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Dipeptidyl-Peptidase IV Secreted by *Aspergillus fumigatus*, a Fungus Pathogenic to Humans

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A dipeptidyl-peptidase IV was purified from the culture medium of the human-pathogenic fungus *Aspergillus fumigatus*. The enzyme has an apparent molecular mass of 95 kDa and contained approximately 10 kDa of N-linked carbohydrate. This glycoprotein is antigenic and has all characteristics of the class IV dipeptidyl-peptidases: removal of Xaa-Pro and to a lesser extent Xaa-Ala dipeptides from the N termini of peptides, including bioactive peptides such as neuropeptide Y, [des-Arg^1] bradykinin, and glucagon-like peptide 1, activity at neutral pH, and presence in the amino acid sequence of the Gly-X-Ser-X-Gly consensus motif of the serine-hydrolases and the putative catalytic triad (Ser^{613}, Asp^{690}, His^{725}) of the dipeptidyl-peptidases. Moreover, the last 200 amino acids displayed 60 to 65% similarity with the other dipeptidyl-peptidases IV from rat, mouse, human, and yeast. However, unlike the other dipeptidyl-peptidases, the dipeptidyl-peptidase IV of *A. fumigatus* is a secreted enzyme with a cleavable signal peptide. Expression of a recombinant dipeptidyl-peptidase IV of *A. fumigatus* has been attained in the yeast *Pichia pastoris*.

Members of the dipeptidyl-peptidase (DPP; EC 3.4.14) family cleave dipeptides from the N termini of peptides or proteins. These enzymes belong to different classes depending on the nature of the dipeptide released as well as their cellular localization (3). A DPP V has been isolated recently from *Aspergillus fumigatus* (3). This DPP V can release the dipeptides His-Ser, Ser-Tyr, and Ala-Ala. It is the first described DPP that is secreted and not membrane associated. A putative role of the DPP V in the immune defense reaction against *A. fumigatus* was indicated by the identity between DPP V and the major nonpurified chymotrypsic antigen currently used for the differential serodiagnosis of patients with aspergilloma (3). In addition, mice surviving infection with *A. fumigatus* have antibodies that recognize only the DPP V specifically in an *A. fumigatus* total extract (20a). A possible role in protection against *Aspergillus* infection was thus suggested. DPPs are recognized as playing an essential role in human physiology. Removal of Xaa-Pro (and to a lesser extent Xaa-Ala) dipeptides from the N termini of peptides may generate biologically active peptides or, conversely, may inactivate peptides by truncation (23, 25, 28). The DPP IV CD26 is a surface differentiation marker involved in the transduction of mitogenic signals in thymocytes and T lymphocytes in human, mouse, and rat and in cell matrix adhesion through specific interactions with fibronectin and collagen (11, 13, 14, 24). During the purification of the DPP V of *A. fumigatus*, we identified a second DPP which belongs to class IV and is antigenic. To better characterize the DPP family, which has been overlooked in fungal pathogens, we undertook the purification, cloning, expression, and biochemical characterization of secreted DPP IV. The ability of this DPP to bind to collagen and to hydrolyze some biological peptides suggests a possible role during lung invasion of *A. fumigatus*.

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MATERIALS AND METHODS

Organism and culture conditions. *A. fumigatus* CBS 144.89 was maintained on 2% malt extract agar slants. Mycelium was obtained from cultures grown in a fermenter for 48 h of culture at 25°C in 1% (wt/vol) yeast extract (Difco). Flask and fermenter culture conditions were as previously described (21).

Purification of DPP IV. *A. fumigatus* DPP IV was purified basically as described for DPP V (3). An ethanol precipitate of a 44-h culture of *A. fumigatus* in a 1% yeast extract medium was dissolved in 50 mM Tris HCl (pH 8.8). After concentration under vacuum, the sample was loaded on a Superdex 75 HR 10/30 (Pharmacia) gel filtration column and eluted with a 150 mM NaCl aqueous solution at 0.8 ml/min. Fractions corresponding to the size range of 80 to 95 kDa were dialyzed, loaded on a MonoQ (Pharmacia) column, and eluted at 0.8 ml/min with a 0 to 0.35 M sodium acetate buffer gradient in 20 mM Tris HCl (pH 8.8). Fractions eluted between 0.25 and 0.35 M sodium acetate were dialyzed and loaded on a ProPac PA1 anion-exchange high-pressure liquid chromatography (HPLC) column (Dionex) and eluted with the same buffer. The presence of DPP IV activity was monitored during the different purification steps by the cleavage of Gly-Pro *p*-nitroanilide (pNA) in the chromatographic fractions (see below).

Protein characterization by SDS-PAGE and Western blotting. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) on 7.5% acrylamide and deglycosylation of *A. fumigatus* DPP IV were performed as described previously (19). Proteins were stained with Coomassie blue or silver nitrate (19).

A pool of serum from aspergilloma patients (kindly provided by J. P. Bouchara, CHR Angers) was used for anti-*Aspergillus* human serum. Monospecific antisera against DPP V of *A. fumigatus* and CD26 from human T cells (monoclonal antibody 1F7; generous gift of C. Morimoto, Dana-Farber Cancer Institute, Boston, Mass.) (9) were also tested on *A. fumigatus* DPP IV (3). Preimmune mouse antiserum and human sera from *Candida*-infected patients were used as control sera. After electrophoresis, samples were electrotransferred to nitrocellulose membranes and immunoblotted as previously described (19), using 1/1,000 dilutions of antisera and their peroxidase-conjugated anti-immunoglobulin G antibodies.

Amino acid sequence determination of peptide fragments of *A. fumigatus* DPP IV. Internal sequences after endolysin digestion of *A. fumigatus* DPP IV excised from an SDS–7.5% polyacrylamide gel and NH_2 terminus peptide sequence were determined as previously described (2, 18).

Cloning and DNA sequencing of *A. fumigatus* DPP IV. We used previously constructed bacteriophage λEMBL3A genomic and λgt11 cDNA libraries of *A. fumigatus* (17). Recombinant plaques of the genomic library were immobilized on nylon membranes (Zeta-Probe; Bio-Rad) and probed with a ^{32}P-labeled oligonucleotide designed according to one internal amino acid sequence (17). Recombinant plaques of the λgt11 cDNA library were transferred on the same membrane and probed with a ^{32}P-labeled randomly primed genomic DNA fragment of the isolated *A. fumigatus* DPP IV. Agarose gel electrophoresis of restricted recombinant bacteriophage λEMBL3A DNA, Southern blotting, and subcloning of genomic DNA fragments into plasmid pMTL21 (7) were performed according to standard protocols (26). Primers were provided by Mi-

TABLE 1. Substrate specificities of the DPP IV of *A. fumigatus* and CD26 of human MOLT4 cells[a]

Substrate	Activity (μg of substrate hydrolyzed)	
	DPP IV	CD 26
Gly-Pro pNA	160	103
Ala-Pro-pNA	200	90
Lys-Pro MNA	400	ND[b]
Arg-Pro pNA	160	ND
Gly-Phe pNA	106	ND
Ala-Ala pNA	60	0
Lys-Ala MNA	0	0
Ser-Tyr NA	0	ND
His-Ser NA	0	ND
Gly-Arg pNA	0	0
Leu-Gly NA	0	ND
Arg-Arg NA	0	ND
N-Acetyl Ala pNA	0	0
Phe pNA	0	ND
Ala-Ala-Phe pNA	0	ND
N-Acetyl Ala-Ala-Ala pNA	0	0
Gly-Gly-Phe pNA	0	ND
NAPNE[c]	Not degraded	ND

[a] The reaction was performed at 37°C for 1 h with 0.5 μg of *A. fumigatus* DPP IV and the monoclonal antibody-purified CD26 (15).

[b] ND, not done.

[c] Not compared because the NAPNE is not a pNA or an arylamide derivative.

crosynth, Balgach, Switzerland. Double-stranded DNA was sequenced by using an automatic sequencer. DNA sequence data were analyzed by using the University of Wisconsin Genetics Computer group program (10).

Expression of *A. fumigatus* DPP IV in *Pichia pastoris*. *P. pastoris* GS115 and the expression vector pHIL-S1 were from a *Pichia* expression kit (Invitrogen). PCR of *A. fumigatus* DPP IV DNA was performed by using two homologous primers based on the genomic DNA sequence: 01 (5′ GC TGC GAT ATC GCC ATT GAC GTC CCT CGT CAA CAA 3′), encoding the N-terminal sequence of the protein (bases 93 to 116); and 02 (5′ C ATG AGA TAT CTA CAG AAC AGA CTT CTT 3′), encoding the C-terminal extremity of the protein (bases 2334 to 2361). cDNA was used as the template. Thirty cycles consisting of a 1-min 95°C melting step, a 1-min 60°C annealing step, and 1-min 70°C extension were run. The PCR product was digested by *Eco*RV and cloned into the *Sma*I site of pHIL-S1, generating plasmid pHIL-S1-DPP IV. *P. pastoris* spheroplasts were transformed with 10 μg of pHIL-S1-DPP IV linearized by *Bgl*II as described in manual version E for the *Pichia* expression kit (Invitrogen). The culture filtrate containing the *A. fumigatus* DPP IV was recovered after 1 to 4 days of expression. Deglycosylation of the recombinant *A. fumigatus* DPP IV was performed by using peptide-*N*-glycosidase F (Oxford Glycosystems) as described previously (19).

Enzymatic activity of the *A. fumigatus* DPP IV. DPP activity was estimated with 0.5 μg of *A. fumigatus* DPP IV and different pNA and arylamide derivatives of mono-, di-, and tripeptides (Sigma) at the final concentration of 0.4 mM in 100 μl of 50 mM Tris HCl (pH 7.5) for 1 h at 37°C (Table 1). The optical density of the pNA coloration was measured at 405 nm, and the fluorescence of the arylamide was measured by using emission and excitation filters at 335 and 405 nm, respectively. The optimum pH was determined by using various buffers containing either 50 mM Tris HCl (pH 6.5 to 9) or 50 mM sodium acetate (pH 3.5 to 6). K_m was determined by using a 50 mM Tris HCl (pH 7.5) buffer containing 0.05 to 2.4 mM substrate. The inhibitory activity of two specific DPP IV inhibitors, lys-4-nitrobenzylocarbonyl-pyrolidide {lys-[Z(NO_2)]-pyrolidide} and lys-[Z(NO_2)]-thizolidide (4, 27), was determined with the two substrates Gly-Pro pNA and Ala-Pro pNA. As a control, H-Gly-Pro-OH (2 μM to 2 mM), which is not a DPP IV inhibitor, was also tested. For inhibitor assays, the DPP IV CD26 from MOLT4 cells was used as a control enzyme. The control enzyme was purified by immunoprecipitation using monoclonal antibody 1F7 (8) and protein A-Sepharose (Sigma) as described previously (15). The reaction buffer for the DPP IV activity of CD26 contained 100 mM HEPES (pH 7.6), 120 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 8 mM glucose, and 1% bovine serum albumin. The inhibition experiments were performed with the specific substrate of DPP IV, Gly-Pro pNA.

The activity of the DPP IV was assayed by using three biologically active peptides: neuropeptide Y (NPY), [des-Arg^1] bradykinin (BK) and glucagon-like peptide (GLP)-1-(7-36) amide. Cleavage of [des-Arg^1] BK (Bachem, Bubendorf, Switzerland) was followed by HPLC using a μBondapak cyanopropyl reverse-phase column (150 by 3.9 mm; Waters, Milford, Mass.) with a linear gradient of 0 to 40% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 1 ml/min. GLP-1-(7-36) amide (gift of B. Thorens, Lausanne, Switzerland) and the cleaved fragments were separated on a Nucleosil phenyl reverse-phase column (125 by 3.0 mm; Macherey-Nagel, Oensingen, Switzerland) with 31% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 1 ml/min. NPY (Novabiochem, Laüfelfingen, Switzerland) and its cleaved fragments were separated on the μBondapak cyanopropyl column with 26.5% acetonitrile in 0.05% trifluoroacetic acid at a flow rate of 1 ml/min. Peptide fragments were detected and quantified by their absorbance at 220 nm. Fragments isolated by HPLC were characterized by electrospray mass spectrometry. Incubations with synthetic peptide substrates were conducted with 50 to 100 ng of *A. fumigatus* DPP IV at 37°C in total volume of 25 to 50 μl of 25 mM triethanolamine (pH 7.8). At the end of the incubation, the assay solutions were acidified with acetic acid and analyzed by HPLC. K_m and V_{max} values for NPY, [des-Arg^1] BK, and GLP-1-(9-36) amide were determined under the following conditions: peptide substrates were incubated at final concentrations ranging from 10 to 100 μM for NPY, 20 to 200 μM for [des-Arg^1] BK, and 10 to 50 μM for GLP-1-(7-36) amide. The substrates and products were quantified after separation by HPLC as described above. K_m and V_{max} were determined from initial velocity measurements plotted versus various substrate concentrations, using the Lineweaver-Burk representation.

Hydrolysis of *N*-acetyl-phenylalanine naphthyl ester (NAPNE), a chymotrypsic substrate, was estimated as described previously (3).

The binding of *A. fumigatus* DPP IV to native collagen was demonstrated by the capacity of the enzyme to be retained on a matrix containing collagen. Briefly, the sample was loaded in a column containing 1 ml of collagen insolubilized on cross-linked 4% beaded agarose (suspension of 1 mg of collagen/ml of packed gel; Sigma). The column was washed with 50 mM Tris HCl (pH 7.5). Elution was performed with 2 volumes of 50 mM Tris HCl (pH 7.5) containing 100, 200, and 350 mM NaCl successively.

Nucleotide sequence accession number. The sequence reported has been assigned GenBank accession no. U87950.

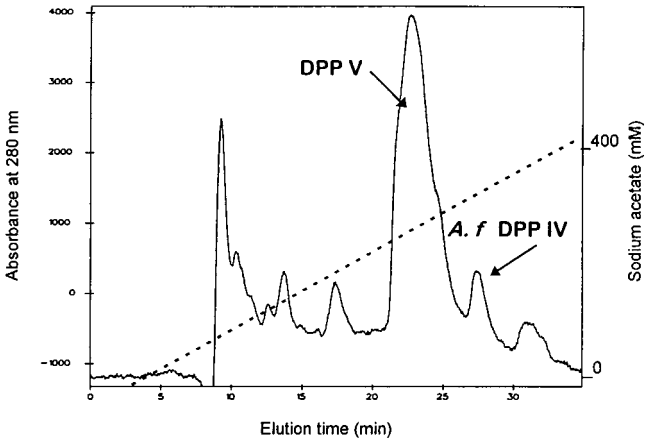

FIG. 1. Separation of *A. fumigatus* (*A. f*) DPP IV by ion-exchange chromatography (ProPac PA1; Dionex) with a sodium acetate linear gradient (0 to 350 mM in 27 min). DPP IV activity was monitored by the cleavage of Gly-Pro pNA and DPP V activity as described previously (3).

RESULTS

Purification and antigenic specificity. Preliminary experiments have shown that culture filtrate of *A. fumigatus* contained two DPP activities: an activity due to the DPP V releasing mainly X-Ala dipeptide but inactive on X-Pro peptides (3) and an activity cleaving X-Pro dipeptides. The 80- to 95-kDa fraction recovered from the Superdex column and subsequently eluted from the MonoQ column at 0.25 to 0.3 M sodium acetate contained both DPP activities. The ProPac PA1 column allowed the separation of the two DPP activities, the fraction containing the X-prolyl DPP activity being eluted at 300 mM sodium acetate (Fig. 1). SDS-PAGE of this fraction on a 7.5% acrylamide gel showed that the fraction was slightly contaminated by the DPP V (characteristically seen as a 87- to 88-kDa doublet [Fig. 2]). The estimated M_r of *A. fumigatus* DPP IV was 95,000 (Fig. 2). The results presented in Fig. 2 showed that the *A. fumigatus* DPP IV is an antigenic protein

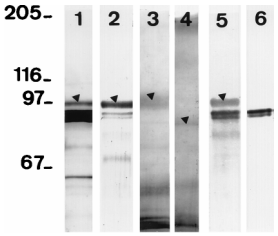

FIG. 2. Protein and antigenic characterization of *A. fumigatus* DPP IV. Lanes 1 to 4, silver nitrate staining of an SDS–7.5% polyacrylamide gel of the MonoQ fraction shearing the X-prolyl DPP activity (lane 1), the most purified *A. fumigatus* DPP IV from the ProPac fraction (lane 2), the recombinant DPP IV (lane 3), and its deglycosylated from (Lane 4). Lanes 5 and 6, immunoblot labeling of *A. fumigatus* DPP IV by a 0.1% dilution of a pool of sera from aspergilloma patients and anti-human peroxidase conjugate (lane 5) and by a 0.1% dilution of an anti-DPP V mouse antiserum and anti-mouse peroxidase conjugate (lane 6). Sizes are indicated in kilodaltons.

recognized by the polyclonal human sera from patients with aspergilloma but did not cross-react with mouse antiserum recognizing specifically DPP V or with monoclonal antibody 1F7, specific for CD26 (data not shown).

Molecular characterization of *A. fumigatus* DPP IV. The N-terminal amino acid sequence as well as the sequence of one internal peptide generated after endolysin digestion of *A. fumigatus* DPP IV were determined to be IDVPRQPYA and KYPVLFTPYGGPGAQ, respectively. One oligonucleotide probe (5′ AAG TAC CCY GTB CTB TTC ACY CCY TAC GGY GG 3′) was designed on the basis of the amino acid sequence KYPVLFTPYGG of the internal peptide and the codon usage for the genes encoding alkaline protease (17), metalloprotease (16), and a restrictocine (20) of *A. fumigatus*. This probe was used to screen 20,000 recombinant plaques of the *A. fumigatus* genomic library, and six positive clones were identified. Restriction enzyme analysis of purified bacteriophage DNA revealed that the six clones carried similar but not identical DNA fragments. However, all six clones contained a 0.6-kb *Sal*I fragment which hybridized with the oligonucleotide probe. Four clones contained a 4.6-kb hybridizing *Hin*dIII fragment, and two clones contained a hybridizing *Hin*dIII fragment over 8 kb which was a chimera of a λEMBL3A arm vector and *A. fumigatus* genomic DNA. The 4.6-kb *Hin*dIII fragment, for which a restriction map is shown in Fig. 3, was cloned in pMTL21, generating plasmid pMTL21-H4.6. An internal *Psp*1406I-*Xba*I fragment including the 0.6-kb *Sal*I fragment was sequenced on both strands (Fig. 4). One long open reading frame (ORF) coding for a polypeptide of 765 amino acids was found, starting 254 bp downstream of the *Psp*1406I site. The sequence of this long ORF and its 5′- and 3′-flanking regions along with the corresponding amino acid sequence is shown in Fig. 4. The N-terminal amino acid sequence of the mature protein is preceded by a putative signal peptidase cleavage site (AAA). However, the absence of a Met residue in the ORF before the N-terminal amino acid sequence of the mature

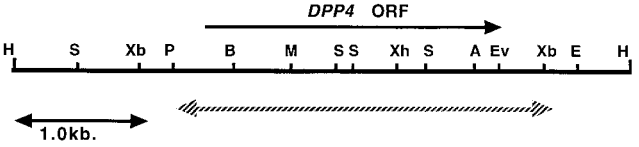

FIG. 3. Restriction map of the 4.8-kb *Hin*dIII genomic DNA fragment obtained from positive recombinant EMBL3 bacteriophages. A, *Asp*718; B, *Bam*HI; E, *Eco*RI; Ev, *Eco*RV; M, *Mlu*I; P, *Psp*1406I; S, *Sal*I; Xb, *Xba*I; Xh, *Xho*I.

protein suggested a possible intron/exon structure at the beginning of the *A. fumigatus* DPP IV gene. Therefore, 100,000 recombinant phage plaques of a previously constructed *A. fumigatus* cDNA library in λgt11 were screened with the genomic DNA of plasmid pMTL21-H4.6. Six clones were identified and purified. The cDNA was extracted from the λgt11 phage DNA after *Not*I digestion and cloned in plasmid pBluescript II KS^+. The nucleotide sequence of the longest cDNA was compared with that of the genomic DNA, and one intron was detected at bp 7 to 59 relative to the ATG encoding the putative Met1 of the DPP IV translation product. The cDNA encoded an 85,816-M_r protein with an isoelectric point, calculated from the cDNA sequence, of 5.73. Comparison of the amino acid sequence with that of the other DPP IV sequences listed in the Swiss-Prot data library revealed similarities, particularly in the last 200 amino acids. Based on the alignments of these 200 amino acids shown in Fig. 5, *A. fumigatus* DPP IV displayed similarities of 60% with rat, mouse, and human DPP IV and 65% with the yeast DPAP B. A similarity score of 47% was observed with the DPP V of *A. fumigatus*. Moreover, this stretch of 200 amino acids also contained the Gly-X-Ser-X-Gly consensus sequence shared by the serine proteases, the serine esterases, and the other DPPs (Fig. 4) (3, 22). The putative catalytic triad of all DPPs IV was also present and arranged in the same topological order (Ser^{613} Asp^{690} His^{725} [Fig. 4]). However, only the DPP IV and DPP V of *A. fumigatus* shared a cleavable signal peptide. The hydropathy profile of the deduced amino acid sequence of the *A. fumigatus* DPP IV showed a highly hydrophobic amino-terminal end corresponding to the signal peptide and a highly hydrophilic carboxy-terminal end.

Expression of *A. fumigatus* DPP IV in *P. pastoris*. After transformation with pHIL-S1-DPP IV, *P. pastoris* secreted *A. fumigatus* DPP IV enzyme in the culture medium upon induction of expression with methanol at a maximal rate of 0.15 mg/ml, with maximum production after 48 h of expression. SDS-PAGE showed that the secreted recombinant *A. fumigatus* DPP IV was the major protein present in the culture medium of *P. pastoris* (Fig. 2). This protein migrated as a broad band between 95 and 100 kDa. However, after deglycosylation by endoglycosidase F, this broad band gave rise to a thin band at 85 kDa (Fig. 2) similar to the one obtained with the deglycosylated native DPP IV (data not shown). The recombinant *A. fumigatus* DPP IV was also recognized by the serum of *A. fumigatus*-infected patients (data not shown).

Activity of *A. fumigatus* DPP IV. Recombinant and native *A. fumigatus* DPP IV exhibited the same substrate specificity. *A. fumigatus* DPP IV showed a hydrolytic activity similar to the other DPPs IV, with a specific degradation of X-Pro dipeptides such as Gly-Pro pNA, Ala-Pro pNA, Arg-Pro pNA, and Lys-Pro methoxy-β naphthylamide (MNA) (Table 1). The reaction using Gly-Pro pNA was linear for 2 h. The apparent K_m at pH 7.5 was determined by using Michaelis-Menten plots. For Gly-Pro pNA and Ala-Pro pNA, the K_m was 0.38 mM; for Arg-Pro pNA, the K_m was 0.15 mM. As a control, CD26, which is a DPP IV, showed the same X-Pro dipeptide substrate specificity (Table 1). The optimum pH of *A. fumigatus* DPP IV was pH 6.5 to 7 (Fig. 6), similar to the optimum pH of CD26 (15). To a lesser extent, Ala-Ala pNA but not Lys-Ala MNA was hydrolyzed by *A. fumigatus* DPP IV (Table 1).

Two specific inhibitors of DPP IV, lys-[$Z(NO_2)$]-pyrolidide and lys-[$Z(NO_2)$]-thiozolidide, were good inhibitors of CD26 and *A. fumigatus* DPP IV: lys-[$Z(NO_2)$]-pyrolidide at 10 μM inhibited *A. fumigatus* DPP IV by 90% and CD26 by 100%; lys-[$Z(NO_2)$]-thiozolidide, at the same concentration, inhibited *A. fumigatus* DPP IV by 77% and CD26 by 95%. As a control,

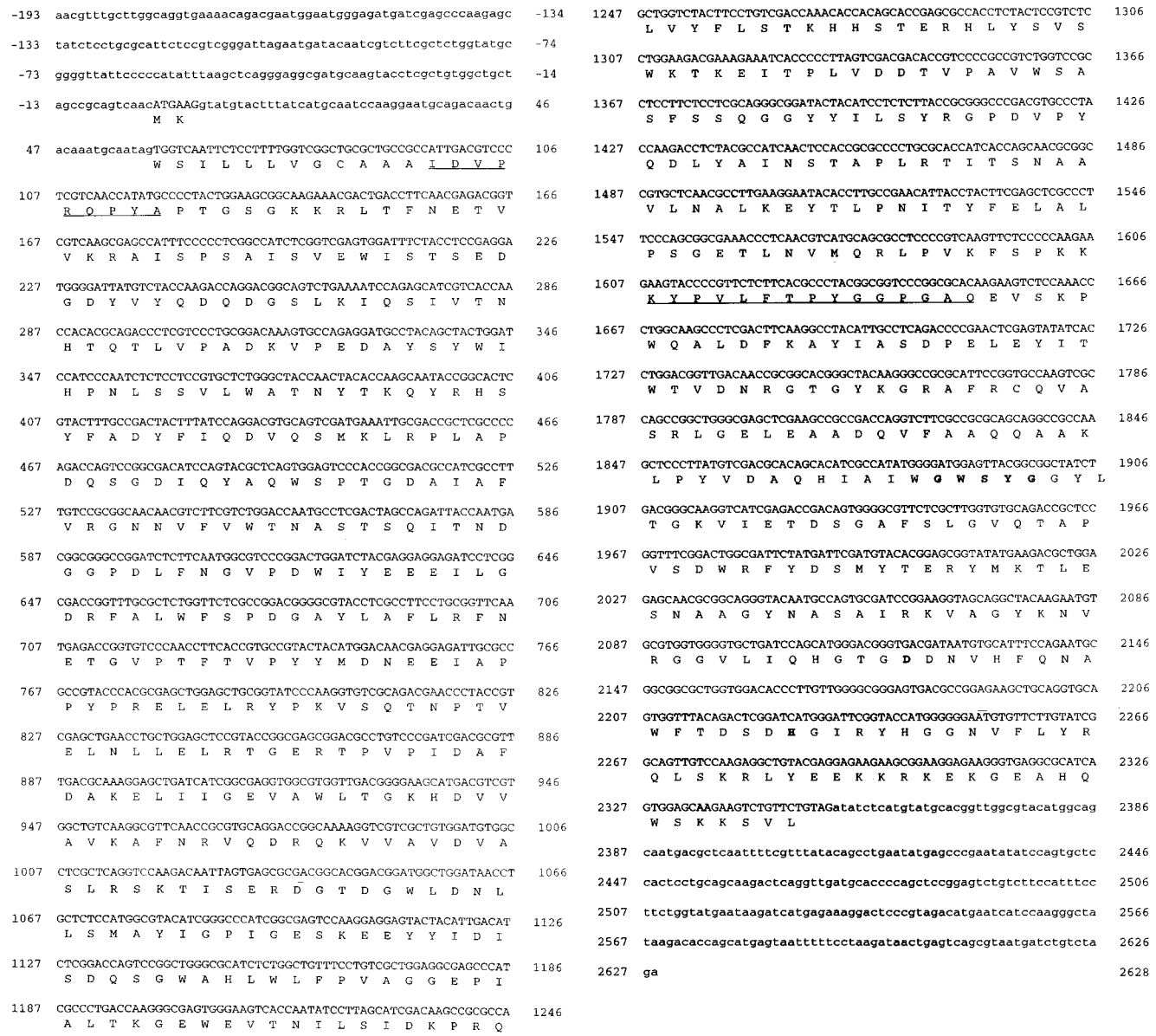

FIG. 4. Genomic nucleotide sequence of the *A. fumigatus* DPP IV and the deduced amino acid sequence. The NH_2 terminus methionine residue is referred to as +1 of the amino acid sequence and A of the initiator ATG codon of the nucleotide sequence. Two introns were identified by comparison with the cDNA sequence of DPP IV and are shown in lowercase. The sequences corresponding to the NH_2 terminus and internal peptides determined for *A. fumigatus* DPP IV are underlined. The Gly^{611}-X-Ser^{613}-X-Gly^{615} consensus and the catalytic triad Ser^{613} Asp^{690} His^{725} are in boldface.

H-Gly-Pro-OH had no effect on the *A. fumigatus* DPP IV or CD26. These results confirmed that *A. fumigatus* DPP IV is a true DPP IV which hydrolyzed preferentially X-Pro dipeptides and reacted with the specific inhibitors of this class of enzymes.

NAPNE, the chromogenic substrate used in the serodiagnosis of aspergilloma which is cleaved by DPP V (3), was not degraded by *A. fumigatus* DPP IV (data not shown), confirming the difference in biochemical activities of these two DPPs from *A. fumigatus.*

Reverse-phase chromatography allowed the separation of intact NPY, GLP-1-(7-36) amide, and [des-Arg^1] BK from their corresponding N-terminally deleted dipeptide fragments (Table 2). The identities of the fragments obtained by cleavage of NPY, GLP-1-(7-36) amide, and [des-Arg^1] BK by *A. fumigatus* DPP IV were confirmed by electrospray mass spectrometry. The average *m/z* products of 3-36 NPY, [des-Arg^1-$Pro^{2,3}$] BK, and GLP-1-(9-36) amide were 3,993, 710, and 3,109, respectively, in close agreement with the calculated $[M+H]^+$ values of 3,991, 710, and 3,071, respectively. The difference observed between the $[M+H]^+$ calculated and experimentally found for GLP-1-(9-36) amide was due to the formation of a K^+ salt. The kinetic data clearly demonstrate that *A. fumigatus* DPP IV cleaved biological peptides more efficiently than chromogenic dipeptidic substrates. K_m values for NPY, [des-Arg^1] BK, and GLP-1-(7-36) amide were 24, 128, and 80 μM, and V_{max} values were 74, 786, 653 pmol/min, respectively.

The *A. fumigatus* DPP IV bound to the collagen agarose column and was recovered mainly in the 200 mM NaCl eluted fraction, whereas no activity was detected in the void volume. This result provided an easy way to separate the two DPPs (V

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mouseDPPIV   518 ......FWYQMILPPHFDK..SKKYPLLLDVYAGPCSQKADASFR.LNWATYLASTENI.
ratDPPIV     525 ......FWYQMILPPHFDK..SKKYPLLIDVYAGPCSQKADAAFR.LNWATYLASTENI.
humanDPPIV   524 ......FWYQMILPPHFDK..SKKYPLLLDVYAGPCSQKADTVFR.LNWATYLASTENI.
yeastDPAPB   565 FGKDILVNSYEILPNDFDETLSDHYPVFFFAYGGPNSQQVVKTF.SVGFNEVVAS..QLN
AfDPPIV      503 .....TLNVMQRLPVKFSP..KKKYPVLFTPYGGPGAQEVSKPWQALDFKAYIASDPELE
AfDPPV       454 ......IHAWVTYPENFDK..SKKYPLIFFIHGGPQGNWADGWSTRWNPKAW....ADQG

mouseDPPIV   568 .IVASFDGRGSGYQGDKIMHAINRRLGTLEVEDQIEA.ARQFVKMGFVDSKRVAIWGWSY
ratDPPIV     575 .IVASFDGRGSGYQGDKIMHAINKRLGTLEVEDQIEA.ARQFLKMGFVDSKRVAIWGWSY
humanDPPIV   574 .IVASFDGRGSGYQGDKIMHAINRRLGTFEVEDQIEA.ARQFSKMGFVDNKRIAIWGWSY
yeastDPAPB   622 AIVVVVDGRGTGFKGQDFRSLVRDRLGDYEARDQISA.ASLYGSLTFVDPQKISLFGWSY
AfDPPIV      556 YITWTVDNRGTGYKGRAFRCQVASRLGELEAADQVFA.AQQAAKLPYVDAQHIAIWGWSY
AfDPPV       502 YVVVAPNPTGSTGFGQALTDAIQNNWGGAPYDDLVKCWEYVHENLDYVDTDHGVAAGASY

mouseDPPIV   626 GGYVTSMVLGSGSG.VFKCG.......IAVAPVS.......RWEYYDSVYTERYMGLPIP
ratDPPIV     633 GGYVTSMVLGSGSG.VFKCG.......IAVAPVS.......RWEYYDSVYTERYMGLPTP
humanDPPIV   632 GGYVTSMVLGSGSG.VFKCG.......IAVAPVS.......RWEYYDSVYTERYMGLPTP
yeastDPAPB   681 GGYLTLKTLEKDGGRHFKYG.......MSVAPVT.......DWRFYDSVYTERYM..HTP
AfDPPIV      615 GGYLTGKVIETDSG.AFSLG.......VQTAPVS.......DWRFYDSMYTERYM..KTL
AfDPPV       562 GGFMINWIQGSPLGRKFKALVSHDGTFVADAKVSTEELWFMQREFNGTFWDAR.......

mouseDPPIV   671 EDNLDHYRNSTVMSRAEHFKQV..EYLLIHGTADDNVHFQQSAQISKVLVDAGV...DFQ
ratDPPIV     678 EDNLDHYRNSTVMSRAENFKQV..EYLLIHGTADDNVHFQQSAQISKALVDAGV...DFQ
humanDPPIV   677 EDNLDHYRNSTVMSRAENFKQV..EYLLIHGTADDNVHFQQSAQISKALVDVGV...DFQ
yeastDPAPB   725 QENFDGYVESSVHNVTA.LAQA.NRFLLMHGTGDDNVHFQNSLKFLDLLDLNGV..ENYD
AfDPPIV      658 ESNAAGYNASAIRKVAG.YKNVRGGVLIQHGTGDDNVHFQNAAALVDTLVGAGVTPEKLQ
AfDPPV       615 .DNYRRWDPSA....PERILQFATPMLVIHSDKDYRLPVAEGLSLFNVLQERGVPSRFLN

mouseDPPIV   726 AMWYTDEDHGIASSTAHQHIYSHMSHFLQQCFSLH.....................
ratDPPIV     733 AMWYTDEDHGIASSTAHQHIYSHMSHFLQQCFSLR.....................
humanDPPIV   732 AMWYTDEDHGIASSTAHQHIYTHMSHFIKQCFSLP.....................
yeastDPAPB   781 VHVFPDSDHSIRYHNANVIVFDKLLDWAKRAFDGQFVK..................
AfDPPIV      717 VQWFTDSDHGIRYHGGNVFLYRQL...SKRLYEEKKRKEKGEAHQWSKKSVL...
AfDPPV       670 ...FPDENHWVVNPENSLVWHQQALGWINKYSGVEKSNPNAVSLEDTVVPVVNYN
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FIG. 5. Alignment of the predicted DPP IV protein (amino acids 503 to 765) of *A. fumigatus* with the corresponding segment of rat and mouse DPP IV, human CD26, yeast DPAP B, and *A. fumigatus* DPP V. Sequences were from the Swiss-Prot data bank (accession no. p27487 [human CD26], p28843 [mouse DPP IV], p14740 [rat DPP IV], and p18962 [yeast DPAP B]) and from the EMBL/GenBank data library for *A. fumigatus* DPP V (accession no. L48074).

and IV) in *A. fumigatus*. All of these results showed that the 95-kDa DPP of *A. fumigatus* exhibited all of the main biochemical characteristics of the DPP IV family and was very different from the DPP V of *A. fumigatus* previously characterized (3).

DISCUSSION

DPPs have not heretofore been considered putative virulence factors of human fungal pathogens. *A. fumigatus* secretes two types of antigenic DPP, IV and V, that may play different roles during infection. First, DPP V is one of the two major antigens of *A. fumigatus* and has long been known as the chymoptrypsic antigen of *A. fumigatus* (3). Second, mice surviving infection with *A. fumigatus* have antibodies that recognize only the DPP V specifically in a total *A. fumigatus* extract (20a), which suggests that this molecule is primarily involved in the protective immunity against *Aspergillus* infection. DPP IV, in contrast to DPP V, is able to bind to and release a dipeptide from collagen and may thus help the fungus in the first step, colonization of the proteinaceous matrix of the lung, which is composed mainly of collagen and elastin. The *A. fumigatus* DPP IV characterized in this study shows the same properties as the other known DPP IV: removal of Xaa-Pro and to a lesser extent Xaa-Ala dipeptides from the N termini of peptides, including bioactive peptides such as NPY, [des-Arg^1] BK, GLP-1-(7-36) amide, and the human growth releasing factor (data not shown), activity at neutral pH, and presence in

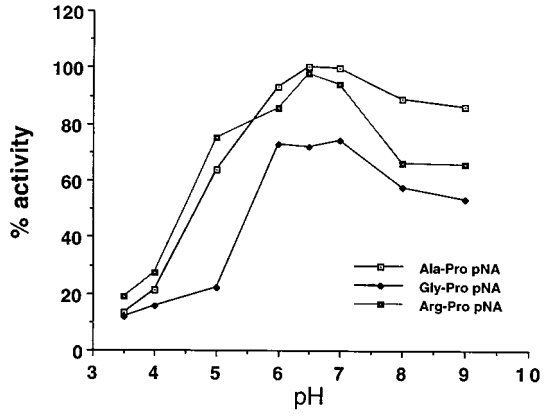

FIG. 6. Influence of pH on the activity of *A. fumigatus* DPP IV. Reactions were performed at 37°C, using Gly-Pro pNA Arg-Pro pNA and Ala-Pro pNA, for 30 min; 100% activity is that at pH 6.5 for Ala-Pro pNA.

TABLE 2. HPLC separation on cyanopropyl or phenyl columns of NPY, GLP-1-(7-36) amide, and [des-Arg^1] BK from their corresponding N-terminally deleted fragments

Peptide or fragment	Retention time (min)
NPY	24.1
3-36 NPY	21.1
[Des-Arg^1] BK	11.6
[Des-Arg^1-Pro^{2,3}] BK	9.0
GLP-1-(7-36) amide	13.7
GLP-1-(9-36) amide	17.4

the amino acid sequence of the Gly-X-Ser-X-Gly consensus motif of the serine-hydrolases and the putative catalytic triad (Ser^{613}, Asp^{690}, and His^{725}) of the DPPs. After removal of the Tyr-Pro sequence from the N terminus of NPY, a peptide involved in cardiovascular regulation, NPY is no longer able to bind to the Y1 receptor (23). GLP-1-(7-36) amide, one of the incretin hormones which potentiates the action of glucose on insulin secretion in the pancreas, is inactivated by DPP IV through removal of the His-Ala dipeptide (25). Similarly, after inactivation of the vasodilatory molecule BK by an aminopeptidase P which liberates the N terminus residue, DPP IV promotes cleavage of the penultimate Pro-Pro sequence from the inactivated [des-Arg^1] BK (28). The K_m values of NPY and of GLP-1-(7-36) amide were higher for *A. fumigatus* DPP IV than those found for mammalian DPP IV; nevertheless, the cleavage specificity is conserved (23, 25, 28). Hydrolysis of NPY, GLP-1-(7-36) amide, and human growth releasing factor by human DPP IV yields biologically inactive metabolites (12, 23, 25). Therefore, the inactivation of these hormone peptides by the DPP IV secreted by *A. fumigatus* may affect the overall physiology of the host.

Complement receptor molecules have been found on the surface of both host cells and fungal pathogens and have been considered to play a significant role in fungal pathogenesis (5). *A. fumigatus* DPP IV and CD26 display significant sequence homologies and show the same enzymatic activity. This is yet another example of homology between receptor molecules found both in a fungus and in its human host. In humans and animals, CD26, either directly or by binding with components of the cell matrix such as fibronectin or collagen, activates T cells (8), in particular $CD4^+$ T cells (24). Moreover, another DPP IV-like enzyme present at the surface of macrophage cells can inactivate the tumor necrosis factor alpha produced after stimulation of the $CD4^+$ Th1 population (1). Activation of the Th2 and inactivation of the Th1 $CD4^+$ T-cell population has been recently shown to favor *A. fumigatus* infection (6). Similarly, binding of *A. fumigatus* DPP IV to collagen may also activate the $CD4^+$ T cells. The availability of recombinant DPP IV and DPP V from *A. fumigatus* will allow us to test the roles of these different DPPs on the cellular immune response during an *A. fumigatus* infection.

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