The *Aspergillus fumigatus* *mepB* gene encodes an 82 kDa intracellular metalloproteinase structurally related to mammalian thimet oligopeptidases

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***Aspergillus fumigatus* produces an 82 kDa intracellular metalloproteinase that hydrolyses the Pz-peptide, 4-phenylazobenzyloxycarbonyl-Pro-Leu-Gly-Pro-Arg, a typical substrate of members of the thimet oligopeptidase family which is ubiquitously distributed across animal species. The *A. fumigatus mepB* gene encoding this 82 kDa metalloproteinase was cloned and sequenced. Analysis of the deduced amino acid sequence of *mepB* showed that the MepB protein is a cytosolic zinc metalloproteinase of the thimet oligopeptidase family (M3) and as such is probably involved in the intracellular degradation of small peptides. An *A. fumigatus* mutant that lacks the MepB Pz-peptidolytic activity was constructed by gene disruption at the *mepB* locus. Analysis of this mutant did not reveal any detectable phenotype.**

Keywords: aspergillosis, metalloproteinase, Pz-peptide, thimet oligopeptidase

INTRODUCTION

In a previous report, we described the purification of an 82 kDa metalloproteinase (referred to as MepB in this paper) from intracellular soluble proteins of *Aspergillus fumigatus*, the main causative agent of pulmonary invasive aspergillosis (Cohen, 1991; Ibrahim-Granet *et al.*, 1994). The purified enzyme was shown to hydrolyse specifically the Pz-peptide, 4-phenylazobenzyloxycarbonyl-Pro-Leu-Gly-Pro-Arg (Pz-PLGPA; Wunsch & Heidrich, 1963) and to cleave native type I collagen (Ibrahim-Granet *et al.*, 1994). Inhibitor studies suggested that the enzyme is a metalloproteinase. A similar activity was also identified in a wide variety of pathogenic fungi, suggesting that it could be involved in a conserved cellular mechanism (Ibrahim-Granet *et al.*, 1996). Because of its ability to cleave native collagen, one of the lung matrix components, MepB was for some time thought to be involved in the physiological catabolism of collagen during infection and to belong to the family of interstitial collagenases (family M10 of Rawlings & Barrett, 1995). This family is one of the largest families of metalloproteinases and includes a variety of enzymes

from organisms as diverse as bacteria (Maeda & Morihara, 1995), plants and animals (Dioszegi *et al.*, 1995). The known enzymes are all secreted endopeptidases and those in mammals, called matrixins, are involved in the proteolytic degradation of collagen and other proteins of the extracellular matrix (Dioszegi *et al.*, 1995). Although MepB is detectable in small amounts in the culture medium, there is no evidence that the enzyme is ever secreted by *A. fumigatus*. However, the cytosolic localization as well as the ability of MepB to cleave the Pz-PLGPA peptide could indicate that this proteinase belongs to the thimet oligopeptidase family (family M3 of Rawlings & Barrett, 1995), the most diverse of the cytosolic metalloproteinase families including endo-oligopeptidase A (Camargo *et al.*, 1979), soluble metalloproteinase or endopeptidase 24.15 (Orlowski *et al.*, 1983) and oligopeptidase M or neurolysin (Checler *et al.*, 1986; Serizawa *et al.*, 1995).

To characterize further the structure of the 82 kDa MepB metalloproteinase, cloning and sequencing of the *A. fumigatus mepB* gene was conducted. The effect of disruption of the *mepB* gene on *A. fumigatus* viability and pathogenicity in a mouse model was investigated.

METHODS

Strains and culture conditions. *A. fumigatus* strains CBS 144-89 and G10 were used for this study. Strains were grown in Sabouraud glucose liquid medium (1% peptone, 2% glucose)

Abbreviation: Pz-PLGPA, 4-phenylazobenzyloxycarbonyl-Pro-Leu-Gly-Pro-Arg.

The GenBank accession number for the nucleotide sequence reported in this paper is U85769.

or in 1% yeast extract liquid medium. Cultures were grown with good aeration at 25 or 37 °C for 42 h.

Escherichia coli LE392 (Sambrook *et al.*, 1989) was used for the propagation of bacteriophage λ EMBL3 (Frischauf *et al.*, 1983). *E. coli* strain PAP105 [$\Delta(lac-pro)$ F'($lacI^{q1}$ $\Delta(lacZ)$ M15 pro^+ Tn10)] was used as the host for recombinant plasmids. Cells were grown at 37 °C in LB (10% NaCl; 10% Bactotryptone, Difco; 5% yeast extract; pH 7.5) medium supplemented with the β -lactam antibiotic carbenicillin (100 μ g ml⁻¹), tetracycline (15 μ g ml⁻¹) or chloramphenicol (25 μ g ml⁻¹) when required.

Amino acid sequencing. The amino-terminal and internal amino acid sequence analyses were performed according to Kawasaki *et al.* (1990). Briefly, the purified 82 kDa metalloproteinase (Ibrahim-Granet *et al.*, 1994) band cut out of an SDS-PAGE gel was digested with endoproteinase Lys-C from *Lysobacter enzymogenes* (Boehringer) and the supernatant was injected onto a DEAE-silica precolumn linked to a C-18 reverse-phase HPLC column. The column was developed with a 0–50% acetonitrile, 0.1% trifluoroacetic acid linear gradient and the A_{206} of the effluent was monitored. Automated peptide sequence analysis was carried out on an Applied Biosystems 470 gas-phase sequencer. Phenylthiohydantoin amino acids were detected with an on-line Applied Biosystems 120 A analyser.

PCR amplification of a segment of the *mepB* gene. The genomic DNA of strain *A. fumigatus* CBS 144-89 prepared according to Girardin *et al.* (1993) was used as a template to amplify a segment of the 82 kDa metalloproteinase gene. A sense primer was based on an internal amino acid sequence (YGHAAN): 5'-TAYGGNCAYGCNGCNAA-3'. The antisense primer (5'-GGNGCYTCNACRAARTC-3') was derived from a conserved amino acid sequence (DFVEAP) from the putative catalytic site of thimet oligopeptidases (Barrett *et al.*, 1995). The amplification protocol consisted of a denaturation step at 95 °C for 5 min, followed by 35 cycles of the following steps: denaturation at 95 °C for 1 min, annealing at 45 °C for 1 min, extension at 72 °C for 3 min. A last elongation step was done at 72 °C for 5 min. This amplification protocol yielded a 309 bp product that was subcloned into the *Sma*I site of pUC19 (Yanisch-Perron *et al.*, 1985) using procedures described below.

Cloning and sequencing of the *mepB* gene. General recombinant DNA techniques and Southern blot analyses were essentially performed as described by Sambrook *et al.* (1989) and Ausubel *et al.* (1992). Transformation of calcium/manganese-treated *E. coli* was as described by Hanahan *et al.* (1991).

The cloned 309 bp PCR product labelled with [α -³²P]dCTP using the Megaprime kit (Amersham) was used to screen a λ EMBL3 *Sau*3AI genomic library of *A. fumigatus* by plaque hybridization (Jaton-Ogay *et al.*, 1992; Girardin *et al.*, 1993). Positive phage plaques were isolated and rescreened to obtain single positive phage isolates. DNAs of two of the 18 positive phages, λ 5.1.1 and λ 8.1.1, were prepared and restriction enzyme analysis with *Eco*RI revealed a fragment of 2.2 kb that hybridized with the 309 bp probe. This fragment was subcloned into pUC19 generating two plasmids, pOG1 and pOG2, with the insert in the opposite orientation. To identify a DNA fragment carrying the 5' region of the *mepB* gene, an *Eco*RI-*Sph*I 600 bp fragment of pOG1 was labelled as described above and used to probe a *Sph*I-*Kpn*I digest of λ 8.1.1. A 3.2 kb *Sph*I-*Kpn*I hybridizing fragment was subcloned into *Sph*I-*Kpn*I-restricted pUC18, yielding plasmid

pOG3. An *Eco*RI-*Bgl*II 1.2 kb fragment from pOG3 was subcloned into *Eco*RI-*Bam*HI-restricted pUC18, yielding pOG4.

cDNAs corresponding to the 3' region of the *mepB* gene were obtained by PCR amplification of DNA of an *A. fumigatus* cDNA library (Jaton-Ogay *et al.*, 1992) using primers hybridizing to the coding strand and to λ gt11. The resulting PCR product was subcloned using the TA cloning kit (Invitrogen).

The DNA sequence of *mepB* was determined by the dideoxy chain-termination method (Sanger *et al.*, 1977) using pOG1, pOG2, pOG3, pOG4 and double-stranded plasmids derived from pOG1 and pOG2 by internal-restriction enzyme-mediated deletions (*Hind*III, *Pst*I, *Sac*I, *Sph*I) as templates. The M13 universal and reverse sequencing primers and a set of appropriate oligonucleotide primers were used. The sequence of a 2563 bp fragment was read on each strand. DNA sequence analysis, comparison of the protein sequence with those in the SWISS-PROT database, and multiple sequence alignments were done using the UWGCG package (Devereux *et al.*, 1984).

Disruption of the *mepB* gene. Disruption of *mepB* was performed in *MepB*-producing G10 (Monod *et al.*, 1993). An 840 bp *Sal*I-*Hind*III fragment of pOG3, corresponding to an internal *Sal*I-*Sph*I fragment of *mepB*, was subcloned into pCB1004, a derivative of pBC SK(+) (Stratagene) carrying the *E. coli* hygromycin phosphotransferase gene (*hph*) that is fused to the promoter region of the *Aspergillus nidulans* *trpC* gene and that can be used for selection of DNA-mediated transformation in *A. fumigatus* (Monod *et al.*, 1993). pCB1004 was a kind gift from Dr J. Sweigard (DuPont Nemours, Wilmington, USA). The resulting plasmid, pOG5 Δ , was linearized with *Hind*III and used to transform protoplasts of *A. fumigatus* strain G10 as previously described by Monod *et al.* (1993). After overnight expression of the *hph* gene, the transformants were selected on minimal medium supplemented with 200 μ g hygromycin B ml⁻¹. After purification to a single colony, the Pz-peptidolytic activity of each transformant was monitored using the assay described below. Disruption of the *mepB* gene in the transformant that lacked Pz-peptidolytic activity was confirmed by Southern analysis. Genomic DNA of strain G10 and of this transformant was prepared as previously described by Girardin *et al.* (1993) and digested with *Pvu*II and *Hind*III. DNA restriction fragments transferred onto nylon membranes (Hybond N⁺; Amersham) were hybridized with an 850 bp *Pst*I fragment of plasmid pOG2 labelled as described above.

Pz-peptidase assay. For preparation of the high-speed supernatant, the mycelium was harvested by filtration, washed with distilled water, and suspended in 50 mM NH₄HCO₃ buffer (pH 8.3). The cells were ruptured with Ballotini beads at 4 °C in a Dyno cell disintegrator (WAB). The homogenate was centrifuged at 4000 g for 10 min at 4 °C to remove fungal cell walls and then at 100000 g for 1 h at 4 °C to remove cell membranes. Protein concentration was measured by the method of Bradford (1976). Pz-peptidolytic activity in the high-speed supernatant was identified using the substrate Pz-PLGPA according to Wunsch & Heidrich (1963) modified as described previously (Ibrahim-Granet *et al.*, 1994).

Mouse infection. Wild-type and *mepB1* strains were inoculated by inhalation into female CPF OF1 (18–20 g) mice. All animals were immunosuppressed by peritoneal injection of 200 μ l cortisone acetate (25 mg ml⁻¹ in 0.9% NaCl; Monod *et al.*, 1993). Injections were given 3 d before infection, on the day of infection and 2 and 4 d after infection. Conidia (10⁶ and 10⁸) of either strain were inoculated intranasally into cohorts of 10 mice. Mortality was evaluated every day for 15 d.

Fig. 1. Restriction maps of phage λ 8.1.1 and plasmids used in this study and outline of the sequencing strategy. A restriction map of a region of phage λ 8.1.1 is shown together with the location and orientation of transcription of the *mepB* gene (thin arrow). Inserts of the different plasmids obtained by subcloning DNA fragments of λ 8.1.1 into pUC18 or pCB1004 are shown as thin lines. The thick arrows represent the region sequenced in each sequencing step. The sequencing was done on both DNA strands with double-stranded DNA. E, *EcoRI*; G, *BglII*; H, *HindIII*; K, *KpnI*; L, *Sall*; Sc, *SacI*; Sp, *SphI*.

RESULTS AND DISCUSSION

PCR amplification and cloning of a fragment of the *mepB* gene

The amino-terminal amino acid sequence (APEHLGRP-PQALPL), and the sequence of two internal peptides (PEP13, YGHAANFNLPQGFIDADGG; PEP19, KLW-TFK) of MepB were determined. Comparison of these amino acid sequences with those in the SWISS-PROT database revealed a significant (approx. 63%) identity of PEP13 with a region of two previously described metalloproteinases: the yscD oligopeptidase of *Saccharomyces cerevisiae* (the product of the *PRD1* gene) (Büchler *et al.*, 1994) and the rat thimet oligopeptidase (Pierotti *et al.*, 1990), two members of the thimet oligopeptidase family (Barrett *et al.*, 1995).

To amplify a segment of the *mepB* gene, two oligonucleotides were designed: a sense primer corresponding to the first six residues of PEP13 (YGHAAN) and an antisense primer synthesized on the basis of the highly conserved sequence (DFVEAP) of the putative catalytic site characterizing the thimet oligopeptidase family (Barrett *et al.*, 1995), assuming that it would be similarly conserved in the *A. fumigatus* MepB proteinase. Using these primers, a 309 bp fragment was amplified from genomic DNA of *A. fumigatus* strain CBS 144-89 by PCR. After purification and subcloning, the nucleotide sequence of the 309 bp PCR product was determined. The deduced amino acid sequence was shown to contain the amino acid sequence of PEP13, indicating that this PCR product was genuinely derived from the *mepB* gene.

Cloning of the *mepB* gene

A genomic library of *A. fumigatus* constructed in λ EMBL3 (Girardin *et al.*, 1993) was screened with the 309 bp *mepB* fragment as a probe. Screening of 2×10^4 phages yielded 18 positive clones. Restriction analysis of the DNAs from two positive phages designated λ 5.1.1 and λ 8.1.1 revealed that these two phages were identical and that they contained a 2.2 kb *EcoRI* fragment that hybridized with the PCR probe. This fragment was subcloned into pUC18 and its nucleotide sequence was determined as outlined in Fig. 1. Furthermore, a 3.2 kb *KpnI*-*SphI* fragment of phage λ 8.1.1 containing the 5'

region of the *mepB* gene was subcloned into pUC18 and part of its nucleotide sequence was determined according to the strategy shown in Fig. 1. The location of introns in *mepB* was confirmed by nucleotide sequencing of a cDNA clone corresponding to the 3' end of the gene (nucleotides 1466–2563). An ORF of 2061 bp interrupted by two introns of 47 and 55 bp, respectively, was identified. It encodes a protein of 716 amino acids with a calculated molecular mass of 81923 Da, close to the estimated molecular size of the purified MepB protein (82 kDa). The amino acid sequences of the PEP13 and PEP19 internal peptides were found in the deduced amino acid sequence of *mepB*. The amino-terminal end of the purified MepB protein was also found in the deduced amino acid sequence immediately downstream of the first methionine residue of the identified ORF, suggesting that MepB is not synthesized as a precursor. The absence of any potential targeting signal in the amino acid sequence of MepB is consistent with a cytoplasmic localization of this enzyme. Moreover, despite the occurrence of two potential N-glycosylation sites at positions 121 (NES) and 442 (NFT) in MepB, the purified protein appears not to be N-glycosylated since it is not bound by an antigalactomannan antibody nor by concanavalin A (data not shown) (Stynen *et al.*, 1992).

Homology of MepB with other metalloproteinases

Comparison of the deduced amino acid sequence of MepB with those found in the non-redundant protein databases revealed homology with different members of the thimet oligopeptidase family (M3), a family of zinc metalloproteinases that are (1) able to hydrolyse the synthetic substrate Pz-PLGPA and other related substrates, (2) located in the cytoplasm or in the mitochondria and (3) involved in intracellular proteolysis of bioactive peptides and of small peptides resulting from protein degradation (Barrett *et al.*, 1995). The MepB protein shares 46.4% identical amino acids with the yeast saccharolysin, the product of the *PRD1* gene (Büchler *et al.*, 1994), approximately 45% identical amino acids with mammalian thimet oligopeptidases (Pierotti *et al.*, 1990; Kawabata *et al.*, 1993; Kato *et al.*, 1994; Thompson *et al.*, 1995), approximately 41% identical amino acids with mammalian neurolysins (Serizawa *et al.*, 1995) and approximately 30% identity

mepd_rat	1	.....KPPAACAGDVVDVTVSPCTV..NHLR.....WDISAQQRALTTQI...PCTECVYDVG...QDFE
neul_rat	1	MIFLCLSTLRLGLRHAGGSRQLTMTLGLKELASPLQAMSSVYAAAGRNVLRL.....WDISPEQIKTRTEQI...PCTECVYDVG...IALK
mepb_afu	1	.....MAPELRRFPQAPLPTAAQSVDADARRI...PCTECVYDVG...VANVKE
prtd_yeast	1	.....MRLLLCKNWFAFPVISPLLYRSLV...SMANTTFPIAPQAPNPGSP...PCTECVYDVG...SKVE
opda_ecoli	1	.....MENPLT...PFELPFSKLPPEHVPAPVKALMDC...PCTECVYDVG...VAGQAP
dcp_ecoli	1	.....TTMNPFLVQSTLPYLA...PCTECVYDVG...PCTECVYDVG...ALNPQM
mepd_rat	57	DVVEYESTIKALADVEVTYTVQRNLEDFPOHVSNNKIRAASTEADKKISEEDVemSMRQDVTQVVMVLOSKIPK...SELKPEAAHYIEL
neul_rat	82	EVLYEUCIQVLADIEVTYIVERTMLDFPOHVSSEDRVRAASTEADKKISREDDiemSMRQDVTQVVMVLOSKIPK...SELKPEAAHYIEL
mepb_afu	49	SALFDSVVKPDAHDENTALESEHLESPYDAVSEEQCIRDAASSKAEELDEP...PCTECVYDVG...KLVDAVLKKNESLDPESSRLIEKE
prtd_yeast	73	SPSVSNVSEFFMKFME...GPITINQITFLDEVSSDKIRDAVNSMMKIDELM...PCTECVYDVG...KLVDAVLKKNESLDPESSRLIEKE
opda_ecoli	47	YLWENLCQPEAEVDV...GRIFSPUSHLISVKNKSP...PCTECVYDVG...KLVDAVLKKNESLDPESSRLIEKE
dcp_ecoli	52	PDENITELAEQSGEL...TRVTSVFFAMTAARENDE...PCTECVYDVG...KLVDAVLKKNESLDPESSRLIEKE
mepd_rat	145	IKLGRNGLHLPQDT.QEKIKIKIKKISL...PCTECVYDVG...KLVDAVLKKNESLDPESSRLIEKE
neul_rat	170	IKMKGRNGLHSEHI...PCTECVYDVG...KLVDAVLKKNESLDPESSRLIEKE
mepb_afu	135	HKDMIRNGLQAPAGKEDDF...PCTECVYDVG...KLVDAVLKKNESLDPESSRLIEKE
prtd_yeast	163	YKDVIESGLDEDEG...PCTECVYDVG...KLVDAVLKKNESLDPESSRLIEKE
opda_ecoli	134	ISDELSGSGHPPKE...PCTECVYDVG...KLVDAVLKKNESLDPESSRLIEKE
dcp_ecoli	139	IQREVLAKAQA...PCTECVYDVG...KLVDAVLKKNESLDPESSRLIEKE
mepd_rat	225	PIIKKCHVPE...PCTECVYDVG...KLVDAVLKKNESLDPESSRLIEKE
neul_rat	250	PVHKKCCVPE...PCTECVYDVG...KLVDAVLKKNESLDPESSRLIEKE
mepb_afu	219	PHNYAKNAE...PCTECVYDVG...KLVDAVLKKNESLDPESSRLIEKE
prtd_yeast	249	PVHKLASSAQ...PCTECVYDVG...KLVDAVLKKNESLDPESSRLIEKE
opda_ecoli	221	PVHTYCNQAL...PCTECVYDVG...KLVDAVLKKNESLDPESSRLIEKE
dcp_ecoli	227	PIIAEMRDRA...PCTECVYDVG...KLVDAVLKKNESLDPESSRLIEKE
mepd_rat	307	QBRVLEIRIAGSAKRGLPF...PCTECVYDVG...KLVDAVLKKNESLDPESSRLIEKE
neul_rat	332	ABREFFLSIRKKECEERGFEY...PCTECVYDVG...KLVDAVLKKNESLDPESSRLIEKE
mepb_afu	300	KEKALDEIRKAGLESRGEPF...PCTECVYDVG...KLVDAVLKKNESLDPESSRLIEKE
prtd_yeast	330	KEIQVQDMRAEGVKKLQGA...PCTECVYDVG...KLVDAVLKKNESLDPESSRLIEKE
opda_ecoli	311	KEIAQIRAFKAKSGVDELQ...PCTECVYDVG...KLVDAVLKKNESLDPESSRLIEKE
dcp_ecoli	313	DEIASQAVIDKQGGFSAQ...PCTECVYDVG...KLVDAVLKKNESLDPESSRLIEKE
mepd_rat	388	.....ANHEDVLYSVRDAASGE...PCTECVYDVG...KLVDAVLKKNESLDPESSRLIEKE
neul_rat	413	.....VNNKSVLYTVKDKATGEV...PCTECVYDVG...KLVDAVLKKNESLDPESSRLIEKE
mepb_afu	389	KGSDIVVHEDVQVSVVH...PCTECVYDVG...KLVDAVLKKNESLDPESSRLIEKE
prtd_yeast	414	.....VHHDVQVQIAVMMDDPKSPNF...PCTECVYDVG...KLVDAVLKKNESLDPESSRLIEKE
opda_ecoli	386	.....VHHPDVVF...PCTECVYDVG...KLVDAVLKKNESLDPESSRLIEKE
dcp_ecoli	389	.....VYHPDVVF...PCTECVYDVG...KLVDAVLKKNESLDPESSRLIEKE
mepd_rat	468	ETVFEHFGHVMHQCSQAFA...PCTECVYDVG...KLVDAVLKKNESLDPESSRLIEKE
neul_rat	493	RTVFEHFGHVMHQCAQTD...PCTECVYDVG...KLVDAVLKKNESLDPESSRLIEKE
mepb_afu	476	RTVFEHFGHVMHQCAQTD...PCTECVYDVG...KLVDAVLKKNESLDPESSRLIEKE
prtd_yeast	497	RTVFEHFGHVMHQCAQTD...PCTECVYDVG...KLVDAVLKKNESLDPESSRLIEKE
opda_ecoli	465	RTVFEHFGHVMHQCAQTD...PCTECVYDVG...KLVDAVLKKNESLDPESSRLIEKE
dcp_ecoli	465	RTVFEHFGHVMHQCAQTD...PCTECVYDVG...KLVDAVLKKNESLDPESSRLIEKE
mepd_rat	538	LKSTQANAGLENLRO...PCTECVYDVG...KLVDAVLKKNESLDPESSRLIEKE
neul_rat	563	LVAISLVNTGLL...PCTECVYDVG...KLVDAVLKKNESLDPESSRLIEKE
mepb_afu	565	LIRKRVNGLD...PCTECVYDVG...KLVDAVLKKNESLDPESSRLIEKE
prtd_yeast	568	LIRKRVNGLD...PCTECVYDVG...KLVDAVLKKNESLDPESSRLIEKE
opda_ecoli	536	MLAAKNYGAALFL...PCTECVYDVG...KLVDAVLKKNESLDPESSRLIEKE
dcp_ecoli	535	MRNASLTFNKGYEMSEL...PCTECVYDVG...KLVDAVLKKNESLDPESSRLIEKE
mepd_rat	614	SEVYSMDHFEHSCPKKE...PCTECVYDVG...KLVDAVLKKNESLDPESSRLIEKE
neul_rat	639	SEVYSMDHFEHSCPKKE...PCTECVYDVG...KLVDAVLKKNESLDPESSRLIEKE
mepb_afu	654	SEVYSMDHFEHSCPKKE...PCTECVYDVG...KLVDAVLKKNESLDPESSRLIEKE
prtd_yeast	649	SEVYSMDHFEHSCPKKE...PCTECVYDVG...KLVDAVLKKNESLDPESSRLIEKE
opda_ecoli	617	ADVLAADAF...PCTECVYDVG...KLVDAVLKKNESLDPESSRLIEKE
dcp_ecoli	617	QOHLADQV...PCTECVYDVG...KLVDAVLKKNESLDPESSRLIEKE

Fig. 2. Alignments for maximal amino acid similarities of *A. fumigatus* MepB metalloproteinase (mepb_afu) with the *S. cerevisiae* yscD/Prd1p proteinase (prtd_yeast; Büchler *et al.*, 1994), the rat thimet oligopeptidase (mepd_rat; Pierotti *et al.*, 1990), the rat neurolysin (neul_rat; Serizawa *et al.*, 1995) and the *E. coli* OpdA and PrC proteinases (opda_ecoli, dcp_ecoli; Conlin & Miller, 1992; Conlin *et al.*, 1992). Identical (upper case letters) and similar (lower case letters) amino acids in the six proteinases have a black background. This alignment was produced with the PILEUP program of the UWGCG package version 8 (Devereux *et al.*, 1984).

with bacterial members of the family of thimet oligopeptidases (Conlin & Miller, 1992; Conlin *et al.*, 1992; Henrich *et al.*, 1993). An alignment of the amino acid sequence of MepB with several of these proteins is shown in Fig. 2.

One of the regions of homology between the six proteinases shown in Fig. 2 is found in the vicinity of two sequences of amino acids that, because of their similarity to the known catalytic sites of other metalloproteinases, are assumed to participate in the catalytic site of thimet oligopeptidases (Matthews *et al.*, 1972; Bode *et al.*, 1993; Büchler *et al.*, 1994; Rawlings &

Barrett, 1995). In the MepB protein, these two amino acid sequences are F₄₇₉HELGH, which contains two histidine residues supposed to represent a partial zinc-binding site and a glutamic acid residue which may polarize the water molecule involved in nucleophilic attack of the scissile peptide bond, and D₅₀₆FVEAP, which contains a second glutamic acid residue that participates in the second zinc-binding site. We can therefore propose that MepB is a zinc metalloproteinase and is a member of the thimet oligopeptidase family. It may serve a function similar to that of the previously described oligopeptidases and is likely to be involved in the degradation of small cytoplasmic peptides orig-

 

Despite the ability of MepB to degrade native collagen and the presence of a serine residue at position 491, separated by six residues from the FHELGH motif, which has been proposed to be a signature of collagenases (Jiang & Bond, 1992) (Fig. 2), the evidence that MepB is a real collagenase is not strong. Given the intracellular location of MepB and the fact that other

 

teolysis, *A. fumigatus* mutants devoid of the MepB protein were constructed by gene disruption. A DNA fragment encoding amino acids 79–361 of MepB was inserted into plasmid pCB1004 which contains the *hph* gene, a selectable marker for transformation of *A. fumigatus*. As shown in Fig. 3(a), upon transformation of *A. fumigatus* protoplasts, integration of the resulting plasmid, pOG5Δ, at the *mepB* locus by a single crossing-over should disrupt the *mepB* gene, resulting in one *mepB* fragment encoding the first 361 amino acids of MepB under the control of the *mepB* promoter and one *mepB* fragment encoding the 637 carboxy-terminal amino acids of MepB but lacking a promoter. Among 25 transformants tested, one was shown to lack soluble Pz-peptidolytic activity and was further studied. Southern hybridization of *PvuII*- and *HindIII*-digested genomic DNA of this mutant strain confirmed that pOG5Δ had integrated at the *mepB* locus since the original *mepB*-hybridizing 7.5 kb *PvuII* and 6.0 kb *HindIII* fragments found in the wild-type strain had been converted into 2.0 kb and 5.2 kb fragments in the mutant strain, respectively (Fig. 3).

Comparison of *A. fumigatus* wild-type and *mepB1* strains showed that the 100000 g supernatant is fully devoid of Pz-peptidolytic activity (data not shown). Therefore, the MepB protein is the only proteinase responsible for the Pz-peptidolytic activity present in the cytoplasm of *A. fumigatus*. This is supported by results showing that no homologue of the *mepB* gene could be detected by low stringency hybridization of *A. fumigatus* DNA using the *mepB* gene as a probe (data not shown). The viability, germination and growth rate of the *mepB1* mutant strain were not significantly different from those of wild-type *A. fumigatus*. Moreover, 64% and 80% mortality were obtained for mice inoculated with the wild-type and *mepB1* strains, respectively (data not shown). Similar results were obtained for genetically engineered mutants of *A. fumigatus* that are deficient in an alkaline proteinase (Alp) and/or in a metalloproteinase (Mep) both secreted by *A. fumigatus*. These mutants behave like wild-type *A. fumigatus* in a mouse model of invasive aspergillosis, causing similar mortality and tissue damage (Monod *et al.*, 1993; Tang *et al.*, 1993; Jatton-Ogay *et al.*, 1994; Smith *et al.*, 1994).

In summary, we have shown in this paper that the 82 kDa proteinase of *A. fumigatus* referred to as MepB is a member of the thimet oligopeptidase family (M3) and, as such, may well be involved in the intracellular degradation of small peptides with bioactivity or resulting from protein degradation (Büchler *et al.*, 1994). Further analysis of the *mepB1* mutant and of the MepB collagenolytic activity will be required to precisely define the function of the MepB protein.

Different filamentous fungi have intracellular soluble metalloproteinases with chromatographic properties similar to those of MepB. One of these proteins, originating from *Trichophyton schoenleinii*, was shown by peptide sequencing to have homology with *A. fumigatus* MepB and other members of the thimet

oligopeptidase family (Ibrahim-Granet *et al.*, 1996). It is therefore likely that, as shown in this paper for *A. fumigatus*, cytoplasmic members of the thimet oligopeptidase family (M3) are found in a wide variety of filamentous fungi, thus confirming the broad distribution of these house-keeping enzymes.

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