

Secreted glutamic protease rescues aspartic protease Pep deficiency in *Aspergillus fumigatus* during growth in acidic protein medium

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In an acidic protein medium *Aspergillus fumigatus* secretes an aspartic endoprotease (Pep) as well as tripeptidyl-peptidases, a prolyl-peptidase and carboxypeptidases. In addition, LC-MS/MS revealed a novel glutamic protease, AfuGprA, homologous to *Aspergillus niger* aspergillopepsin II. The importance of AfuGprA in protein digestion was evaluated by deletion of its encoding gene in *A. fumigatus* wild-type D141 and in a *pepΔ* mutant. Either *A. fumigatus* Pep or AfuGprA was shown to be necessary for fungal growth in protein medium at low pH. Exoproteolytic activity is therefore not sufficient for complete protein hydrolysis and fungal growth in a medium containing proteins as the sole nitrogen source. Pep and AfuGprA constitute a pair of endoproteases active at low pH, in analogy to *A. fumigatus* alkaline protease (Alp) and metalloprotease I (Mep), where at least one of these enzymes is necessary for fungal growth in protein medium at neutral pH. Heterologous expression of AfuGprA in *Pichia pastoris* showed that the enzyme is synthesized as a preproprotein and that the propeptide is removed through an autoproteolytic reaction at low pH to generate the mature protease. In contrast to *A. niger* aspergillopepsin II, AfuGprA is a single-chain protein and is structurally more similar to G1 proteases characterized in other non-*Aspergillus* fungi.

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INTRODUCTION

Aspergillus fumigatus is an important opportunistic pathogen that is the main causative agent of invasive aspergillosis in neutropenic patients (Latgé, 1999). In composts, this fungus plays an important role in the decomposition of organic material and in recycling environmental carbon and nitrogen (Beffa *et al.*, 1998). Like many other ascomycetes from the soil, *A. fumigatus* grows well in media containing protein as the sole nitrogen and carbon source and shows secreted proteolytic activity (Reichard *et al.*, 1990; Monod *et al.*, 1991; Sriranganadane *et al.*, 2010). At neutral pH, the

fungus secretes two endoproteases, an alkaline protease (Alp) of the subtilisin family (Reichard *et al.*, 1990; Monod *et al.*, 1991) and a metalloprotease (Mep) of the fungalisin family (Monod *et al.*, 1993a, b; Jatton-Ogay *et al.*, 1994). In addition, *A. fumigatus* secretes leucine aminopeptidases (Laps), which are non-specific monoaminopeptidases, dipeptidyl-peptidases (DppIV and DppV) and a prolyl-peptidase (AfuS28) as exopeptidases (Beauvais *et al.*, 1997a, b; Monod *et al.*, 2005; Sriranganadane *et al.*, 2010). *alpΔ mepΔ* knockout mutants are unable to grow in a medium containing protein as the sole nitrogen source at neutral and high pH while single *alpΔ* and *mepΔ* mutants produced 30 and 70 % of the proteolytic activity of the wild-type strain, respectively (Monod *et al.*, 1993a, b; Jatton-Ogay *et al.*, 1994).

In a protein medium at low pH, *A. fumigatus* was found to secrete another set of proteases, including the aspartic

Abbreviation: pNA, p-nitroaniline.

A supplementary figure showing the amino acid sequence alignment of *A. fumigatus* GprA (MER107323) with glutamic proteases from related taxa is available with the online version of this paper.

protease Pep as an endoprotease (Reichard *et al.*, 1995), tripeptidyl-peptidases (Tpp) of the sedolisin family (SedB to SedD) (Reichard *et al.*, 2006), and AfuS28 and carboxypeptidases of the S10 family as exoproteases (Sriranganadane *et al.*, 2010). In addition, we identified by MS an as yet uncharacterized putative glutamic endoprotease, AfuGprA (XP_748619, MER107323; encoding a gene at the locus AFUA_3G02970) (Monod *et al.*, 2009; Sriranganadane *et al.*, 2010), homologous to *Aspergillus niger* proteinase A or aspergillopepsin II (Takahashi, 2004) and to *Scytalidium lignicolum* scytalidopepsin B (Oda, 2004). *A. niger* aspergillopepsin II is not inhibited by pepstatin and was previously considered as a non-pepsin-type acid protease (Sasaki *et al.*, 1995; Takahashi, 2004). The active site of scytalidopepsin B and aspergillopepsin II was identified with a catalytic dyad formed by residues Glu (E) and Gln (Q) (Fujinaga *et al.*, 2004; Yabuki *et al.*, 2004; Kataoka *et al.*, 2005). This finding led to the establishment of a novel family of proteases called eqolisins (Fujinaga *et al.*, 2004) or glutamic peptidases (Yabuki *et al.*, 2004; Kataoka *et al.*, 2005) separate from the aspartic proteases (see the MEROPS proteolytic enzyme database, <http://merops.sanger.ac.uk>).

A. fumigatus pep mutants were constructed in order to test the role of Pep in virulence (Reichard *et al.*, 1997). However, no difference in pathogenicity was observed between the wild-type strain and *pep* mutants. Mortality curves were not statistically different, and histopathological studies of lungs from infected guinea pigs showed a similar extent of mycelium growth. In a control analysis, we found that *A. fumigatus pep* mutants grew well at low pH in a protein medium. Because this result suggested that other endoproteases could rescue a lack of Pep activity, here we characterized AfuGprA and investigated its function with respect to protein digestion. We show that AfuGprA and Pep constitute a pair of endoproteases in which one of them is necessary for fungal growth in a protein medium at low pH.

METHODS

Fungal and bacterial strains. *A. fumigatus* D141 (NRRL 6585; US Department of Agriculture, Peoria, IL) and an isogenic *pepΔ* mutant (Reichard *et al.*, 1997) were used in this study. All plasmid subcloning experiments were performed in *Escherichia coli* XL1 Blue. *Pichia pastoris* GS115 (Invitrogen) was used to produce recombinant AfuGprA.

Growth of *A. fumigatus* on solid media. *A. fumigatus* was grown on 2% (w/v) malt agar medium (Oxoid) for production of spores, and on BSA agar medium and soy protein agar medium at pH 7.0 or pH 4.0. BSA agar medium contained 1.2% (w/v) yeast carbon base (YCB; Difco), 0.2% (w/v) bovine serum albumin (Fraction V; Serva) and 1.5% (w/v) agar. Nine hundred millilitres of a solution containing YCB and agarose was adjusted to pH 7.0 or pH 4.0 with 1 M NaOH or 1 M HCl, respectively, and sterilized by autoclaving at 120 °C. A BSA solution (2.0%, w/v, 100 ml) with pH adjusted to 7.0 or 4.0 using 1 M NaOH or 1 M HCl, respectively, was sterilized by filtration and added to cooled YCB-agarose and the mix was poured

into Petri dishes. Soy protein agar medium contained 0.2% (w/v) soy protein (Supro 1711; Protein Technologies International) and 1.5% (w/v) agar. To test the growth of *A. fumigatus* at low pH, soy protein was dissolved in 68 mM citrate buffer (pH 4.0). Neutral and acidic soy protein media were sterilized by autoclaving at 120 °C and poured into Petri dishes.

Liquid cultures of *A. fumigatus*. To promote production of proteolytic activity at neutral pH, *A. fumigatus* was grown in liquid medium containing protein as the sole nitrogen source [1.2% (w/v) BSA supplemented with 1.2% (w/v) YCB or 0.2% (w/v) soy protein]. The pH was adjusted to 7.0 with 1 M NaOH and was observed to increase slightly to pH 7.5 during growth of the fungus. To test proteolytic activity at low pH in BSA liquid medium, 1.2% (w/v) BSA supplemented with 1.2% (w/v) YCB was adjusted to pH 4.0 with 1 M HCl. To test proteolytic activity at low pH in soy protein liquid medium, 0.2% (w/v) soy protein was dissolved in 68 mM citrate buffer (pH 4.0). One hundred millilitre flasks containing 50 ml of medium were inoculated with approximately 10^7 spores each and incubated for 96 h at 30 °C on an orbital shaker at 200 r.p.m. The pH of the medium was measured at 48 h, 72 h and the end of the incubation and was found to remain constant.

Gene replacement. *AfugprA* replacement was performed in *A. fumigatus* D141 and in the D141 *pepΔ* mutant. The plasmid *pΔgprA* was designed to function as a one-step gene replacement vector in transformation experiments and was constructed using pSK397 (Krappmann *et al.*, 2005, 2006). The *pΔgprA* plasmid contained *AfugprA* replaced by the *E. coli* hygromycin B phosphotransferase gene (*hph*) under the control of a truncated *gpd* promoter and the *trpC* terminator of *Aspergillus nidulans* (Fig. 1). For plasmid construction, two approximately 1.2 kb fragments covering the 5'- and 3'-flanking regions of *AfugprA* and small portions (<100 bp) of this gene (*Nflr* and *Cflr*, respectively) were PCR-amplified using genomic *A. fumigatus* DNA as a template. P1/P2 and P3/P4 primers, which contained additional restriction sites at the 5' extremity for subsequent cloning steps (Table 1), were used to amplify *Nflr* and *Cflr*, respectively. *Nflr* and *Cflr* were subsequently digested with *NotI/XmaI* and *PacI/HinDIII*, respectively, and were inserted end-to-end into a modified pBluescript II SK(+) (Stratagene) in which a *PacI* restriction site has been previously added in the multiple cloning site. The pBluescript construction generated contained, between unique *NotI* and *PacI* sites, *Nflr* and *Cflr* in the same orientation as in the *A. fumigatus* genome, and two different *SfiI* sites located between *Nflr* and *Cflr* for subsequent directive cloning (Kämper, 2004). Thereafter, the cassette containing the hygromycin-resistance gene was excised with *SfiI* from pSK397 and cloned into the *SfiI* site of the pBluescript construction to generate *pΔgprA*.

Transformation of wild-type *A. fumigatus* D141 and the D141 *pepΔ* mutant was accomplished according to a protocol that has been used for *A. nidulans* and *A. fumigatus* (Tilburn *et al.*, 1983). Transformation of 10^7 protoplasts with 5 µg *PacI* and *NotI* double-digested *pΔgprA* typically yielded 100–200 hygromycin-resistant colonies. After overnight *hph* expression, the transformants were incubated on agar, based on GYE medium [1% (w/v) glucose, 0.5% (w/v) yeast extract] containing 200 µg hygromycin ml⁻¹ (Sigma), and selected after 5 days of incubation at 20 °C followed by an overnight incubation at 42 °C. Transformants initially identified as hygromycin-resistant were picked and subcultured again on agar containing hygromycin.

A. fumigatus gprAΔ mutants were identified by PCR of genomic DNA from various numbers of the hygromycin-resistant colonies as previously performed for selection of other protease- and transporter-deficient mutants (Reichard *et al.*, 2006; Léchenne *et al.*, 2007). Two pairs of specific *ΔgprA* primers (P5/P6 and P7/P8, Table 1) were used to verify correct *gprA* replacement. In each primer pair,

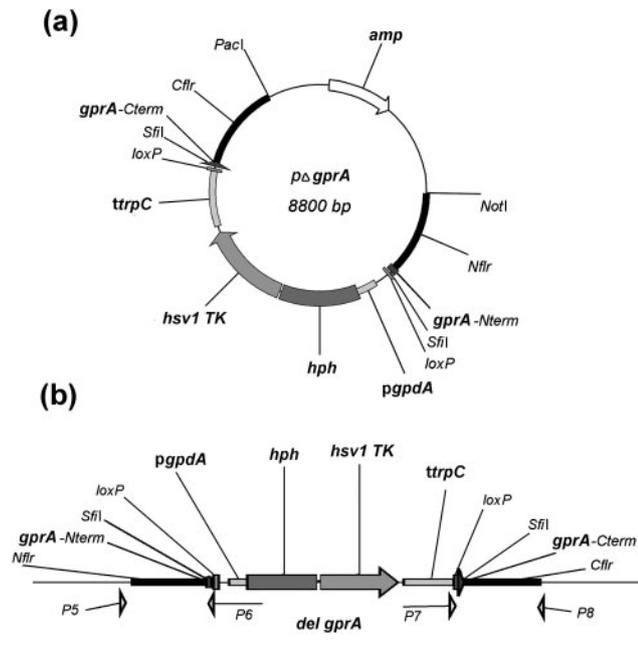


Fig. 1. (a) Map of $p\Delta gprA$ used for *AfuGprA* replacement. (b) Genomic DNA of generated *A. fumigatus gprAΔ mutants. The transforming DNA was excised from $p\Delta gprA$ via double digestion with *NotI* and *PacI*. Screening for homologous integration was done by PCR with primer pairs P5/P6 and P7/P8 (see Methods). *amp*, ampicillin-resistance gene; *gprA-Nterm*, 3'-extremity of *AfuGprA*; *gprA-Cterm*, 5'-extremity of *AfuGprA*; *hph*, *E. coli* hygromycin B phosphotransferase gene; *Nflr* and *Cflr*, 1.2 kb fragments covering, respectively, the N-terminal and C-terminal flanking regions including small portions of *AfuGprA* (<100 bp); *hsv1 TK*, thymidine kinase-encoding sequence from the herpes simplex virus type 1; *loxP*, specific recombinase binding sites of plasmid pSK397 (18–19); *pgpdA*, truncated *A. nidulans gpd* promoter; *ttrpC*, terminator region of the *A. nidulans trpC* gene.*

one primer hybridized with a part of the former transformation plasmid sequence and the other primer hybridized with genomic DNA near the desired homologous integration locus (Fig. 1). The predicted PCR product sizes were 1343 and 1350 bp with P5/P6 and P7/P8 primer pairs, respectively, if the transforming DNA was integrated at the homologous site. Two *A. fumigatus* D141 *gprA*Δ and two D141 *pepA gprA*Δ mutant strains were identified among 20 and 30 hygromycin-resistant colonies, respectively, which were tested for gene disruption.

Recombinant protease production. To construct *P. pastoris* strains producing GprA, DNA encoding N-terminal and C-terminal portions of the protein was amplified by PCR with a standard protocol using homologous sense and antisense primers (P9 and P10, Table 1) and 200 ng *A. fumigatus* genomic DNA as no intron was predicted in the gene encoding *AfuGprA* (*AfuGprA*). The PCR product was digested with *XhoI* and *BglII*, for which a site was previously designed at the 5' end of the primers and inserted into pKJ113 (Borg-von Zepelin *et al.*, 1998) digested with *XhoI/BamHI* to generate the expression plasmid p*AfuGprA*. Transformation of *P. pastoris* GS115 with *EcoRI*-linearized plasmid DNA, selection of transformants and production of recombinant *AfuGprA* in methanol medium were performed as previously described (Borg-von Zepelin *et al.*, 1998).

Purification of heterologously produced *AfuGprA*. *AfuGprA* was purified at 4 °C as follows. Secreted proteins from 250 ml *P. pastoris* culture supernatant were concentrated by ultrafiltration to 6 ml using a Centricon Plus-70 (10 kDa cut-off; Millipore). Thereafter, the concentrate was desalted with a PD10 column (Amersham Pharmacia) and applied to a DEAE-Sepharose column which had previously been equilibrated with 100 mM Tris/HCl buffer (pH 9.5). After washing the column with the same buffer, the recombinant protein was eluted with 100 mM Tris/HCl buffer (pH 8). Enzymic activity was tested with resorufin-labelled casein as a substrate (see below) and active fractions were pooled.

Protein gel electrophoresis. Extracts were analysed by SDS-PAGE with a separation gel of 12% polyacrylamide (Laemmli, 1970). The gels were stained either with 0.1% Coomassie brilliant blue R-250 (Bio-Rad) in ethanol/acetic acid/water (40:10:50, by vol.) or with silver nitrate according to Chevillet *et al.* (2006).

Protein identification by LC-MS/MS. Identification and confirmation of heterologously expressed proteins were obtained by a LC-MS/MS analysis approach. Recombinant enzyme at pH 6.0 or after 60 min incubation at room temperature in formic acid buffer (pH 4.0) was subjected to regular SDS-PAGE (12%). Coomassie-blue-stained bands were excised from the SDS-PAGE and in-gel digested with sequencing-grade chymotrypsin (Promega) as described by Shevchenko *et al.* (1996) and Wilm *et al.* (1996). Extracted peptides were analysed on a hybrid linear trap LTQ-Orbitrap mass spectrometer (Thermo Fisher) interfaced via a TriVersa Nanomate (Advion Biosciences) to an Agilent 1100 nano HPLC system (Agilent Technologies). Solvents used for the mobile phase were 95:5 (v/v) H₂O/acetonitrile with 0.1% (v/v) formic acid (solvent A) and 5:95 (v/v) H₂O/acetonitrile with 0.1% (v/v) formic acid (solvent B). Peptides were loaded onto a trapping microcolumn (ZORBAX 300SB C18; 5 mm × 300 μm i.d., 5 μm; Agilent) in 97:3 (v/v) H₂O/acetonitrile plus 0.1% (v/v) formic acid at a flow rate of 10 μl min⁻¹. After 5 min, they were back-flush eluted and separated on a reversed-phase nanocolumn (ZORBAX 300SB C18 column; 75 μm i.d. × 15 cm, 3.5 μm; Agilent) at a flow rate of 300 nl min⁻¹ with a gradient from 5 to 85% acetonitrile in 0.1% formic acid: 5 min at 0% solvent B, from 0 to 25% B in 35 min, 25 to 50% B in 15 min, 50 to 90% in 5 min, 90% B for 10 min, 90 to 0% in 5 min and 15 min at 0% (total time: 90 min).

For spraying, a 400-nozzle ESI Chip (Advion Biosciences) was used at a voltage of 1.65 kV, and the mass spectrometer capillary transfer temperature was set at 200 °C. In data-dependent acquisition controlled by Xcalibur 2.0.7 software (ThermoFisher), the six most intense precursor ions detected in the full MS survey performed in the Orbitrap (range 350–1500 *m/z*, resolution 60 000 at *m/z* 400) were selected and fragmented. MS/MS was triggered by a minimum signal threshold of 10 000 counts, carried out at relative collision energy of 35% and with isolation width of 4.0 amu. Only precursors with a charge higher than 1 were selected for collision-induced dissociation (CID) fragmentation, and fragment ions were analysed in the LTQ linear trap. The *m/z* of fragmented precursors was then dynamically excluded, with a tolerance of 0.01 amu, from any selection for 120 s.

From *raw* files, MS/MS spectra were exported as *mgf* (Mascot Generic File, text format) files using the *extract_msn.exe* script (Thermo Fisher) with the following settings: peptide mass range, 500–5000; minimum total ion intensity threshold, 500; minimum number of fragment ions, 15; minimum signal-to-noise ratio needed for a peak to be written, 3.

Samples were analysed using Mascot 2.2 (Matrix Science). Mascot was set up to search a custom-built database containing the sequences of the glutamic protease *AfuGprA* and of contaminants (enzymes, keratins). Semi-specific cleavage at F, L, W and Y (not before P) was

Table 1. Primers used in this study

Primers P1–P4 were used to construct *Nflr* and *Cflr* fragments. Primers P5–P8 were used for screening *A. fumigatus gprΔ* mutants. For location of those primers in the *A. fumigatus gprΔ* mutant, see Fig. 1. Primers P9 and P10 were used for *AfugprA* gene expression in *P. pastoris*.

Primer	Sequence (5'–3')*	Location	PCR product size with cloning sites
P1	TATAT GCGGCGCC CATATTCGTCTGGCTCACC	Upstream sequence in <i>AfugprA</i>	1192 bp (<i>Nflr</i> fragment)
P2	ATATTACCGGGGG CCTGAGTGGCC TTGACGC TGTCTGTGAGG	Complement of <i>AfugprA</i> , <i>N-terminal region</i>	<i>NotI</i> – <i>SfiI</i> – <i>XmaI</i>
P3	ATATTA AAAGCTTGGCCATCTAGGCC CAGCGGCAA GGTTTTGACTT	Downstream sequence in <i>AfugprA</i>	1219 bp (<i>Cflr</i> fragment)
P4	TATCG TAAATTA AAATGAACCGCCTTCAGAGCTA	Complement of <i>AfugprA</i> , <i>C-terminal region</i>	<i>HindIII</i> – <i>SfiI</i> – <i>PacI</i>
P5	GGGCTGAAGGAAGAATACCC	Upstream sequence in <i>AfugprA</i>	1343 bp
P6	AGGTGATATCGGCCTGAGTG	pSK397	
P7	CGTTTACCCAGAATGCACAG	Downstream sequence in <i>AfugprA</i>	1350 bp
P8	GCACAGTGTATCGGACGAC	pSK397	
P9	GTT CTCGAGCC ATCGCTGCCCTCACA	<i>AfugprA</i>	782 bp
P10	GTT GGATCCT TAGACATACTTAACAGTGAC	Complement of <i>AfugprA</i>	<i>XhoI</i> – <i>BamHI</i>

*Nucleotides shown in italics and bold represent cloning sites.

used as the enzyme definition, with a maximum of two missed cleavages allowed. Mascot was searched with a fragment ion mass tolerance of 0.50 Da and a parent ion tolerance of 10 p.p.m. The iodoacetamide derivative of cysteine was specified in Mascot as a fixed modification. Deamidation of asparagine and glutamine, oxidation of methionine and protein *N*-acetylation were specified as variable modifications.

Proteolytic activities. Endoproteolytic activity was measured with 0.02% (w/v) resorufin-labelled casein as a substrate (Roche Diagnostics) at different pHs in sodium citrate buffer (50 mM final concentration; pH 2.0–7.0) in a total volume of 0.5 ml (Borg-von Zepelin *et al.*, 1998; Reichard *et al.*, 2006). After incubation at 37 °C, the undigested substrate of the enzyme/substrate mix was precipitated by trichloroacetic acid (4%, v/v, final concentration) and separated from the supernatant by centrifugation. Five hundred microlitres of Tris/HCl buffer (500 mM; pH 9.4) was added to the collected supernatant (neutralization step) and the A_{574} of the mixture (1 ml) was measured. For practical purposes, 1 mU activity was defined as producing an increase in absorbance of 0.001 min⁻¹ in a proteolytic assay (1 ml) at optimal pH for activity. Activities were measured in culture supernatant without and with the presence of the aspartic protease inhibitor pepstatin at a concentration of 10 µg ml⁻¹.

Tripeptidyl-peptidase and AfuS28 activities were measured as previously described, using Phe-Pro-Ala-pNA and Ala-Ala-Ala-pNA (Bachem), respectively, as substrates (Reichard *et al.*, 1997; Sriranganadane *et al.*, 2010). The reaction mixture contained 80 µl culture supernatant, 5 mM substrate and 50 mM citrate buffer (pH 4) in a total volume of 100 µl. After incubation at 37 °C for 0.5–4 h, the reaction was terminated by adding 5 µl glacial acetic acid. The *p*-nitroaniline (pNA) released was measured by spectrometry as a change in A_{405} . A control with a blank substrate and blank culture broth was carried out in parallel. Enzyme activities were expressed in mU (nmol released pNA min⁻¹).

Phylogenetic analyses. Amino acid sequences of a selection of putative glutamic proteases from *Aspergillus* spp., and previously characterized proteases from the ascomycetes *Talaromyces emersonii*, *Botryotinia fuckeliana*, *Sclerotinia sclerotium*, *Scytalidium lignicolum* and *Cryphonectria parasitica* (MEROPS G01 family) were aligned

using CLUSTAL W as implemented in BioEdit Sequence Alignment Editor software (Hall, 1999). Phylogenetic analyses were performed using the following reconstruction methods and parameters: PhyML (Guindon & Gascuel, 2003) with SH-like approximate likelihood ratio test, four substitution rate categories, and estimation of gamma distribution parameter and proportion of invariable sites; BIONJ (Gascuel, 1997), using a Dayhoff PAM substitution matrix; TNT (Goloboff *et al.*, 2008) with sectorial search and tree fusing options; and MrBayes (Ronquist & Huelsenbeck, 2003) with GTR likelihood model and 100 000 Markov chain Monte Carlo generations. Phylogenetic trees were edited by using Dendroscope (Huson *et al.*, 2007).

RESULTS

Importance of AfuGprA in extracellular protein digestion

To evaluate the importance of AfuGprA in protein digestion at low pH, *AfugprA* was replaced in *A. fumigatus* wild-type D141 and *pepΔ* mutant as described in Methods. *A. fumigatus* wild-type D141, and *pepΔ*, *gprΔ* and *pepΔ gprΔ* mutants were first tested for their ability to grow using protein solid and liquid media at neutral and low pH (Fig. 2). Two different protein sources, BSA and soy protein, which is a heterogeneous peptide mixture (Fig. 3c), were used.

No phenotypic difference was observed between wild-type and mutant strains at neutral pH. At low pH, *A. fumigatus gprΔ* could not be differentiated from the wild-type D141 strain using the two protein sources. In contrast, *A. fumigatus pepΔ gprΔ* was unable to grow on BSA agar medium at low pH whereas *A. fumigatus pepΔ* showed only slightly reduced colony size (Fig. 2). The four *A. fumigatus* strains showed comparable growth rate on soy protein agar medium at low pH (Fig. 2).

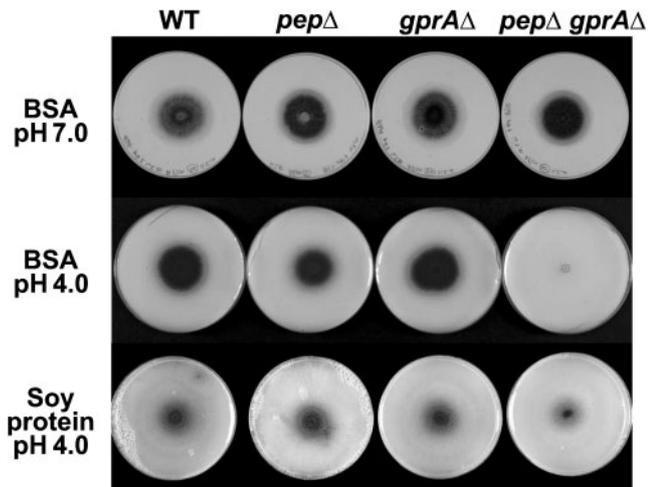


Fig. 2. Growth of *A. fumigatus* wild-type D141 (WT), and *pepΔ*, *gprAΔ* and *pepΔ gprAΔ* mutants on BSA and soy protein agar medium.

Similar results were obtained with liquid cultures at low pH: *A. fumigatus* wild-type D141, and *pepΔ* and *gprAΔ* mutants grew well in BSA and soy protein liquid medium; and only residual growth of *A. fumigatus pepΔ gprAΔ* without clarification of the medium was observed with BSA liquid medium, while the fungus grew well with soy protein (data not shown). After 96 h growth in the latter medium, the secreted activity of the *gprAΔ* and *pepΔ* mutants was approximately 70 and 20% of that of *A. fumigatus* wild-type D141, respectively (Fig. 4). In the presence of the aspartic protease inhibitor pepstatin, activity of the *gprAΔ*

mutant was totally inhibited and activity of the wild-type strain was reduced to the rate of that of the *pepΔ* mutant. *A. fumigatus pepΔ gprAΔ* soy protein culture supernatant was completely devoid of extracellular endoproteolytic activity. Comparable tripeptidyl-peptidase and AfuS28 activities were found to be secreted at low pH by *A. fumigatus* wild-type D141, and *pepΔ*, *gprAΔ* and *pepΔ gprAΔ* mutants in soy protein medium, as well as by *A. fumigatus* D141, *pepΔ* and *gprAΔ* in BSA medium (mean \pm SD 7.6 ± 1.0 and 0.03 ± 0.01 mU, respectively). The production of these exoproteases was apparently not changed by deleting *pep* and *gprA*.

Characterization of AfuGprA

SDS-PAGE results of culture supernatants of *A. fumigatus* wild-type D141, *pepΔ*, *gprAΔ* and *pepΔ gprAΔ* are shown in Fig. 3. The absence of a 36 kDa band in *A. fumigatus pepΔ* and a 26 kDa band in *A. fumigatus gprAΔ* mutants identified Pep and GprA, respectively. AfuGprA was characterized as a recombinant enzyme using *P. pastoris* as an expression system. A yield of $25 \mu\text{g ml}^{-1}$ recombinant protein in culture supernatant was obtained. Recombinant AfuGprA in *P. pastoris* culture supernatant showed an apparent molecular mass of 30 kDa, which is higher than that of AfuGprA secreted by *A. fumigatus* at pH 4 (Fig. 5, lane 1). However, after pre-incubation at pH 4.0, recombinant AfuGprA showed the same molecular mass as native AfuGprA (compare Fig. 5, lane 2, and Fig. 3). These results suggest that AfuGprA, like other G1 proteases, is made as a preproprotein and that the propeptide was cleaved and removed to generate the active enzyme through an autoproteolytic reaction.

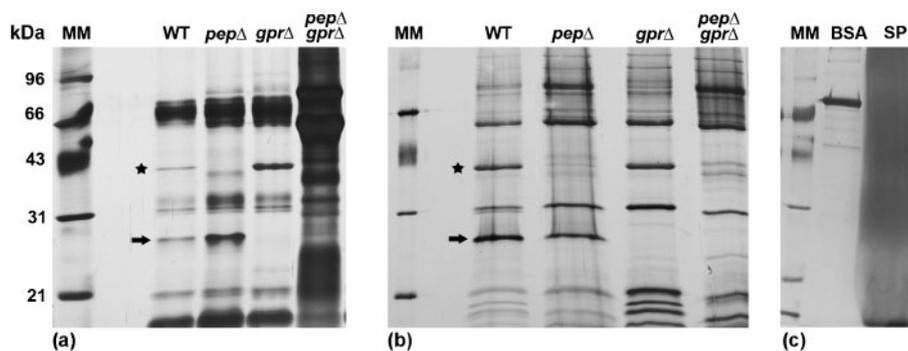


Fig. 3. (a, b) Identification of Pep and GprA in culture supernatant of *A. fumigatus* wild-type D141 (WT) and *pepΔ*, *gprAΔ* and *pepΔ gprAΔ* mutant strains growing in BSA (a) and soy protein (b) acidic liquid medium (pH 4.0). Flasks (200 ml) containing 50 ml medium were inoculated with approximately 10^7 spores and incubated for 96 h at 30°C on an orbital shaker at 200 r.p.m. Supernatant extracts were concentrated by ultrafiltration and acidic precipitation as previously described (Sriranganadane *et al.*, 2010). The precipitate from 2.0 ml culture supernatant was resuspended in $10 \mu\text{l}$ SDS-PAGE buffer, and subsequently loaded on SDS-12% polyacrylamide gels. (c) SDS-12% polyacrylamide gels with $1 \mu\text{l}$ non-digested BSA and soy protein liquid medium ($1 \mu\text{g}$ protein). The gels were stained with silver nitrate according to Chevallet *et al.* (2006). The bands corresponding to Pep and GprA are marked with an asterisk and an arrow, respectively, in panels (a) and (b). MM, molecular mass markers.

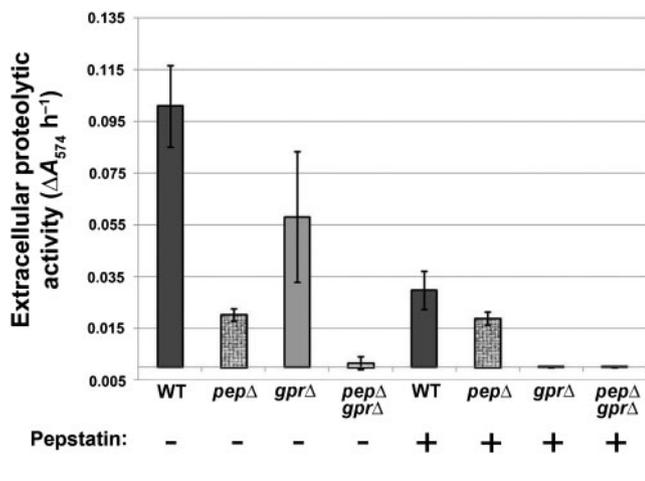


Fig. 4. Secreted endoproteolytic activity of *A. fumigatus* wild-type D141, and *pep* Δ , *gprA* Δ and *pep* Δ *gprA* Δ mutants in soy protein medium (pH 4.0) after 96 h growth at 30 °C. Activities from 50 μ l culture supernatant were measured as described in Methods using resorufin-labelled casein as a substrate in the absence and in the presence of pepstatin (10 μ g ml⁻¹). Means \pm SD of values recorded in three independent experiments are indicated.

The deduced amino acid sequence of the *AfugprA* open reading frame shows a 16–18 amino acid hydrophobic signal peptide with a putative signal peptidase cleavage site Ala-Ile-Ala in accordance with von Heijne's rule (von Heijne, 1986)

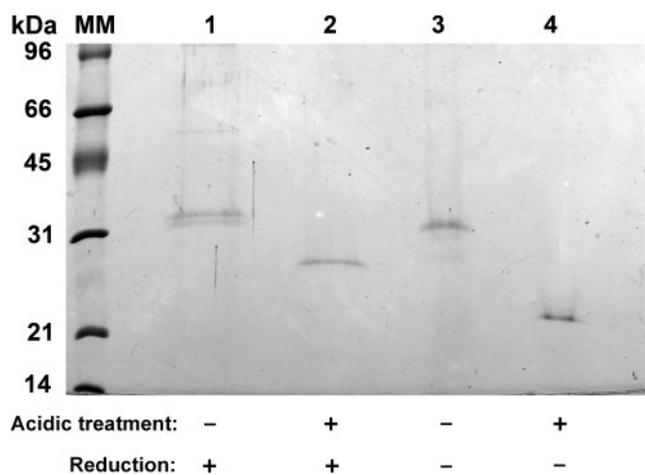


Fig. 5. SDS-PAGE (9% gel) of recombinant AfuGprA secreted by *P. pastoris*. The gel was stained with Coomassie blue. Approximately 0.3 μ g recombinant reduced (lanes 1 and 2) and non-reduced (lanes 3 and 4) AfuGprA was loaded on the gel. AfuGprA reduction was performed after heat treatment in SDS-PAGE buffer containing 2% (v/v) β -mercaptoethanol. In lanes 2 and 4, recombinant AfuGprA was incubated at pH 4.0 for 30 min at 30 °C. The double band observed for native proGprA attests to two different signal peptidase cleavage sites (see Fig. 6). MM, molecular mass markers.

(see Supplementary Fig. S1, available with the online version of this paper). The AfuGprA proprotein generated after signal peptidase cleavage comprised 252 amino acids, with a theoretical mass of 26.34 kDa. Therefore, the molecular mass of reduced proAfuGprA was overestimated in SDS-PAGE gels as it migrates as a 30 kDa protein. Of particular note, a similar observation was made with *Botrytis cinerea* secreted G1 protease (Rolland *et al.*, 2009). Non-reduced proAfuGprA and AfuGprA showed a higher electrophoretic mobility than reduced proAfuGprA and AfuGprA, respectively, after a 2 min heating treatment in SDS-PAGE buffer (Fig. 5, lanes 3 and 4).

Identification of the heterologously produced AfuGprA was also confirmed using electrospray ionization (ESI)-LC-MS/MS. Chymotryptic digestion was performed in gel slices containing the 30 kDa protein secreted by *P. pastoris* at neutral pH, and the 26 kDa protein obtained after treatment at pH 4.0 (Fig. 5). MS analysis allowed the detection of different AfuGprA peptides matching with residues 42–269 in the 30 kDa secreted protein. Peptides matching with residues 61–269 but not with residues 1–61 were detected in the 26 kDa protein (Fig. 6). These results confirmed that active AfuGprA was generated after cleavage of a prosequence at low pH at residue 61 or a few residues before as suggested from the alignment with other glutamic proteases. Gln and Glu residues of the catalytic dyad of AfuGprA were identified at positions 120 and 206, respectively (Fig. S1).

Characterization assays of the recombinant AfuGprA were done after purification by ion-exchange chromatography. The enzyme was found to be active between pH 2.0 and 6.0 with an optimum activity at pH 5.0 on resorufin-labelled casein. The specific activity of AfuGprA was 2.2 mU (μ g protein)⁻¹ at pH 5.0. The enzyme was not inhibited by pepstatin (aspartic protease inhibitor), E64 or iodoacetamide (cysteine protease inhibitors), EDTA or phosphoramidon

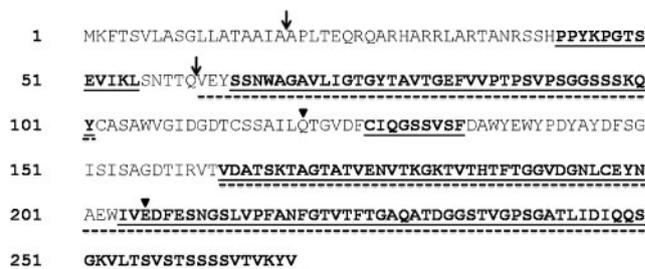


Fig. 6. Amino acid sequence of PreproAfuGprA. MS-detected peptides generated by chymotryptic digestion of proAfuGprA secreted by *P. pastoris* and mature AfuGprA after incubation at low pH are underlined with full and dashed lines, respectively. Vertical arrows indicate two potential signal peptidase cleavage sites at amino acid positions 16 and 18, and the cleavage site in the proprotein generating the mature enzyme. Active-site residues Gln and Glu forming the catalytic dyad are indicated by solid triangles.

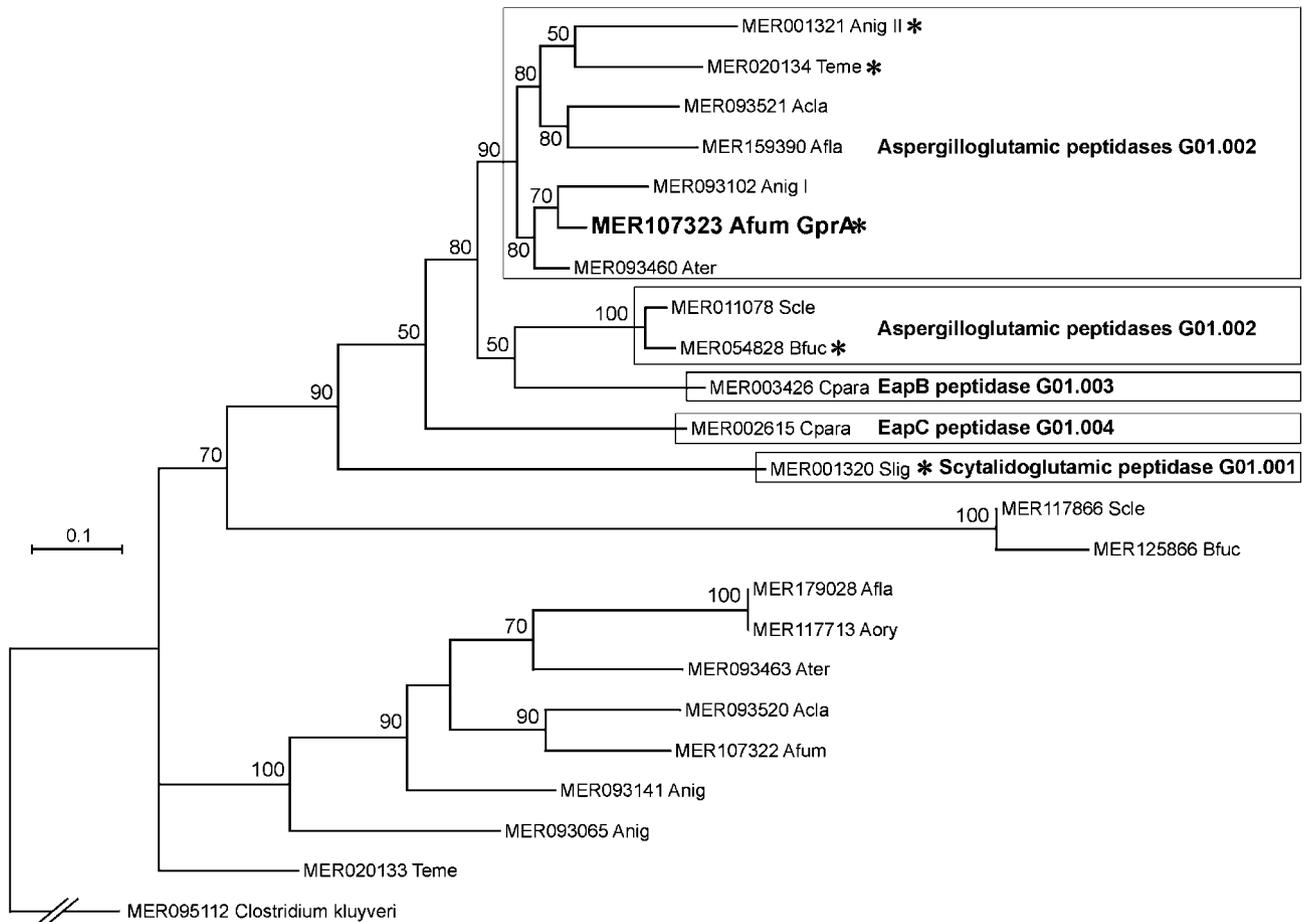


Fig. 7. Phylogenetic position of *A. fumigatus* GprA with respect to other fungal glutamic proteases obtained by a maximum-likelihood analysis using PhyML. Asterisks indicate proteases that have been characterized biochemically. A glutamic protease from *Clostridium kluveri* (*Firmicutes*, *Bacteria*) was used a potential outgroup. MEROPS identifiers and names of type proteases are indicated. Numbers at nodes are approximate likelihood-ratio test values of branch support. The scale bar indicates the number of expected changes per site. Abbreviations: Afum, *Aspergillus fumigatus*; Anig, *Aspergillus niger*; Acla, *Aspergillus clavatus*; Ater, *Aspergillus terreus*; Afla, *Aspergillus flavus*; Teme, *Talaromyces emersonii*; Scle, *Sclerotinia sclerotium*; Bfuc, *Botryotinia fuckelania*; Cpara, *Cryphonectria parasitica*; Slig, *Scytalidium lignicolum*.

(metalloprotease inhibitors), or PMSF, antipain, chymostatin, leupeptin or aprotinin (serine protease inhibitors). Being resistant to class-specific inhibitors of aspartic, cysteine, metallo- and serine proteases, AfuGprA behaves like other previously described glutamic proteases (Oda, 2004; Takahashi, 2004; O'Donoghue *et al.*, 2008).

DISCUSSION

Two endoproteases, Pep and GprA, are secreted by *A. fumigatus* in an acidic protein medium. We have shown that either *A. fumigatus* Pep or GprA was necessary for fungal growth in BSA medium at low pH. AfuGprA deficiency alone did not affect *A. fumigatus* growth while a slight growth rate reduction of the single *pepΔ* mutant was detected. These results apparently contrast with those obtained with *T. emersonii*, growth of which is strongly

inhibited with glutamic peptidase antagonists (O'Donoghue *et al.*, 2008). However, the G1 inhibitors tested could affect functions in the fungus other than G1 protease activity.

During the process of protein digestion the main function of the endoproteases is to produce large numbers of free-end peptides on which exopeptidases may act. Synergism of non-specific amino peptidases (Lap at neutral pH, Seds at low pH) and prolyl peptidases (DppIV or AfuS28 at neutral pH, AfuS28 at low pH) allows sequential degradation of large peptides previously generated by endoproteolysis into amino acids, and di- and tripeptides (Byun *et al.*, 2001; Sriranganadane *et al.*, 2010). Large peptide cleavage by further exoproteolytic activity is also essential for fungi using protein as sole nitrogen and carbon source as only amino acids and short peptides can be assimilated by transporters. When using soy protein as source, which is a heterogeneous peptide mixture, exoproteolytic activity alone was sufficient for fungal growth.

Two major proteases, aspergillopepsin A (Pep) and aspergillopepsin B (aspergillopepsin II), were found in the culture filtrate of *A. niger* (Koaze *et al.*, 1964). An *A. niger* mutant deficient in the secretion of both proteases was generated by UV mutagenesis (Mattern *et al.*, 1992). This strain, in which a mutation is located in a gene encoding a regulator of extracellular protease genes called PrtT (Punt *et al.*, 2008), has only 1–2% extracellular protease activity at pH 5.3. In contrast, *A. fumigatus pepΔ gprAΔ* produces substantial proteolytic activity and grows well in a protein medium at this pH because of the secretion of Alp and Mep (data not shown). Both enzymes are still active at pH 5.3 (Monod *et al.*, 1991, 1993b). The *A. fumigatus* genome contains an orthologue of *A. niger* PRTT, which was shown to control the expression of multiple secreted proteases such as Alp, Mep, Pep, SedB and DppIV (Sharon *et al.*, 2009; Bergmann *et al.*, 2009). However, functional PrtT dependence of *A. fumigatus* secreted proteases was investigated at neutral pH only, and the role of PrtT in protein gene expression was not addressed at low pH. Virulence studies were not within the scope of the present investigations, but *A. fumigatus prtT* deletion mutants were shown to be not attenuated in virulence in immunosuppressed mice (Sharon *et al.*, 2009; Bergmann *et al.*, 2009).

Being a single-chain protein, mature AfuGprA resembles other previously characterized fungal glutamic proteases from *T. emersonii*, *S. lignicolum* and *B. fuckeliana*. However, it differs from *A. niger* aspergillopepsin II, which is a two-chain protein composed of a 39-residue light chain and a 173-residue heavy chain. Both chains of aspergillopepsin II are non-covalently bound and originate from a single precursor of 282 amino acids which contains a 14 amino acid intervening peptide (Fig. S1) that is absent in the AfuGprA preproprotein. The heterodimer results from autoproteolytic cleavage at the level of the intervening peptide as for heterodimer yapsins, where an autocleavage occurs within an internal loop of the polypeptide chain (Gagnon-Arsenault *et al.*, 2006). In fact, none of the currently available sequences of fungal glutamic proteases possesses the intervening peptide found in *A. niger* aspergillopepsin II. This two-chain glutamic protease seems therefore to be an exception.

Sequence searches in the GenBank and MEROPS databases, sequence alignments and phylogenetic analyses indicated that putative orthologues of AfuGprA occur in all *Aspergillus* species (Fig. 7). Two G1 proteases are found in the *A. fumigatus* genome, GprA (MER107323) and a second G1 protease (MER107322) which was not detected in the present study. AfuGprA belongs to a robust clade including all aspergilloglutamic peptidases (MEROPS identifier G01.002) from *Aspergillus* spp. and *T. emersonii*. AfuGprA is related most closely to MER093102 of *A. niger* (76% identity, 89% similarity), a putative protease. Characterized aspergilloglutamic peptidases from *Sclerotinia sclerotium* and *B. fuckeliana*, as well as glutamic proteases from *C. parasitica* and *Scytalidium lignicolum* (G01.001, G01.003

and G01.004), branch basally with respect to *Aspergillus* G01.002 peptidases. It may be noted that most fungal species possess at least two paralogous genes encoding glutamic proteases. Each species typically harbours an aspergilloglutamic-like protease belonging to the G01.001–G01.004 clades and a glutamic protease designated ‘unassigned’ in the MEROPS database and branching at the base of the tree. None of these unassigned glutamic proteases has been characterized experimentally to date. The relatively high amino acid sequence divergence between the glutamic proteases found in a given genome (only 37% identity between *A. fumigatus* GprA and *A. fumigatus* unassigned protease MER107322) together with the scattered distribution of sequences in the phylogenetic tree suggests that these paralogues arose by ancient gene duplication.

In conclusion, we have shown that secreted glutamic protease rescues aspartic protease Pep deficiency in *A. fumigatus* and that exoproteolytic activity is not sufficient for complete protein hydrolysis and fungal growth in an acidic protein medium. Pep and GprA constitute a pair of acidic endoproteases analogous to the *A. fumigatus* Alp/Mep pair (Jaton-Ogay *et al.*, 1994) at neutral pH, in which expression of either one of them was shown to be sufficient for fungal growth in a protein medium.

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