

Cloning and disruption of the gene encoding an extracellular metalloprotease of *Aspergillus fumigatus*

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Summary

Aspergillus fumigatus secretes a serine alkaline protease (ALP) and a metalloprotease (MEP) when the fungus is cultivated in the presence of collagen as sole nitrogen and carbon source. The gene encoding ALP was isolated and characterized previously. We report here the cloning and the sequencing of the gene encoding MEP. Genomic and cDNA clones were isolated from *A. fumigatus* libraries using synthetic oligonucleotides as probes. Stretches of the deduced amino acid sequence were found to be in agreement with the N-terminal amino acid sequence of MEP and with internal peptide sequences. The amino acid sequence of the enzyme contains a putative active-site sequence HEYTH homologous to the active site of other bacterial and eukaryotic zinc metalloproteases. Sequence analysis reveals that MEP has a pre-proregion consisting of 245 amino acid residues preceding the 388 amino acid residues of the mature region (molecular mass of 42 kDa). An *alp mep* mutant, deficient in proteolytic activity at neutral pH *in vitro*, was constructed and tested for pathogenicity in a murine model. No difference in pathogenicity was observed between the wild-type strain and the *alp mep* double mutant, suggesting that ALP and MEP are not essential for the invasion of the lung tissues by *A. fumigatus*.

Introduction

The opportunistic fungus *Aspergillus fumigatus* is the causative agent of a number of diseases in humans, including

allergic bronchopulmonary aspergillosis, aspergilloma and invasive aspergillosis (Bodey and Vartivarian, 1989). The last has become an increasingly important threat to neutropenic patients undergoing bone-marrow transplantation and chemotherapy. Several reports suggest that the virulence of *A. fumigatus* is associated with the production of secreted proteases (Kothary *et al.*, 1984; Miyaji and Nishimura, 1977). We showed that *A. fumigatus* secretes two proteases when the fungus is cultivated in the presence of collagen as the sole nitrogen and carbon source, a serine alkaline protease (ALP) and a metalloprotease (MEP) (Monod *et al.*, 1993b). ALP was isolated and characterized by several groups and was considered as a potential virulence factor of the fungus (Reichard *et al.*, 1990; Monod *et al.*, 1991; Frosco *et al.*, 1992; Larcher *et al.*, 1992; Kolatukudy *et al.*, 1993). This enzyme has a molecular mass of 33 kDa, cleaves elastin and collagen and belongs to the subtilisin family. The gene encoding ALP (ALP) was isolated (Jatón-Ogay *et al.*, 1992) and inactivated by targeted gene disruption in pathogenic wild-type strains (Tang *et al.*, 1992; Monod *et al.*, 1993a). Surprisingly, both isogenic ALP-producing and non-producing *A. fumigatus* strains invade lung tissues, causing comparable mortality in immunosuppressed mice (Monod *et al.*, 1993a; Tang *et al.*, 1993). MEP was isolated from the ALP-non-producing mutant cultures that still showed a significant proteolytic activity (Monod *et al.*, 1993b). The molecular mass of the enzyme was determined to be 40 kDa from electrophoretic mobility on SDS-PAGE. MEP cleaves collagen but does not cleave elastin and was totally inhibited by specific inhibitors of metalloproteases such as EDTA, 1,10-phenanthroline, and phosphoramidon.

We report the isolation and the nucleotide sequence of both genomic DNA and cDNA encoding MEP (MEP). An *alp, mep* mutant, deficient in proteolytic activity at neutral pH *in vitro*, was then constructed and tested for pathogenicity in a murine model.

Results

Isolation of genomic DNA clones encoding the extracellular metalloprotease of *A. fumigatus*

The N-terminal amino acid sequence of MEP, as well as the sequence of an internal peptide generated after cyanogen bromide cleavage, were determined. Two oligonucleotide probes (probes A and B) were designed on the basis of

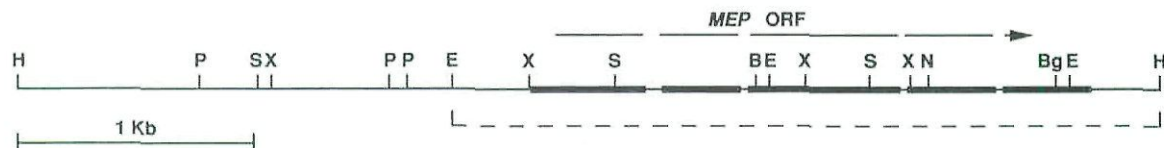


Fig. 1. Restriction map of the 5.0 kb *HindIII* genomic DNA fragment obtained from positive recombinant λ EMBL3 bacteriophages. The *MEP* cDNA is indicated by the filled-in region. The part of this map for which the nucleotide sequence is reported in Fig. 2 is dotted. B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hind*III; N, *Nco*I; P, *Pst*I; S, *Sal*I; X, *Xho*I.

these sequences and of the codon usage for the genes encoding ALP (Jatón-Ogay *et al.*, 1992) and the restrictocin (Lamy and Davies, 1991) of *A. fumigatus* (Table 1). A mixture of probes A and B was used to screen the *A. fumigatus* genomic library and eight positive clones were identified. Seven clones that hybridized with probe A or B used separately were selected for further analysis. Restriction enzyme analysis of purified bacteriophage DNA revealed that the seven clones had a common 5.0 kb *HindIII* fragment that hybridizes with probes A and B (data not shown). This fragment, for which a restriction map is shown in Fig. 1, was subcloned in pMTL21 generating the plasmid pMTL21-H5. The sequences hybridizing with the probes A and B were localized on two *Eco*RI subfragments of 1.4 and 1.3 kb, respectively. The sequence of these two *Eco*RI fragments together with an additional 400 bp *Eco*RI–*HindIII* fragment is shown in Fig. 2. The 1.3 kb *Eco*RI fragment contained a stretch of 24 base pairs encoding a putative active site of a zinc-dependent metalloprotease (Jongeneel *et al.*, 1989) consisting of a HEXXH motif after three hydrophobic amino acids.

Table 1. Amino acid sequences and oligonucleotide probes used to screen the *A. fumigatus* genomic library.

Probe	Amino acid sequence	Oligonucleotide probe
A	ADYQVYAWGINDPTEGERT ^a	TAC CA(A/G) GT(T/C) TA(T/C) GC(T/C) TGG GG(T/C) AT(T/C) AA(T/C) GAC CC
B	ALQPCNPNFVQARDAILD ^b	CAG CC(T/C) TGC AA(T/C) CC(T/C) AA(T/C) TT(T/C) GT(T/C) CA(A/G) GC

a. N-terminal amino acid sequence of MEP.

b. N-terminal amino acid sequence of a peptide generated after cyanogen bromide cleavage of MEP. Oligonucleotide probes were based on the underlined amino acid sequences.

Northern blot analysis of RNA isolated from *A. fumigatus* grown in collagen or glucose and peptone medium

Supernatants of *A. fumigatus* cultures contained MEP only when the fungus was grown in a medium containing collagen as sole nitrogen and carbon source, but not in Sabouraud medium containing glucose and peptone (Monod *et al.*, 1993b). Northern blots of RNA isolated from fungal mycelia grown in either medium were probed with genomic DNA of *MEP* (1.3 and 1.4 *Eco*RI fragments) and consistently an mRNA transcript (2.4 kb) was observed only when the fungus was grown in collagen medium (Fig. 3). No *MEP* signal was observed with DNA upstream from the *Xho*I site in base pair position –92 used as a probe (data not shown).

Isolation of a cDNA clone encoding MEP

In absence of a cDNA library constructed from *A. fumigatus* grown in collagen medium, a λ ZAP library was constructed in AC broth (Difco) in which proteolytic activity was not detected (data not shown) (Moser *et al.*, 1992). Because of the low frequency of *MEP* cDNA in this library, approximately 100 000 recombinant phage plaques were screened with the 1.3 and 1.4 kb *Eco*RI fragments. Only one clone was identified and purified. A pBluescript-II SK plasmid carrying *MEP* cDNA, pK100, was excised *in vivo* from the hybridizing λ ZAP bacteriophage using the f1 helper bacteriophage (Short *et al.*, 1988), and further restriction analysis of pK100 showed a cloned insert of 2.4 kb. The nucleotide sequence of the cloned cDNA was compared with that of the genomic DNA. The 5' extremity of the cloned cDNA starts in base pair position –87, and four introns were detected (base pair positions 357–406, 802–853, 1401–1452, 1868–1920) (Fig. 2).

Fig. 2. Nucleotide sequence of *MEP* genomic DNA. The sequences of both strands were determined. The 5' and 3' (polyA) extremities of cloned cDNA are indicated by arrow heads at base pair –87 and 2276, respectively. The sequences in brackets are introns and are not present in *MEP* cDNA. The deduced amino acid sequence is shown below the nucleotide sequence. The N-terminal amino acid sequence of the mature enzyme begins at amino acid residue 246 and is preceded by a vertical bar and a horizontal arrow. The primary sequence motif HEXXH found in zinc-dependent metalloproteases is boxed. The putative cleavage sites within the signal sequence for secretion are indicated by a vertical arrow. The amino acid sequences determined by protein sequencing are underlined. The GenBank accession number is Z30424.

-451 GAATTCAGCAGCAAGAGCAGCGGACATCCGACGGACAGGTCCCTGAAATCTTCATGAAGATTGCGTCCCTCGCCCACTTCCAATCAACCA -362
 -361 TGCAGATCGTTATCTCCACCAATTCTCAGCGGGCGTGCACCTCGATTGTGCATACAGTCCGCAATGCTTATAGGTGAACACTATGCAGTGT -272
 -271 CTCTGCTAATGCAACTGTCTCGGAGTGCAGGGCGGGGAACCGGAAGATATCGACTTGCAATATCCCATGATGTGAATAGCTCCGGACTGG -182
 -181 GTCGGGCCAAGAACGACGCGTTCTACGCTGGATCACAATGAGAATGGCATGGAGTAGATAGGGGATGCAGAACCAAAACGCCAGATGCT -92
 -91 CGAGTATAAATACTCGCTGAAGCTCAGAGAGTCGCTAGAAGCATCCGCTTTTGAATCCGGCATCGTTCTTCGCCAGATACATCTCTCAA -2
 1 20
 -1 CATGCGCGGCTCTTCTTGTGCTGGGGCTCTTGGCCCTACCTGCTTCTTGTCTTTGCCCCATCCCGCTCACCAGTCTTACGGACTCAATCGTCG 88
 M R G L L L A G A L A L P A S V F A H P A H Q S Y G L N R R
 40 60
 89 TACTGTTGATCTGAATGCTTTCGCTTGAAGTCCCTGGCCAAGTACGTCAATGCCACAGAGACCGTCATTGAGGCCCGTCTCTCTTTG 178
 T V D L N A F R L K S L A K Y V N A T E T V I E A P S S F A
 80
 179 CCCTTTCAAGCCGCAAAGCTATGTGGAGGTGGCGACGCAACATGTCAAGATGATTGCCCTGATGCGACTTTCCGCGTGGTCGACGATCA 268
 P F K P Q S Y V E V A T Q H V K M I A P D A T F R V V D D H
 100
 269 CTATGTTGGTGACAACGGCGTGGCCATGTCCATTTCCGCCAGACGGCCAAAGGTCTCGATATCGACAATGCTGACTTCAATGTCAACET 358
 Y V G D N G V A H V H F R Q T A N G L D I D N A D F N V N
 120
 359 ACGTTATGATTCCGGTCCGCTGTATCTCTCTTGTGCTGACAGGGGTGCAAGTCGGAAAAGATGGAAGGTCTTTTCGTATGGCAACTCTTTTC 448
 V G K D G K V F S Y G N S F
 140 160
 449 TATACCGGACAGATCCCCAGCTCGGCTGCATTGACCAAGCGTGAAGTCTCTCCGACCTGTGACTGCTCTCAAGGGAACACAAATACACTA 538
 Y T G Q I P S S A A L T K R D F S D P V T A L K G T T N T L
 180
 539 CAGCTGCCCATCACTGTGGATAGCGCTCGCTCCGAGAGCACAGAGGAGAGGAGTGTATGTTTCAAGGGGTCTCTGGCACTGTCTCT 628
 Q L P I T V D S A S S E S T E E K E S Y V F K G V S G T V S
 200 220
 629 GATCCCAAAGCCAAACTGGTATATCTTGTCAAGACGATGGAACCTCGCCTTGGCATGGAGAGTTGAGACCGACATTTGATAGCAACTGG 718
 D P K A K L V Y F V K D D G T L A L A W R V E T D I D S N W
 240
 719 CTGTTGACCTACATCGATGCCAAGAGCGGCGAGGAGATCCACGGTGTGTGGACTATGTTGCTGAAGCCGACTACCAAGTATATGTAGTA 808
 L L T Y I D A K S G E E I H G V V D Y V A E A D Y Q V Y
 260
 809 TGGGACCAACCTCCAAAGAGGCTGACTGACATGGTGCACCGCATGGGGCATTAATGACCCAAAGAGGGCGAGCGGACGGTGTATC 898
 A W G I N D P T E G E R T V I
 280
 899 AAGGATCTTGGGACTCGGTACAGAAATTCACCTGGATCAGTGTGGCAGCACAATTTATACCACTTCGCGCGGCAACACGGCATCGCG 988
 K D P W D S V T E F T W I S D G S T N Y T T S R G N N G I A
 300 320
 989 CAATCCAAATCCAGCGGTGGGCGGAGCTACCTGAACAACCTACCGCCTAGCAGCTCGAGCCTGTCAATCAAGTATCCCTATAGTGTGAGC 1078
 Q S N P S G G P S Y L N N Y R P S S S S L S F K Y P Y S V S
 340
 1079 TCGTCCGCGCCATCATCTACATCGACGCATCGATCATCCAGCTCTTCTACACGGCCAATATCTACCATGATCTGCTTTACACCTCGGGC 1168
 S S P P S S Y I D A S I I Q L F Y T A N I Y H D L L Y T L G
 360 380
 1169 TTCACCGAAAAGCGCGTAACTTCGAGTACAACACTAACGGGCAAGCGGCCCTGGGAAATGACTATGTCTATCTGAACGCCAGGACGGC 1258
 F T E K A G N F E Y N T N G Q G G L G N D Y V I L N A Q D G
 400
 1259 TCTGGCACCAACAACGCCAACTTTGCCACTCTCTGTATGGCCAGCCCGCCGATGCGCATGTACGTCTGGACCGAGTTCGACGCCGTAC 1348
 S G T N N A N F A T P P D G Q P G R M R M Y V W T E S T P Y
 420
 1349 CGCGACGGCTCTTTGAGGCTGGAATCGTGATCCACGAGTATATCAAGGTCTACGTATTACCTTGTGGTTTATTGACACAGCACTC 1438
 R D G S F E A G I V I H E Y T H G L
 440
 1439 ACCCTTTTTCAGTTTTCACACCGACTCAGTGGCGGCCCTGCCAACTCCAACTGCCTGAACGCCCTCGAGTCAGGCGGCATGGGCGAAG 1528
 S N R L T G G P A N S N C L N A L E S G G M G E G
 460 480
 1529 CTGGAGTGATTTTCATGGCGACGGCCATCCGCTCTGAAGCCCGCGCACAAGCGCTCGACCGACTACACCATGGGCGAATGGGCTTCCAACCG 1618
 W S D F M A T A I R L K P G D K R S T D Y T M G E W A S N R
 500
 1619 GGCAGGCGGAATCCGCGACTACCGTACTCGACTCGCTGAGCACCACCTCTAACGTACACCAGCGTCAACTCGCTGAACGCGGTGCA 1708
 A G G I R Q Y P Y S T S L S T N P L T Y T S V N S L N A V H
 520 540
 1709 CGCCATTGCGACGGTCTGGGCATCCATGCTGTACGAGGTGTGTGGAATTTGATCGATAAGCAGCGCAAGATGATGCGCGAAGCCTAC 1798
 A I G T V W A S M L Y E V L W N L I D K H G K N D A P K P T
 560
 1799 CCTGCGCATGGGGTGGCGACGGCAGTACCTTGGCATGAACTAGTTCATGGATGGGATGGCATTTACGTGCGGAGAAATCCTTCA 1888
 L R D G V P T D G K Y L A M K L V M D G M A L
 580
 1889 CCGGTTATGTTGACACCGCTAATGACAAACAGTCAACCATGCAACCCCAACTTTGTCAGGCCGTCGACGCCATCTCGATGCTGATACT 1978
 Q P C N P N F V Q A R D A I L D A D T
 600 620
 1979 GCTCTGACTGGTGGCGAGAACCAGTGTGAGATTGGACGGCAATTTGCCAAGCGGGATTTGGGTGCTGGGCGAAGTATAGCTCAAGGAAC 2068
 A L T G G E N Q C E I W T A F A K R G L G A G A K Y S R N
 2069 CGGGTGGGAAGTACTGAGGTCCCGAGTGTCTGTTGAGTGACCATGACCGAGCAACAGCAGGACCGGGAGTGAATGAGAATAATG 2158
 R V G S T E V P S G V C ***
 2159 ACAATCTCGGCACGGTTACCTTGTCTCCGCAAGATCTTGTAAATAAGCGGGGAGAAATAGGGTGAATTCACACGCGCTCTGAGAGC 2248
 CACAAGTAACCAATCTGGACTTCTGGAACCTATCTACTCCGTAAGTGTGAGGGTGATTGTAGACGATCCACCTTAATTCTACTAAT 2338
 2339 AGTAATCCAATCTTCTGGAAATGACACGAGATTCTGAACCAATTGCGCCCCCTGAATTGCTAGCCCAAGAAATTAGCCGCTTACGG 2428
 2429 CCCAGGAACAGCTCTGAACACGGCCAATCACTGTCCATACTAGGAGAAATGGACTGTCCCAAGAGGTTACGGGAGGACACAGTTCACGG 2518
 2519 GCCTTGAGATGACAGTCGATCCAGTGTGCTCTCTTTGTGATCATGGGTTGTGCGGCTGCCACGACTGTGGTTCATTTCCGGGGATGA 2608
 2609 TAGTAGAAGCTT

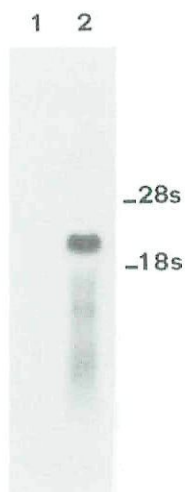


Fig. 3. Northern blot of RNA from a culture of *A. fumigatus* grown in Sabouraud medium (lane 1) and in collagen medium (lane 2) probed with genomic DNA of *MEP*. The apparent molecular sizes of the RNA hybridizing with the probes were estimated to be 2.4 kb based on the position of the 28 s and 18 s ribosomal RNA. RNA agarose gel electrophoresis was performed in the presence of $10 \mu\text{g ml}^{-1}$ of ethidium bromide. The 18 S and 28 S RNA bands visualized after exposure of the blotted membranes to u.v. radiation attested that the RNA was not degraded.

Sequence analysis

Northern blot analysis and the nucleotide sequence of cDNA indicated that *MEP* is encoded by nucleotides starting from the ATG codon 451 bp downstream of a *EcoRI* site (Fig. 2). The *N*-terminal amino acid sequence of the mature enzyme obtained by amino acid sequencing (Table 1) was preceded by a polypeptide of 245 amino acid residues, suggesting that the protein was synthesized as a precursor of 633 amino acid residues. Its calculated molecular mass was 68 656 Da. Inspection of the *N*-terminal amino acid sequence of the precursor beginning from the Met-1 residue suggested the existence of a signal peptide with an hydrophobic core of 10 amino acid residues and two putative signal peptidase cleavage sites in accordance with the -3-1 von Heijne's rule (von Heijne, 1986) after the Ala-14 and Ala-18 residues (Fig. 2). The mature protein sequence generated after cleavage of the 245 amino acid residue polypeptide from the 633 amino acid residue precursor predicts a molecular mass of 42 064 Da. The deduced amino acid sequence of *MEP* was further verified by amino acid sequencing of the *N*-terminus of five internal peptides obtained after trypsin digestion of the protein (Fig. 2).

Total *A. fumigatus* RNA was translated *in vitro* in rabbit reticulocyte lysate and products immunoprecipitated using a polyclonal anti-*MEP* specific rabbit antiserum were analysed on SDS-PAGE (Fig. 4). The immunoprecipitated products migrated as two protein bands with estimated sizes of 70 and 40 kDa, corresponding to the

putative 633 amino acid residue precursor and the mature form of the enzyme, respectively.

MEP disruption

MEP disruption was performed in ALP-producing G10 and ALP-non-producing $\Delta 18$ strains (Monod *et al.*, 1993a). The plasmid p Δ *MEP* was designed to function as a one-step gene disruption vector in the transformation experiment and was constructed using the pAN8-1 and pMTL21-H5 plasmids as described in Fig. 5. The p Δ *MEP* contained *MEP* disrupted by the phleomycin-resistant gene of *Streptococcus hindustanus* under the control of the *GPD* promoter and the *TRPC* terminator of *Aspergillus nidulans* (Fig. 6A). Transformation of protoplasts of strains G10 and $\Delta 18$ with the 7.3 kb *HindIII* fragment of p Δ *MEP* generated 31 and 53 *A. fumigatus* colonies resistant to phleomycin, respectively. One G10 transformant, M1, showed 70% of G10 collagenic activity (data not shown). This activity was totally inhibited by PMSF, suggesting that M1 was *MEP* deficient and produced only ALP. Three $\Delta 18$ transformants, MA1, MA2, MA3, were completely devoid of extracellular proteolytic activity, as indicated by the absence of degradation of the collagen of the medium during growth as well as the lack of azocoll degradation by the culture filtrate (Fig. 7). SDS-PAGE of proteins secreted by the four transformants M1, MA1, MA2 and MA3 showed the absence of the 40 kDa band (Fig. 8) identified as *MEP* (Monod *et al.*, 1993b). The four transformants were retained for restriction fragment analysis. Genomic DNA of *A. fumigatus* G10 and $\Delta 18$, and of the four transformants not producing active *MEP*, was digested with *EcoRI* or *HindIII*, electrophoresed and probed with the 1.4 and 1.3 kb *EcoRI* fragments of pMTL21-H5 containing *MEP*. The band pattern of *MEP*-non-producing strains was consistent with the correct replacement of *MEP* by the



Fig. 4. *In vitro* translation and immunoprecipitation of *MEP* translation product. *MEP* translation product was immunoprecipitated using polyclonal anti-*MEP* specific rabbit antiserum (1) and preimmune serum (2) and analysed on 9% SDS-PAGE. Molecular mass markers are on the left margin.

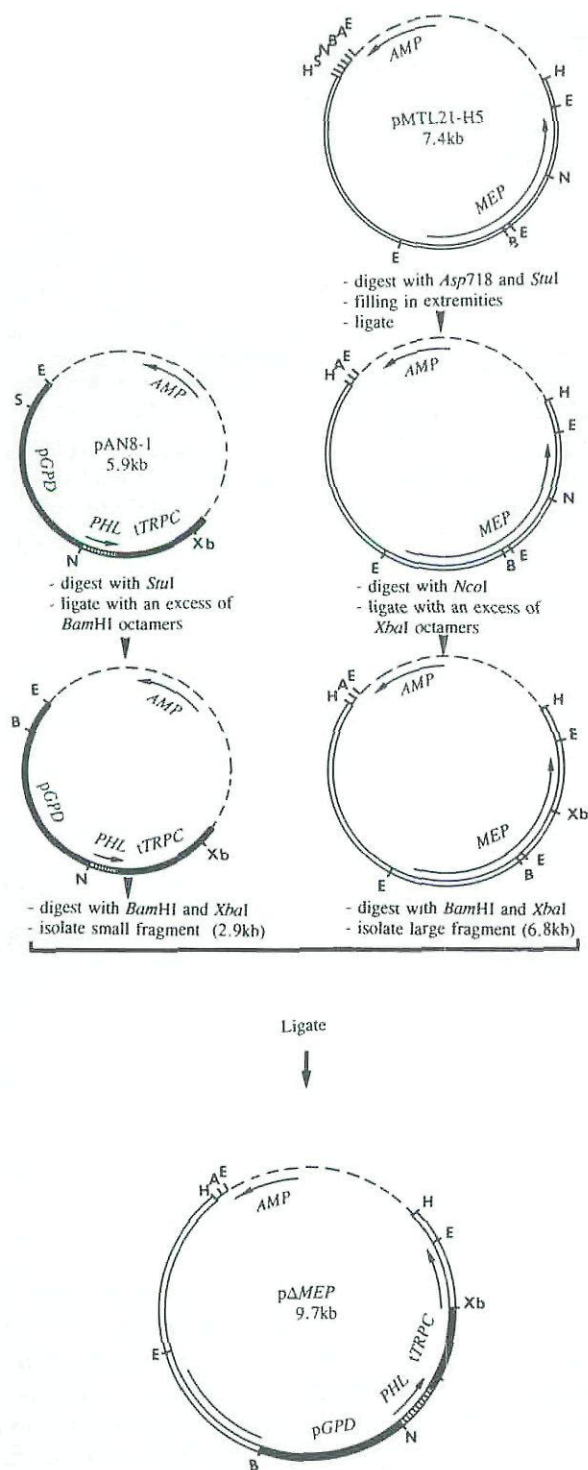


Fig. 5. Construction of plasmid pΔMEP. The plasmid pMTL21-H5 was obtained by cloning a 5.0 kb *Hind*III fragment carrying *MEP* in pMTL21. The plasmid pAN8-1 has been described elsewhere (Mattern *et al.*, 1988). Thick lines, double lines, dotted lines represent *A. nidulans*, *A. fumigatus* and *E. coli* DNA, respectively. *pGPD* is the 5' expression signal of the *A. nidulans* *GPD* gene; *tTRPC* is the terminator region of the *A. nidulans* *TRPC* gene. *A*, *Asp* 718; *B*, *Bam*HI; *E*, *Eco*RI; *H*, *Hind*III; *N*, *Nco*I; *S*, *Sst*I; *Xb*, *Xba*I.

mepΔ::PHL construct in chromosomal DNA (Fig. 6B and C).

Infectivity of *alp mep* mutants

The parental strain (G10) and the *alp mep* mutant (MA1) were injected at two dosages (10^8 and 10^5 per mouse) to mice submitted to two different immunosuppression regimens. In both cases, similar mortality was observed with wild-type strain and double-mutant strain (Fig. 9). With the second regimen, maximal mortality was attained with the lowest concentration one week after injection, whereas with the first regimen and a high conidia concentration, mortality occurred much more rapidly. All dead animals had lungs infected by *A. fumigatus*. Fungi isolated from lung tissues were strains that did not produce ALP and MEP and which were resistant to both hygromycin and phleomycin.

Histopathological studies of lungs from infected mice showed a similar extent of mycelial growth in both parental and mutant strains (Fig. 10). Invasion of the lung tissues by both strains occurred through the bronchial epithelium after extensive development of the fungus inside the bronchia and bronchioles. No colonization of the alveolar space of the lung was noticed before the death of the mouse. When the collagen and elastin matrix was crossed by fungal hyphae, no apparent proteolytic degradation was seen around the hyphae of either mutant or wild-type fungi.

Discussion

Analysis of the deduced amino acid sequence of MEP reveals that the mature protein is composed of 388 amino acid residues. Its estimated molecular mass is 42 kDa, which is 2 kDa larger than the size evaluated from the electrophoretic mobility of the protein purified on SDS-PAGE. In addition, the theoretical *pI* of this protein is 4.9, which is close to the value determined experimentally (Monod *et al.*, 1993a). The MEP amino acid sequence contains the consensus sequence of a metalloprotease represented by the motif I-V-I-H-E-Y-T-H-G-L-S, in which the two histidine residues co-ordinate the cation Zn^{2+} in the active centre and the glutamate is involved in the bond-breaking process (Jongeneel *et al.*, 1989). The three amino acid residues before the first histidine and after the second histidine are generally represented by hydrophobic or small and neutral amino acids in the active site of a metalloprotease. The sequence found in MEP is in agreement with the above motif.

A pre-proregion consisting of 245 amino acid residues was observed with the MEP sequence analysis. Two proteins, corresponding to the putative precursor and mature form of the enzyme, were produced when MEP

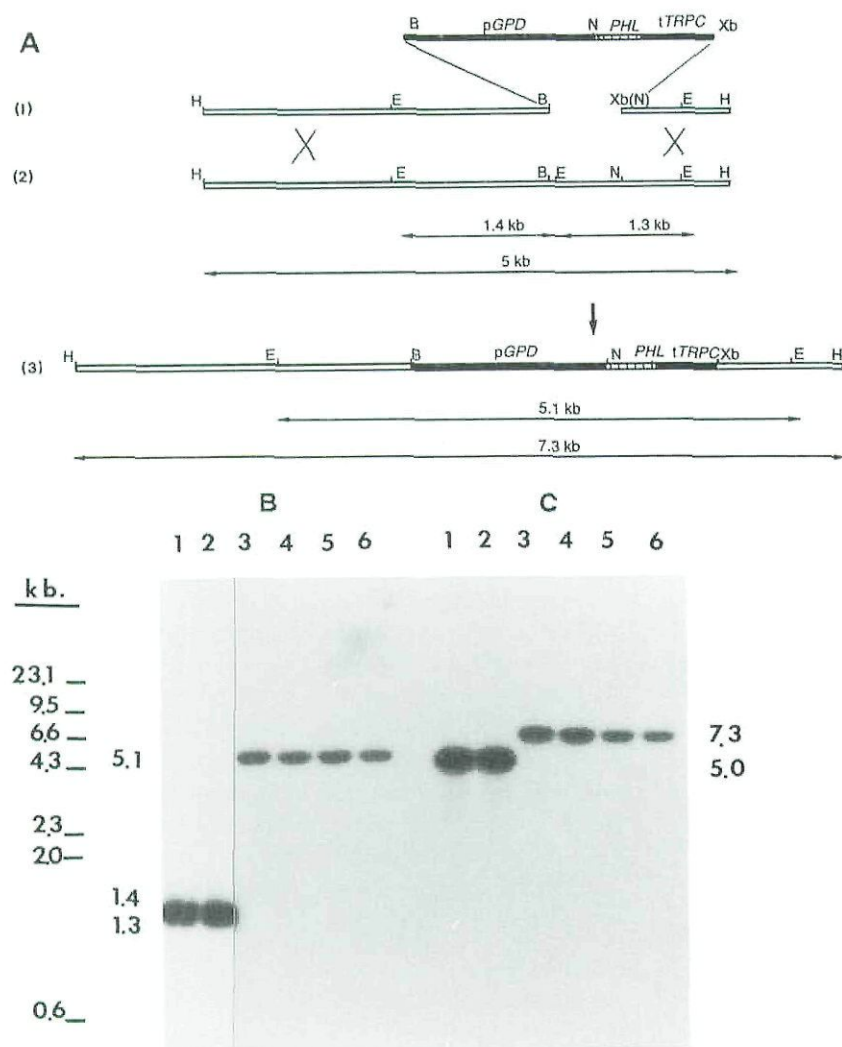


Fig. 6. A. Disruption of *MEP* in *A. fumigatus*: (1) map of transformant pΔMEP *Hind*III fragment containing *MEP* disrupted by the *PHL* gene; (2) genomic DNA of the *MEP* recipient strain; (3) genomic DNA of *mep* transformants. Thick lines and double lines represent *A. nidulans* and *A. fumigatus* DNA, respectively. pGPD is the 5' expression signal of the *A. nidulans* GPD gene; tTRPC is the terminator region of the *A. nidulans* TRPC gene. The cross-hatched area represents the phleomycin-resistance gene (*PHL*). The size of restriction of the *Eco*RI and *Hind*III fragments before and after gene disruption are shown. The diagrammed double crossover event produces the *mep* desired mutation. B, *Bam*HI; E, *Eco*RI; H, *Hind*III; N, *Nco*I; Xb, *Xba*I. B and C. Southern blot analysis from *A. fumigatus* *MEP*-producing strain and from *MEP* non-producing transformants. Chromosomal DNA was subjected to electrophoresis after cleavage with *Eco*RI (B) and *Hind*III (C) and transferred to a Gene Screen Plus Membrane. The filters were hybridized with the labelled 1.3 kb and 1.4 kb *Eco*RI fragments of the plasmid pMTL21-H5. Lane 1, recipient G10 (*ALP MEP*); lane 2, recipient Δ18 strain (*alp MEP*); lane 3, transformant M1 (*ALP mepΔ::PHL*); lane 4, 5 and 6, transformant MA1, MA2 and MA3, respectively (*alp mepΔ::PHL*).

was translated *in vitro*. This finding suggests that *MEP* processing is achieved by an autoproteolytic activity and is similar to that of other microbial secreted proteases (Power *et al.*, 1986; Miyazaki *et al.*, 1989; Hiramatsu *et al.*, 1989; Terada *et al.*, 1990; Tatsumi *et al.*, 1991). However, it cannot be formally excluded that rabbit reticulocyte lysate contains a specific protease which cleaves the 68 kDa protein. A pre-proregion has also been reported for the *Aspergillus oryzae* neutral protease II, which is the only other secreted metalloprotease sequenced in *Aspergillus*. This pre-proregion has 175 amino acid residues and precedes the mature enzyme which consists of 177 amino acid residues (Tatsumi *et al.*, 1991).

In terms of molecular mass, pI and effect of pH on activity, *MEP* resembles other metalloproteases, such as *Aspergillus sojae* and *A. oryzae* neutral proteases I (Sekine *et al.*, 1972; Nakadaï *et al.*, 1973), *Bacillus thermoproteolyticus* thermolysin (Fujii *et al.*, 1983) and related enzymes of other bacteria (reviewed by Häse and Finkelstein, 1993).

However, the *A. sojae* and *A. oryzae* neutral proteases I, like *MEP*, differ from the *B. thermoproteolyticus* thermolysin by losing their activity at temperatures above 60°C after 10 min. The genes of the *A. sojae* and *A. oryzae* proteases I have not been isolated and sequenced, while thermolysin and other bacterial zinc-dependent metalloproteases have been extensively characterized. The deduced amino acid sequence of the mature *MEP*, as well as the pre-proregion, was compared with those of proteins available in the NCBI database. Except for the zinc signature of metalloproteases, consisting of 11 amino acid residues spanning the HEXXH motif, no significant homology was found. In contrast to *ALP* and *Bacillus sp.* subtilisins (Tatsumi *et al.*, 1989), *MEP* and *B. thermoproteolyticus* thermolysin do not appear to be closely related.

Two *A. fumigatus* metalloproteases of 82 and 43 kDa have been recently described (Ibrahim-Granet *et al.*, 1994; Markaryan *et al.*, 1994). The 82 kDa enzyme is intracellular and has a higher molecular mass than the pre-proform of

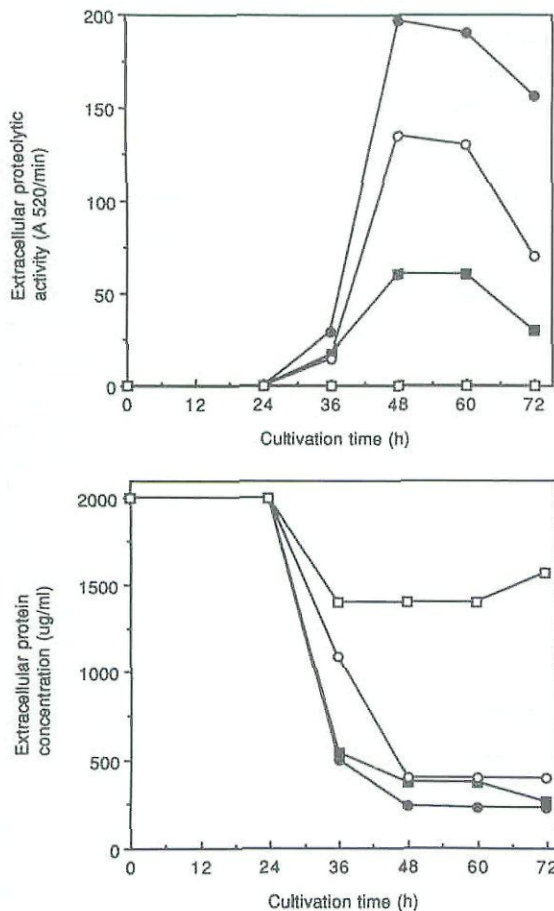


Fig. 7. Extracellular proteolytic activity (A) and protein concentration (B) of G10 (ALP MEP) (●), Δ18 (alp MEP) (■), M1 (ALP mep) (○), MA1 (alp mep) (□) strains. The fungi were grown in collagen medium at 30°C. Proteolytic activities and protein concentrations were measured as described in the *Experimental procedures*.

MEP. The 43 kDa enzyme is secreted and has a molecular mass and a pH optimum of activity comparable to those of MEP (Monod *et al.*, 1993b), but differs by having elastinolytic activity and by retaining 50% of its activity after 1 h at 60°C. The *N*-terminal amino acid sequence obtained by amino acid sequencing of the metalloprotease described by Markaryan *et al.* (1994) is very similar to that of MEP, the first 13 amino acid residues being identical. The presence of an additional gene encoding a metalloprotease was investigated by Southern analysis of *Eco*RI-digested genomic DNA of five different *A. fumigatus* strains. The 1.3 and 1.4 kb *Eco*RI fragments, encoding MEP, were used as a probe and the hybridization was performed at low-stringency conditions. Only 1.3 and 1.4 kb bands were detected (data not shown), suggesting that *A. fumigatus* does not contain a DNA sequence encoding a second enzyme closely related to MEP. Therefore MEP and the 43 kDa metalloprotease described by Markaryan *et al.* (1994) appear to be the same enzyme and the differences

observed between these two proteins should be evaluated in several other *A. fumigatus* strains to make sure they are consistent. If not, the protease isolated by Markaryan *et al.* (1994) could be an enzyme homologous to MEP secreted by a strain different from those isolated in Europe.

MEP was disrupted in wild-type and in *A. fumigatus* strains containing *alp*. The *alp mep* strains were completely devoid of extracellular proteolytic activity in collagen medium (Fig. 7), while *mep* ALP strains had 70% of the proteolytic activity of the wild-type strain. The *Streptomyces hindustanicus* PHL gene was for the first time used for targeted disruption of a gene in *A. fumigatus*. A high majority (> 90%) of transformants resistant to phleomycin still revealed MEP secretion. The integration events generating these transformants were not investigated further, since it is known that integration of foreign DNA in *Aspergillus* can occur at chromosomal positions other than the targeted homologous site (Miller *et al.*, 1985). However, transformants with the MEP gene disrupted by the phleomycin-resistance gene occurred at frequencies similar to those obtained in previous gene-disruption experiments, using the *Escherichia coli* hygromycin phosphotransferase gene (*HPH*) as selective resistant marker (Monod *et al.*, 1993a).

Extensive colonization of the lung tissue by *A. fumigatus* has suggested the key role of fungal proteases during infection. The role of ALP was eliminated when ALP-non-producing mutants, constructed by targeted gene disruption, were shown to cause comparable mortality and to invade lung tissues in immunosuppressed mice, as well as the parental strain (Monod *et al.*, 1993a; Tang *et al.*, 1993). These results differ from those of Kolattukudy *et*

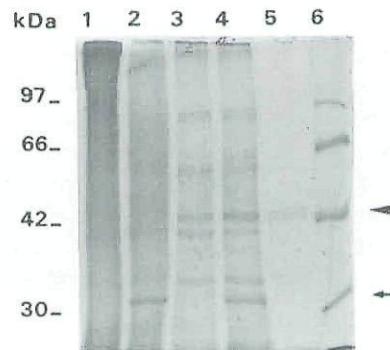


Fig. 8. Secreted protein preparations of *A. fumigatus* strains grown for 48 h at 30°C in collagen medium. The proteins of 1 ml of culture supernatant were precipitated with TCA before loading on a 9% SDS-PAGE gel. The gel was stained with Coomassie brilliant blue R250. Lane 1, 2, 3 and 4, protein profile of MA1 (*alp mep*), M1 (*ALP mep*), Δ18 (*alp MEP*) and wild-type (*ALP MEP*) strains, respectively. Lane 5, purified MEP. Lane 6, molecular mass markers: phosphorylase *b*, 97 kDa; bovine serum albumin, 66 kDa; ovalbumin, 42 kDa; and bovine carbonic anhydrase, 30 kDa. Arrow and arrow head indicate ALP (33 kDa) and MEP (40 kDa), respectively.

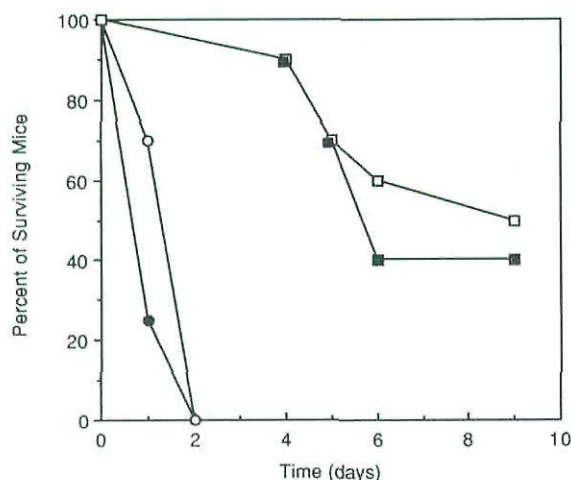


Fig. 9. Acute and chronic experimental aspergillosis in mouse infected with a high dose of conidia (10^8) and regimen 1 (○), or a low dose of conidia (10^5) and regimen 2 (□) of the *alp mep* mutant (open symbols) and the wild-type strain (closed symbols).

al. (1993), who observed that an ALP-deficient mutant obtained by nitrosoguanidine treatment was less virulent than the wild-type strain of *A. fumigatus*. Mutagenic treatments, however, are known to cause multiple undefined mutations which could be responsible for the defect in virulence. Using targeted gene disruption, no difference of pathogenicity was observed between isogenic wild-type strains and *alp mep* mutants totally deficient in proteolytic activity in collagen medium. Mortality curves (Fig. 9) and sections of lung from mice experimentally infected with both mutants and parental wild-type strains were similar (Fig. 10). Therefore, it must be concluded that the secreted *A. fumigatus* proteases, identified *in vitro* and active at neutral pH, are not essential factors for the invasion of the tissues. Our results with an opportunistic organism such as *A. fumigatus* contrast with those obtained with a wild-type strain of the pathogenic bacteria *Legionella pneumophila* and an isogenic metalloprotease-deficient mutant constructed by targeted gene disruption (Moffat *et al.*, 1994). The protease-deficient mutant of *L. pneumophila* was still pathogenic for guinea-pigs, but death was delayed and the bacterial colonization of the lung was limited. Histopathological studies also showed a reduction in the necrosis of the tissue as well as an increase in macrophage infiltrates in animals infected by the mutant.

Recently, the lack of vessel wall elastolysis in human invasive aspergillosis was demonstrated by Denning *et al.* (1992), with tissues obtained at autopsy. In experimentally infected mice, a heavy saprophytic colonization of the lung occurs rapidly after the death of the animal (unpublished results). For this reason, histopathological studies were conducted during the early stages of the disease to see if a different degradation pattern could be seen at

the beginning of the infection and during the saprophytic colonization of the lung after mouse death. In both cases, no apparent degradation of the collagen was seen, even in the tissue surrounding the bronchia which seems to be the target organ for infection and is a different microenvironment from the blood vessel. An analysis of growth conditions in this very peculiar microenvironment may help to elucidate the nature of the factors regulating the infection process of *A. fumigatus*. Since proteases do not appear to play a role, another hypothesis for virulence of *A. fumigatus* could be advanced. One can imagine that *A. fumigatus* has a ecological niche in the bronchia characterized by nutrient and temperature conditions favourable to its saprophytic growth, since this fungus accounts for only a small proportion of the airborne mould spores but is the most frequently isolated fungus from lung and sputum (Mullins and Seaton, 1978; Schmitt *et al.*, 1991). Consequently invasive aspergillosis could be circumstantial when the host phagocytic defences have been weakened by immunosuppressive treatments.

Experimental procedures

Strains and growth media

A. fumigatus strains G10 and $\Delta 18$ were used. G10 is a spontaneous nitrate reductase mutant (*nia*) of strain CBS 144-89 with a reversion frequency of less than 1×10^8 . $\Delta 18$ is a G10 mutant in which the gene encoding ALP was inactivated by gene disruption (Monod *et al.*, 1993a). The strains were grown in Sabouraud liquid medium or, to promote production of MEP, in 0.2% collagen medium at 30°C, shaking at 150 r.p.m. for 2 days (Monod *et al.*, 1991). *E. coli* LE392 was used for the propagation of bacteriophage λ EMBL3 (Promega). All plasmid subcloning experiments were performed in *E. coli* strain DH5 α .

N-terminal and internal amino acid sequence analysis of MEP

MEP was produced by *A. fumigatus* $\Delta 18$ strain grown in liquid culture as previously described (Monod *et al.*, 1993b). MEP and MEP fragments obtained by cyanogen bromide treatment were separated by Tricine-SDS-PAGE (Schagger and von Jagow, 1987) and transferred onto PVDF-Immobilon membranes (Millipore). After staining with Coomassie brilliant blue, polypeptide bands were cut out and analysed by a gas-phase sequencer 470A (Applied Biosystems) with on-line PTH-derivative analyser 120A.

Tryptic fragments were generated by in-gel digestion of the MEP band resolved on a 15% polyacrylamide gel, essentially as described by Rosenfeld *et al.* (1992). The fragments were separated on a Brownlee Aquapore RP-300 (7 μ m) reversed-phase column using an acetonitrile gradient (0–80% in 65 min) in 0.01% trifluoroacetic acid (TFA). The manually collected fragments were sequenced on a gas-phase sequencer as described above.

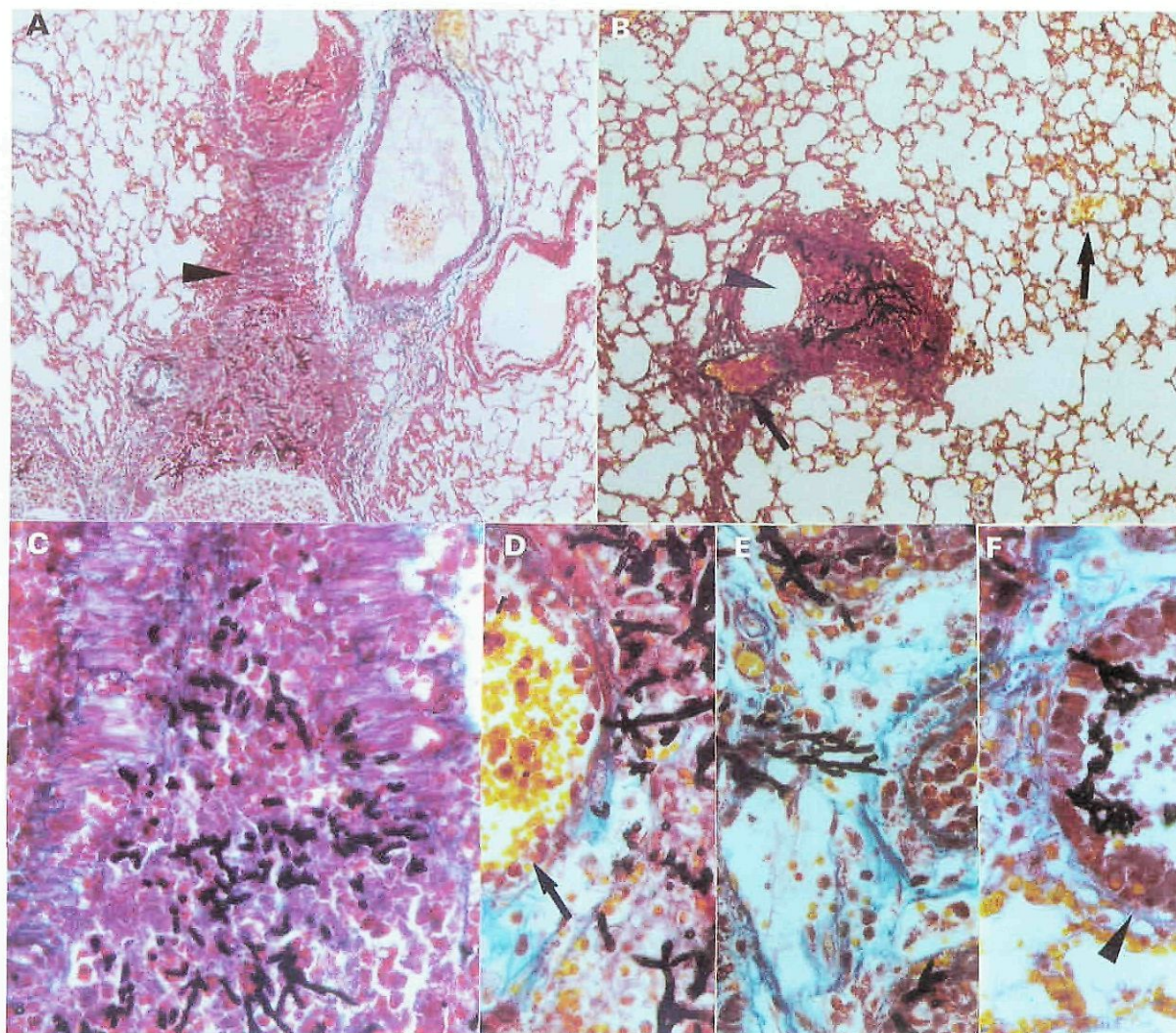


Fig. 10. Sections of lung from experimentally infected mice. A, B. Early stages of infection showing similar mycelial development inside the bronchia for the wild-type (A) and mutant (B) strains. Note the absence of fungus in the alveolar space. Arrowhead: bronchia. Arrow: blood vessel. C, D, E, F. Details of lung thin sections made from mice infected with wild-type strain and showing the absence of evident degradation of collagen and elastin matrix (stained in blue) when infiltrated by *A. fumigatus* mycelium. C. Tangential section of a bronchia crossed by hyphae. D, E, F. Hyphae crossing the collagen–elastin matrix in lung fixed after mouse death. Similar results were seen with the *alp mep* mutant. Combined Grocott's–Roos' stain. Magnification: A, B $\times 125$, C–F $\times 500$.

DNA manipulation

We used previously constructed λ EMBL3 genomic and λ ZAP cDNA libraries of *A. fumigatus* (Jaton-Ogay *et al.*, 1992; Moser *et al.*, 1992). Recombinant plaques of the genomic library were immobilized on nylon membranes (Zeta-Probe, Bio-Rad) and probed with ^{32} P-labelled oligonucleotides (Amersham) (Table 1) as described (Jaton-Ogay *et al.*, 1992). Agarose gel electrophoresis of restricted recombinant bacteriophage λ EMBL3 DNA, Southern blotting, subcloning of genomic DNA fragments into pMTL21 (Chambers *et al.*, 1988) and pAN8-1 (Mattern *et al.*, 1988) plasmids were performed according to standard protocols (Sambrook *et al.*, 1989). The plasmid

pAN8-1 carrying the *S. hindustanus* phleomycin-resistance gene (*PHL*) was kindly provided by Dr P. J. Punt (TNO Medical Biological Laboratory, Rijswijk, The Netherlands).

Screening of the cDNA library was performed using ^{32} P-labelled genomic DNA fragments as the probe. Labelling of DNA was performed using a random-primed DNA labelling kit (Boehringer) and [α - ^{32} P]-dATP. A pBluescript-II SK plasmid was excised from λ ZAP bacteriophages using f1-helper bacteriophages (Short *et al.*, 1988).

Double-stranded DNA was sequenced using a Sequenase Version 2.0 sequencing kit (USB) according to the supplier's instructions. DNA was annealed with SL primer, reverse primer or synthetic oligonucleotide primers (Microsynth).

RNA extraction and Northern blotting

A. fumigatus was grown in Sabouraud and collagen media at 30°C for 16 and 44 h, respectively. The mycelium of 500 ml culture was collected on filter paper and incubated for 30 min at 37°C in 20 ml of an osmotic solution (1.2 M MgSO₄, 10 mM sodium phosphate, pH 5.8) containing 1 mg ml⁻¹ of Novozym 234 (Calbiochem). Enzymatically lysed mycelium was collected by centrifugation in a swinging bucket rotor at 6000 × g for 5 min and total RNA was extracted following the guanidinium thiocyanate phenol/chloroform protocol of Chomczynski and Sacchi (1984) with a further purification by an anion-exchange resin column (QIAGEN System). Gel electrophoresis of RNA with 10 µg ml⁻¹ ethidium bromide in the agarose gel and Northern blotting were performed following standard protocols (Sambrook *et al.*, 1989). After the RNA transfer was complete, the membrane filters (Zetaprobe, Bio-Rad) were air-dried and exposed to u.v. radiations from a u.v. illuminator for 2 min. The 18 s and 28 s RNA bands were visible at this stage and positioned on the membrane with a pencil. The filters were prehybridized at 65°C for 5 min in a solution containing 7% SDS, 0.5 M NaH₂PO₄ pH 7.2 and 1 mM EDTA. Hybridization was performed overnight in the same solution containing ³²P-random-primed labelled probes. The membranes were exposed to X-ray film after two washes in 2 × SSC, 0.1% SDS at 65°C.

In vitro translation and immunoprecipitation of MEP

Translation was performed for 90 min at 37°C in a final volume of 50 µl containing 5 µg total *A. fumigatus* RNA, 25 µl rabbit reticulocyte lysate and 20 µCi [³⁵S]-methionine. Immunoprecipitation was performed on the translation mixture as previously described (Franzoso *et al.*, 1991) using polyclonal anti-MEP rabbit antibodies. Rabbit antiserum directed against MEP was prepared as described previously (Monod *et al.*, 1991) with 100 µg of purified enzyme (Monod *et al.*, 1993b).

A. fumigatus transformation

The transformation procedure of *A. fumigatus* was based on methods previously described (Tilburn *et al.*, 1983; Malardier *et al.*, 1989). After overnight expression of the *phl* gene, the transformants were selected on minimal medium containing 20 µg ml⁻¹ of phleomycin (CAYLA).

Proteolytic assay and protein concentration in culture supernatant

Collagenic activity was measured as previously described (Monod *et al.*, 1991) using azocollagen (Calbiochem) as substrate. Protein concentrations were measured in culture supernatant by the method of Bradford *et al.* (1976) with a Bio-Rad dye reagent (No. 500-0006) with casein as the protein standard.

Mouse infection

Male IOPS OF1 mice, 18–20 g (Ifa Credo) were used for infection experiments. All animals were cortisone-pretreated: 5 mg

cortisone acetate (200 µl of a 0.9% NaCl aqueous solution containing 25 mg ml⁻¹ cortisone acetate) were given intraperitoneally for each injection. Two immunosuppression regimens were used: (1) injection at day -3 and day 0 and (2) injection at day -3, 0, +2, +4. Wild-type and transformant strains were inoculated intranasally into cohorts of 10 mice at a dosage of 10⁸ conidia per mouse with regimen (1) and 10⁵ conidia per mouse with regimen (2). Mortality was evaluated every day for 10 d. *Aspergillus* strains were reisolated, on malt agar, from lung homogenates of infected mice. Cortisone-pretreated control mice received intranasal injection of saline solutions only. Control mice survived for at least 2 weeks after challenge.

Animal work was performed in the Pasteur Institute following the Helsinki declaration and the recommendations of the French Ethical Committee for Life Sciences and Health.

Histopathology

Mice infected with *alp mep* MA1 mutant and G10 strain were killed 3 to 4 days after intranasal infection (10⁵ conidia per mouse, cortisone regimen 2). Lungs dissected from euthanased mice were fixed in 10% buffered neutral formalin under vacuum and processed to paraffin wax. Tissue sections were stained with the Grocott's methenamine silver nitrate method, where fungal hyphae appear black (Grocott, 1955). Sections were then counterstained with a one-step polychrome method (Roos, 1992) which stained in blue the collagen and elastin lung matrix.

Acknowledgement

We thank M. Suter and R. Cramer (Swiss Institute of Allergy and Asthma Research, Davos, Switzerland) for providing the *A. fumigatus* cDNA bank, P. J. Punt and C. A. M. J. J. van den Hondel for providing plasmid pAN8-1, and P. Shaw and H. Pooley for critical review of the manuscript and assistance with the English. This work was supported by the Swiss National Foundation for Scientific Research, Grant 31-36248.92.

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