDS-PAGE analysis of the purified preparation identified a single protein of molecular mass in excess of 45 kDa (data not shown), while QTOF electrospray MS analysis indicated that the molecular mass of the recombinant protein was approximately 45 529 Da. This value is similar to the molecular mass of native ChiB1 (Escott et al., 1998). Recombinant ChiB1 exhibited chitinase activity on a glycol chitin gel and polyclonal antibodies raised against the pure protein identified a single band on a Western blot (data not shown); no immunoreactive protein was detected using pre-immune serum from the rabbit used for antibody production. When the deduced amino acid sequence of recombinant ChiB1 was subjected to primary structure analysis using the proteomics server of the Swiss Institute of Bioinformatics (SIB; http://ca.expasy.org/), predicted molecular mass and pI values for ChiB1 were 43 653 Da and 4.93, respectively. The difference between theoretical and measured molecular mass values is most likely attributable to post-translational modification of the ChiB1 polypeptide in *A. fumigatus* and in the heterologous host *P. pastoris*. A protein motif search, using the Scan Prosite program at the SIB website, identified several potential sites for post-translational modification, each with a high probability of occurrence; included among these is an Asn N-glycosylation site containing residues 266–269. It is of interest to note, in this regard, that purified native ChiB1 reacted positively with the carbohydrate stain Periodic acid–Schiff’s reagent (Escott et al., 1998).

**Kinetic properties of recombinant ChiB1**

**Anomeric form of the reaction products.** The anomers of ChiB1 products from (GlcNAc)_6 substrate at pH 6-0 were analysed using HPLC (TSK Amide 80 column). The major products were (GlcNAc)_2 and (GlcNAc)_4 and the two anomers of each product eluted separately as a function of time (Fig. 2). After a 5 min incubation virtually all of the newly formed disaccharide was formed as the β-anomer. Over a much longer incubation period (up to 90 min) the β-anomer gradually mutarotated to the α-anomer. Similarly, after a 5 min incubation, the ratio of α:β anomer for the (GlcNAc)_4 reaction product was approximately 1:1, but after 90 min this ratio was close to the standard mutarotation equilibrium value for GlcNAc oligomers of approximately 5:2 (Fukamizo et al., 2001). These results indicate that ChiB1 has a retaining mechanism of action typical of family 18 chitinases with the β-conformation of the glycosidic linkage preserved in the reaction products. The predominance of the (GlcNAc)_2 product indicates that ChiB1 preferentially cleaves (GlcNAc)_6 between the second and third sugars from the non-reducing end of this substrate.

**Reaction time-course.** The rate of product formation for various oligosaccharide substrates was followed by HPLC (gel filtration) as a function of time. The results confirmed that (GlcNAc)_6 is hydrolysed mainly to (GlcNAc)_2 and (GlcNAc)_4 with much less cleavage to (GlcNAc)_3 + (GlcNAc)_5 (Fig. 3a). The presence of (GlcNAc)_6 indicates that transglycosylation also occurred and may have involved condensation of two tetramers or of a dimer with the hexamer substrate. The pentamer was cleaved primarily to trimer and dimer and the presence of a small amount of heptamer indicated transglycosylation (pentamer+dimer; Fig. 3b). Finally, almost all of the (GlcNAc)_4 substrate was cleaved to dimer although a small amount of (GlcNAc)_5 product was detected (Fig. 3c).
A very low level of (GlcNAc)$_6$ suggested transglycosylation (tetramer + dimer or trimer + trimer).

**Confirmation of chiB1 gene disruption by Southern hybridization**

Ninety hygromycin-resistant transformants were analysed by restriction enzyme digestion of genomic DNA and Southern hybridization. Fifteen of these transformants arose by gene replacement, as shown by a shift of the hybridizing DNA fragment from 3.7 kb in the wild-type strain to 5 kb in the mutant strains (Fig. 1d; three mutants shown). When the transformants were hybridized with a probe for the hygromycin resistance determinant, a single band was identified in each case (data not shown), indicating that integration occurred at a single chromosomal locus.

**Loss of ChiB1 chitinase activity by the ΔchiB1 transformant and comparative analysis of growth and morphogenesis of the parental and ΔchiB1 strains**

Mutant and wild-type strains were cultured for 8 h in Vogel's N medium containing crab shell chitin and culture filtrates isolated as described in Methods. Chitinolytic activity in culture filtrates was assayed using a zymogram procedure and the presence/absence of ChiB1 was determined using antibodies raised against recombinant ChiB1 and Western blotting. A chitinase of approximately 45 kDa was detected in the wild-type, but not in the mutant strain containing the disrupted chiB1 gene (Fig. 4, lanes 1 and 2). The results of the Western blot analysis confirmed disruption of chiB1: the ChiB1 protein was detected in the wild-type, but not in the mutant strain (Fig. 4, lanes 3 and 4).

Several days after the initiation of batch cultures, zymogram and Western blot analyses indicated strong induction of ChiB1 activity in the wild-type organism (Fig. 5a, b); no such enzyme activity or immunoreactive protein was detected in the mutant strain (data not shown). ChiB1 clearly makes a major contribution to the extracellular chitinolytic activity of *A. fumigatus* as the wild-type organism secreted far higher levels of chitinolytic activity detected using 4MU-(GlcNAc)$_2$ and 4MU-(GlcNAc)$_3$ substrates (Fig. 5c). It therefore seems likely that the chiB1 mutant will grow less effectively than the wild-type strain in a medium containing chitin as sole carbon source. After 6 days, the hyphae of both wild-type and mutant organisms began to disintegrate. The dry weight yield for
each culture was monitored continuously and the decrease in dry weight noted towards the end of the 16-day period was the same for the mutant and wild-type. This suggests that chitinolysis involving ChiB1 is not a rate-limiting step during fungal cell-wall autolysis. Disruption of ChiB1 had no detectable effect on growth or morphology of *A. fumigatus* as the mutant and wild-type strains appeared identical when cultured under a range of growth conditions (data not shown).

**DISCUSSION**

The deduced amino acid sequence for the ChiB1 protein indicates this enzyme is a member of glycohydrolase family 18. Our kinetic analyses with recombinant ChiB1 reveal that, in common with other family 18 chitinases, ChiB1 has a retaining mechanism of action in that the β-glycosidic linkage of the chitin substrate is preserved in the product. These studies also indicate that, as for the related chitinase CiX1 of *C. immitis*, polysaccharides bind to an extended

**Fig. 3.** Time-courses of GlcNAc oligosaccharide hydrolysis by ChiB1. (a) shows the hydrolysis of (GlcNAc)$_6$. Hexamers are shown as filled circles, dimers as filled squares, trimers as filled triangles and tetramers as open circles. Octamers generated by transglycosylation are shown as open diamonds. In (b), (GlcNAc)$_5$ is shown as open squares, dimers as filled squares, trimers as filled triangles and heptamers, formed by transglycosylation, as open diamonds. In (c), (GlcNAc)$_4$ is shown as open circles, dimers as filled squares, trimers as filled triangles and hexamers, formed by transglycosylation, as closed circles. The experiment was repeated with essentially the same results.

**Fig. 4.** Extracellular ChiB1 expression in wild-type and ΔchiB1 disruptant strains. Organisms were cultured in Vogel's N medium containing sucrose for 20 h, mycelia were harvested, resuspended in Vogel's N medium containing chitin and cultured for a further 8 h as described in Methods. Culture supernatants were assayed using the zymogram procedure (lanes 1 and 2) and Western blotting using anti-ChiB1 raised in rabbits (lanes 3 and 4). Wild-type (lanes 1, 3); ΔchiB1 (lanes 2, 4). All tracks contained 12 μl culture supernatant.
site on ChiB1 and are preferentially cleaved between the second and third sugars from the non-reducing end of the substrate. Over a prolonged incubation period, products of transglycosylation were also observed. Similar results were obtained by Xia et al. (2001), who showed that native ChiB1 promotes transglycosylation. Morphogenetic roles have been proposed for chitinases with transglycosylase activity. It is possible that these enzymes contribute to cell-wall synthesis and modification by catalysing cross-linking of chitin polymers to other wall components. However, the conditions used to demonstrate transglycosylase activity during the present and other studies are likely to differ markedly from those for chitinases present in the fungal cell wall. Thus, as suggested by Fukamizo et al. (2001), the transglycosylase activity demonstrated for ChiB1 and CiX1 of C. immitis may be dependent upon the high concentrations of the products of chitinolytic activity found under laboratory conditions and may not occur in nature.

The results of our gene disruption studies argue against a major morphogenetic role for ChiB1. Growth and morphogenesis for all three of the mutant strains harbouring a disrupted chiB1 gene appeared identical to growth and morphogenesis of the wild-type organism under a range of growth conditions. However, the levels of lytic activity detected in the growth medium of the wild-type strain using 4MU-(GlcNAc)2 and 4MU-(GlcNAc)3 substrates were markedly higher than the levels of activity detected in a chiB1 disruptant during a 16-day batch culture (Fig. 5c). These results suggest strongly that ChiB1 is secreted into the growth medium in response to nutrient depletion, indicating that the principal role for this enzyme is the degradation and recycling of fungal chitin during autolysis. ChiB1 is a family 18 chitinase of the fungal/bacterial class and the results obtained during the present study are similar to those obtained by Reichard et al. (2000). These workers were unable to detect any effect on endosporulation or virulence of the pathogenic fungus C. immitis following disruption of CTS1, a gene encoding a fungal/bacterial chitinase in this organism. Taken together these results suggest that family 18 chitinases of the fungal/bacterial class may not themselves represent targets for antifungal agents. However, mechanisms that regulate the activities of these or other chitinases may be exploitable in the design of novel drugs as excessive expression of chitinolytic activity during normal growth may trigger cell lysis. Currently we are investigating the transcriptional and post-translational regulation of A. fumigatus chitinase activities.

Family 18 chitinases of the fungal/plant class appear to have morphogenetic roles in S. cerevisiae and A. nidulans (Kuranda & Robbins, 1991; Takaya et al., 1998a). During the present study we cloned a gene, chiA1, which encodes a chitinase of the fungal/plant class in A. fumigatus (GenBank accession no. AY217659). The deduced amino acid sequence was found to have 50·3% identity with the deduced sequence of ChiA, a fungal/plant chitinase from A. nidulans (Takaya et al., 1998a) and 36·8% identity with the deduced sequence for the C. immitis fungal/plant chitinase CTS2 (Pishko et al., 1995). Further analysis of the deduced amino acid sequence for A. fumigatus ChiA1, using the DGPI facility at the SIB website, identified putative GPI-anchor and cleavage sites at the C terminus of the protein; no such sites were identified for A. fumigatus ChiB1. GPI-anchoring of fungal chitinases may provide at least a partial explanation for the close association of chitinases with membrane and cell-wall fractions isolated from A. fumigatus and other species (Dickinson et al., 1991; Rast et al., 1991; Hearne et al., 1998). When the gene encoding ChiA, a closely related chitinase from A. nidulans, was disrupted, the mutant strain exhibited a decreased rate
of germination of conidia and a lower hyphal growth rate when compared to the wild-type organism (Takaya et al., 1998a). Analysis of the deduced amino acid sequence of A. nidulans ChiA indicates that this protein is also likely to have GPI-anchor and cleavage sites at the C terminus. Recently, Mouyna et al. (2000) demonstrated that a glucanosyl-transferase, with an important role in A. fumigatus cell-wall biosynthesis, is GPI-anchored to the cell membrane. Moreover, Bruneau et al. (2001) identified a further nine GPI-anchored proteins in A. fumigatus; five of these proteins are homologues of putatively GPI-anchored proteins from Candida albicans or S. cerevisiae with apparent roles during cell-wall construction or maintenance. Fungal/plant chitinases like ChiA1, GPI-anchored to the fungal cell membrane or wall, may also perform essential roles during growth and morphogenesis in filamentous fungi.

A. fumigatus ChiA1 contains a serine/threonine-rich domain located primarily at the C terminus of the protein (no such domain is present in ChiB1). Frieman et al. (2002) found that a serine/threonine-rich region of Epalp1, a GPI-anchored cell-wall adhesin of Candida glabrata, was required for the projection of the N-terminal ligand-binding domain of this protein into the external environment. It is possible that the serine/threonine-rich domain of ChiA1 performs a similar role in projecting the N-terminal active site of the GPI-anchored enzyme into, or through, the cell wall of A. fumigatus.

Future gene disruption experiments with chiA1 and genes encoding other chitinases of the fungal/plant class should allow us to identify precise roles for these enzymes in A. fumigatus. However, as the A. fumigatus genome sequencing project nears completion it should be noted that the elucidation of roles for individual chitinases is likely to be complicated by the identification of at least 11 putative active site domains for 18 chitinases in the A. fumigatus TIGR database.

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