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Nucleotide sequence of a genomic and a cDNA clone encoding an extracellular alkaline protease of *Aspergillus fumigatus*

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1. SUMMARY

An *Aspergillus fumigatus* extracellular alkaline protease (ALP) which is an enzyme of the subtilisin family is a potential virulent factor of the fungus. The gene encoding ALP was isolated from a genomic library made from DNA of an *A. fumigatus* isolate. The nucleotide sequence of this gene was compared to that of a cDNA encoding *A. oryzae* ALP and to that of a cDNA encoding *A. fumigatus* encoding the mature ALP protein. Mature *A. fumigatus* ALP contains 282 amino acids and is encoded by three exons. The pre-proenzyme has a leader sequence of 121 amino acids.

2. INTRODUCTION

Invasive aspergillosis is mainly caused by the opportunistic fungus *Aspergillus fumigatus*, and occasionally by other *Aspergillus* species such as *A. flatus* and *A. terreus*. A positive correlation between proteolytic activity and invasivity in animals of the responsible organism suggests that the virulence of *A. fumigatus* is related to its proteolytic activity [1,2]. A collagen inducible alkaline exoprotease (ALP) of *A. fumigatus* was recently isolated by several groups [3–5]. *A. fumigatus* ALP differed from *A. oryzae* ALP in having a higher isoelectric point and a two-fold higher specific activity against collagen and elastin. The high substrate specificity of *A. fumigatus* ALP towards collagen could facilitate invasion in various tissues. Hence, ALP may be of crucial importance for the pathogenicity of the fungus [4].

In this report we describe the isolation and the nucleotide sequence of both a genomic and a

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cDNA clone encoding ALP. The availability of this clone will allow the determination of the role of the gene product in the pathogenesis provoked by the fungus and will be of help in the elucidation of the molecular basis of the regulation of the extracellular alkaline protease gene (*alp*).

3. MATERIALS AND METHODS

3.1. Strains and growth media

A. fumigatus strains CHUV 192-88 [4] and ATCC 42202 were used. The strains were grown in Sabouraud liquid medium or, to promote production of ALP, in collagen medium [4]. *Escherichia coli* LE392 was used for the propagation of bacteriophage λ EMBL3 (Promega, Madison, WI). All plasmid subcloning experiments were performed in *E. coli* strain DH 5 α using standard procedures [6].

3.2. Determination of the N-terminal amino acid sequence of ALP

ALP was produced by *A. fumigatus* strain CHUV 192-88 grown in liquid culture as previously described [4]. The ALP-enriched protein fraction was then separated by Tricine-SDS-PAGE [7] and transferred onto PVDF-Immobilon membranes (Millipore Bedford, MA). After staining with Coomassie blue, the protein band was cut out and analysed by a gas phase sequencer 470A (Applied Biosystems) with on-line PTH-derivative analyser 120A.

3.3. Construction of genomic library

A genomic DNA library was prepared using DNA from *A. fumigatus* isolate CHUV-192-88. The isolated DNA [8] was partially digested with *Sau*3A. DNA fragments ranging from 12 to 20 kb were isolated from low melting agarose (Biorad, Richmond, CA) and inserted into bacteriophages using the λ EMBL 3 *Bam*HI arm cloning system (Promega).

3.4. Screening of the genomic library

Approximately 50 000 recombinant plaques of the genomic library were immobilized on nylon membranes (Zeta-Probe, Biorad). These filters

were probed with ^{32} P-labelled oligonucleotides (Amersham, Rahn, Zürich) [6] (Table 1) in a solution containing 5 $\times$ SSC, 7% SDS, 10 $\times$ Denhardt's, and 20 mM NaH₂PO₄ (pH 7.0), at 50°C for 24 h. The membranes were exposed to X-ray films after a first wash in 3 $\times$ SSC, 5% SDS, 25 mM NaH₂PO₄ (pH 7.0) and a second wash in 1 $\times$ SSC, 1% SDS, at 50°C. Positive plaques were purified and the DNA was isolated [9]. Agarose gel electrophoresis of restricted recombinant bacteriophage λ EMBL3 DNA, Southern and Northern blotting were performed according to standard protocols [6].

3.5. DNA sequencing

Double-stranded DNA subcloned into plasmid pMTL21 [10] was sequenced using a sequenase version 2.0 sequencing kit (USB, Cleveland, OH) as recommended by the supplier.

3.6. Isolation of poly(A)⁺ mRNA and synthesis of cDNA

For the isolation of mRNA, a 4-day-old culture of *A. fumigatus* ATCC 42202 was used. Total RNA was isolated according to Teeri et al. [11]. The isolation of poly(A)⁺ mRNA was performed according to Sambrook et al. [9] or using the Poly(A) QuikTM mRNA isolation kit (Stratagene, La Jolla, CA). 5 μ g poly(A)⁺ RNA was used to synthesize cDNA with the ZAP-cDNATM synthesis kit (Stratagene).

3.7. PCR amplification and cloning of cDNA encoding ALP

PCR was performed in a volume of 100 μ l. The sample contained the appropriate buffer, 200 μ M each of the ALP cDNA or ALP leader primers (Table 1), 1 ng of non-packaged cDNA and 1 U of VentTM DNA polymerase (Bio Labs, Beverly, MA). The samples were subjected to 30 cycles of amplification consisting of the following steps: 1 min at 94°C, 2 min at 55°C and 1 min at 72°C, followed by a final incubation at 72°C for 10 min. The PCR products were double-digested with *Bam*HI and *Bgl*II and cloned into plasmid pMTL21 [10] for restriction analysis and sequencing.

4. RESULT AND DISCUSSION

4.1. Probe design

ALP was shown to be a serine protease of the subtilisin family which includes *A. oryzae* alkaline protease [12], *Saccharomyces cerevisiae* protease B [13], *Yarrowia lipolytica* alkaline extracellular protease [14], and *Tritirachium album* proteinase K and R [15,16]. These enzymes share a high degree of amino acid sequence similarity near their active-site (Gly-Thr-Ser-Met-Ala-Thr/Ser-Pro-His-Ile/Val-Val/Ala). The nucleotide sequences encoding the amino acids (aa) of the active site were compared, and also shown to be highly conserved. An oligonucleotide probe (Probe A, Table 1) was thus synthesized to detect clones containing this conserved region of subtilisin-like enzymes in the genomic library made from *A. fumigatus* DNA.

The 13 aa residues of the N-terminus of the mature protein were determined to be Ala-Leu-Thr-Thr-Gln-Lys-Gly-Ala-Pro-Trp-Gly-Leu-Gly. On the base of this aa sequence, together with information on the nucleotide sequence encoding the N-terminal sequence of other proteases of the subtilisin family [12–16], a second oligonucleotide (Probe B, Table 1) was designed.

4.2. Cloning and DNA sequencing of the *A. fumigatus* alp

A mixture of probes A and B was used to screen the genomic library. Sixteen hybridizing clones were identified. Among these clones, only three hybridized when probes A or B were used separately. These three clones were selected for further analysis. Restriction enzyme analysis of purified DNA revealed that the three clones had a common 2.2-kb *Sph*I fragment which hybridized with probes A and B (date not shown). The nucleotide sequence of this fragment was determined (Fig. 1). It contained a sequence of 39 base pairs (bp) encoding 13 aa identical to the 13 N-terminal aa determined by amino acid sequence of *A. fumigatus* ALP. This strongly suggested that the 2.2-kb *Sph*I fragment contained the coding sequence of the N-terminal end of mature ALP. However, a stop codon in-frame was present downstream of the putative 13 N-terminal aa of ALP (TGA, position bp 874–876, Fig. 1), indicating the presence of an intron. The deduced aa sequences of the three possible reading frames determined by the nucleotide sequence of the *Sph*I fragment were therefore compared to the aa sequence of other fungal subtilisin proteases [12–16]. The deduced aa sequence from a cDNA encoding *A. oryzae* ALP [12] fur-

Table 1

List of oligonucleotide probes and PCR-primers

Primers/probes	Amino acid sequence	Sequence used
Probe A	G T S N A T P H (I/V) (V/A)	GGT ACC TC(C/A) ATG GC(T/C) AC(T/C) CC(C/T) CAC (A/G)TT G
Probe B	Q K G A P W G L G	CAG AAG GG(T/C) GC(C/T) CC(C/A) TGG G(T/C) CT(G/T) GG
5'ALP	A L T T Q K G	^{Bam} HI 5'CGGATCC GCC TTG ACC ACT CAA AAG GG 3'
3'ALP	L A Y N G N A STOP	^{Bgl} II 5'GCAGATCT TTA AGC ATT GCC ATT GTA GGC AAG 3'
5'ALP-Leader	M L S I K R T S	^{Bam} HI 3'CGGATCC ATG CTT TCT ATC AAA CGC ACT TCG 3'

Probe a, conserved region of subtilisin-like enzymes near their active site; Probe B, N-terminal end of ALP; 5'- and 3' end of cDNA clone of mature ALP; 5' Leader, 5' end of cDNA clone of ALP precursor.

^{SphI} -296 GCATGCATCTATGCTCTCCGACGGTGTGCGATGCAGAGTTGCA - 252 -251 ATGTTACCTCTTGTAGTTCACATGCTCTCCGATGCATCTTCG - 207 -206 GATTGTCTCCATCTTGTCTCGCGTGGTGACTAGGAGATTCTGA - 162 -161 GGCATGAAATGGCAAGTGTGGAAGAACATGAATGGAACCGA - 117 -116 AGAGACAAAAGTATATAAACCCTGCTGTCTCCAGAGATCTGT - 72 - 71 GTCACACGACCATTCAGTCCAAATCTCCAGCGCTGATCCGCCA - 27 - 26 TCTAAAGCTTGTCTAGAGTCTCGGCATGCTTTCTATCAACGCA 19 10 30 70 80 20 CTTGCGTCTCTTGGAGCTGTCTGCCAGCGCTCTTTGGCGCGC 64 T S L L L G A V L P A V F G A 65 CTGTCCAGAAATCTGTCTGTCTGCTCAGAAATCTCTGGCAAGT 109 P V Q E T R R A A Q K I P G K 110 ACATCGTGACCTTCAACCGCGCACCGATACAGTACCATCTGAGT 154 Y I V T F K P G T D T A T I E 155 CTCACACTCTTTGGGCACTGTCTTCAACAAACGCAATCTGGAGC 199 S H T L L W A T D L H K R N L E 200 GTGTGATACCATAGCGCGCAACCTCCCTCGGTGCTGAGGAAGA 244 R R D T T S G E P P V G I E K 245 GCTCAAGATCAAGGATTTCCGCGCTACGCTGGCTCTTCGATG 289 S Y K I K D F A A V A G S F D 290 ACGGCACCATCGAGAAATCCGCAAGAGGGGAGACTTCAGTACTC 334 D A T I E E I R K R G D 335 TTCAGAACCCGACATATAAGACAAAGTGGCTTACAAGTGCAC 379 ***** 380 AGTTTGGCCATGTTGAGGAGGACCAATCTGGTACTCTGACGCT 424 V A H V E E D Q I Y W Y L D A 425 TGACCACTCAAAAGGCGCCCATGGGCGCTGGCAGCATTTCCC 469 L T T Q K G A P W G L G S I S 470 ACAAGGACAAAGCAAGCACCAGTATCTACGACACCCAGCGCTG 514 H K G Q A S T D Y I Y D T S A 515 GCGCAGGACCTATGCTACGTTGTGACAGTGGCATCAATGTCA 559 G A G T Y A Y V V D S G I N V 560 ACCACGTCGACTTCGAGAGCGCGCATCGCTGGCATCAACGCGG 604 N H V E F E S R A S L A Y N A 605 CTGGTGGCAGCATGTGTCAGACATCGGCCACGGAACGACGCTG 649 A G G S H V D S I G H G T H V 650 CTGGTACCATTTGGCGGCAAGACCTACGAGTGGCCAGGAAGCCA 694 A G T I G G K T T G V A K K T 695 ACCTCTGTCTGCTCAAGGTTCTCCAGGCGAGTCTCTAGCACCT 739 N L L S V K V F Q G E S S T 740 CCATCATCTTGCAGCGCTTCAACTGGGCTGTCAATGACATTGTGA 784 S I I L D G F N W A V N D I V	250 785 GCAAGGGTGGTATAGAAGGCTGGCATCAACATGACGCTTCTA 829 S K G R T K K A A I N...S L 830 AGATGACCTCTTGTCTCGTCAACTGACTGAAAGTAAACGGCGCT 874 * 875 GATGGATAATGACATCTGTGCTTACTCTTATGCTTCAACACG 919 ** G G G Y S Y A F N N 920 CTGTGAGAACGCTTTCGATGAAGGTCTCTTCTCTGCTGCTG 964 A V E N A F D E G V L S V V A 965 CTGGAACGAGAACTATGTATCCCCGTTTGAAGAAATTCACAA 1009 A G N E N 1010 ATGAACATTGCAAGTTTGGTGGGACTAACATGTTGTGCCAGT 1054 S 1055 ATGCTCAAAATACCAAGCCCTGTTCCGCTCTCAACGCTTACGG 1099 D A S N T S P A S A P N A L T 1100 TTGCTGGATCAACAGCAAGCAACCGCGCTCTCTTCTCAACT 1144 V A A I N...S N A R A S F S N 1145 ATGTTCTCGTGTGCTGACATCTTGTCTCCGCTCAGGATACCTTT 1189 Y G S V V D I F A P G Q D I L 1190 CGGCTGGATGGCTCCACCACTGGCCACCAACCATCTCCGGTA 1234 S A H I G S T T A T N T I S G 1235 CTTCCATGCCCCCTCACAATGTTGGCTATCCGCTGTACTTGA 1279 T S M A T P H I V G L S V V L 1280 TGGTCTTGAGAACCTCTCTGGCCCTCTGCGATGACCGCTCGCA 1324 M G L E N...S G P A A V T A R 1325 TCAAGAGCTGGCCACCAAGTGTGTTACCAAGCTTAAGGGCA 1369 I K E L A T N G V V T N V K G 1370 GCGCCACCAAGCTTGGCTACAAATGCTTAAGCTTATGTG 1414 S P N K L A Y N G N A *** 1415 AAGAAGATAGTTTACAGAAACTGCGCAGGGCGAGTCTTACCA 1459 1460 GATGATGGTGGGGGAGTAGGCGAGCAGGAGGTCTATGACGCT 1504 1505 GAATCTATGAGCCTACATATATTAACAAAGCAATGGAATGATT 1549 1550 GTGTTATTATGCTGTGACCTGTAAACAAATTAACCTTTATTCG 1594 1595 GCTTGCATGTAGTTCGGAACAAAGCTAATCATCGAGATGGGAGC 1639 1640 GATGGAATCACACTTCAACATGTCCGCTAAAGTATGTGAACCT 1684 1685 CCACTGTCTTTCGCTACGGCTGACGCTACGCTCCTCCTGA 1729 1730 CATTAACTCTCAAGGACAGCAGCAAGATTTGGCGATGAAGAACG 1774 1775 TCGGAAGTGCCTCCCAAGGAATGGGAAGACATATTCATCCGTG 1819 ^{SphI} 1820 AGGAATGTTGATGCTGCTGAACCTTCTGACCTTCCAAATGAAGCA 1864 1865 TGC
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Fig. 1. Nucleotide sequence of the *A. fumigatus alp* gene. The deduced amino acid (aa) sequence is shown below the nucleotide sequence. The N-terminal aa sequence of the mature enzyme begins at aa residue 122 and is preceded by a vertical bar and a horizontal arrow. The 13 aa sequence from N-terminus of the protein deduced by aa sequencing and the conserved aa sequence around the active site of the enzyme are underlined. Two putative cleavage sites within the signal sequence for secretion are indicated by arrows. The sequences in brackets are introns and are not present in cDNA clones generated by PCR. A potential TATA box (pos. -103 to -97) is indicated. *** indicates stop codons. Potential N-linked glycosylation sequences N-X-S are dotted. The DNA sequence reported here has been submitted to the EMBL database and the accession number is Z11580.

ther suggested intron/exon structures in position 325-382, 827-889 and 979-1050 (Fig. 1) in the *A. fumigatus alp* gene. All putative introns start and terminate by consensus sequences for RNA

splice junctions [17]. To verify the intron/exon structure of this gene we performed PCR using *A. fumigatus* cDNA as the target. Using ALP-cDNA primers (5'-ALP and 3'-ALP, Table 1)

only one fragment of 846 bp was amplified. The nucleotide sequence of this fragment confirmed the proposed intron/exon structure and contained an ORF of 282 aa. Interestingly, no difference was found comparing the corresponding nucleotides of the genomic *alp* and cDNA *alp* even though two different *A. fumigatus* strains were used for library construction. In a second PCR experiment the intron/exon structure in the putative leader (position bp 1–420, Fig. 1) was determined using the 5'-ALP-leader and the 3'-ALP primers (Table 1) for amplification. The predicted DNA fragment of 1209 bp was amplified and again the intron/exon structure was verified.

4.3. Northern blot analysis of RNA isolated from *A. fumigatus* grown in collagen or Sabouraud medium

Supernate of *A. fumigatus* contained ALP when the fungus was grown in collagen medium, but not when grown in Sabouraud medium [4]. We thus expected to find high amounts of ALP-specific mRNA from *A. fumigatus* grown in collagen medium. The 562-bp *Bgl*II–*Sal*I fragment (pos. –77 to 485, Fig. 1) was used to probe Northern blots of RNA isolated from fungal mycelia grown in either medium. A mRNA transcript of 1.6 kb was observed only when the fungus was grown in collagen medium (Fig. 2). No signal was observed with DNA upstream from the *Bgl*II, indicating that the transcription of the gene starts after this site. A sequence TATAAAT (25 bp upstream the *Bgl*II site) constitutes a possible TATA box [18] (Fig. 1).

4.4. Sequence analysis

Based on Northern blot analysis and on the nucleotide sequence of cDNA, ALP is encoded by 1209 nucleotides starting from the ATG codon 79 bp downstream of a *Bgl*II site (Fig. 1). The N-terminal aa sequence of the mature ALP obtained by aa sequencing was preceded by a polypeptide of 121 aa suggesting that the protein was synthesized as a precursor of 403 aa. Inspection of the N-terminal aa sequence of the precursor beginning from the Met1 residue (pos. 1, Fig. 1) suggested the existence of a signal peptide in the protease precursor polypeptide with an hydrophobic core of 9 aa. Two putative signal pepti-

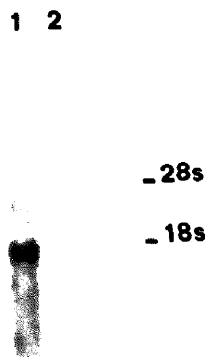


Fig. 2. Northern blot of RNA from a culture of *A. fumigatus* grown in collagen (lane 1) or Sabouraud medium (lane 2) probed with the 0.6-kb *Bgl*II–*Sal*I fragment (pos. –77 to 485, Fig. 1). The apparent molecular size of the RNA hybridizing with the probe was estimated to be 1.6 kb based on the position of the 28 and 18S ribosomal RNA.

dase cleavage sites in accordance with the 3–1 Von Heijne's rule [19], indicated each by an arrow in Fig. 1, were found after the Gly and Ala residues at positions 20 and 21, respectively. The mature protein molecule generated after cleavage of the 121-aa polypeptide from the 403-aa precursor had a calculated molecular mass of 28.5 kDa. The size estimated from the electrophoretic mobility of the protein on SDS-PAGE is 33 kDa [4]. The primary structure of the mature protein contains four potential N-linked glycosylation sequences Asn-X-Ser. However, on SDS-PAGE gels, the protein did not stain with PAS reagent, indicating that the enzyme is not glycosylated.

There are 46 aa substitutions (17% of aa) in *A. fumigatus* ALP compared to the mature sequence of *A. oryzae* ALP (Fig. 3). In particular five neutral aa in *A. fumigatus* ALP are substituted by a Lys in *A. oryzae* ALP and six Asn are substituted by an acidic aa. The theoretical pI of

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A_oryzae      G   S   Q   E   V   D   E
A_fumigatus_1 ALTYRGATMGLSGTGHKGQASTQVLYDTSAGAGCTAYVVDSCINMNIIVE

51      G   K   Q   S   A   Y   I   A   G   I
FESHASLAYNAAGSHVDVSGHCTHWAGTJGKRTGVAKKTNLLSVKVFQ

101      V   A   K   S   K   D
GESSTSIILLGPFNAVNDIVSKRTKAAINKMSLOGGYSTAFNNAVENA

151      E   Q   G   Q   D   I   Q   N   F   K
FDEGVLSVVAAGHNSDA*NTSPASAPHALTPVAATKKBARASFSNYGVV

201      V   S   L   A   A   D
VDIFAPGGDILSANIGSTTATNTISGTSMAFPHVGLSVVLNGLENIISGP

251      K   K   D   K   L
AAVTAIKIKELATNOVVTNVKGSFNKLAYNGNA
    
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Fig. 3 Comparison of *A. fumigatus* and *A. oryzae* ALP aa sequences. The aa substitutions found in *A. oryzae* mature ALP are indicated above the *A. fumigatus* ALP aa.

A. fumigatus and *A. oryzae* ALP are 7.2 and 6.5, respectively. The difference of aa composition accounts for the differences of *pI*, optimum pH for collagen and elastin digestion, and specific activity for both substrates [4].

To our knowledge, this is the first report of an *A. fumigatus* gene encoding for a potential virulence factor. We intend to disrupt this gene in *A. fumigatus* in order to examine the pathogenicity of *alp*-deficient *A. fumigatus* mutants in vivo. Experiments are also underway for the examination of the maturation of the protein and the control of transcription of this gene at the molecular level.

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