Identification and expression of an allergen Asp f 13 from *Aspergillus fumigatus* and epitope mapping using human IgE antibodies and rabbit polyclonal antibodies

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The *Aspergillus* genus of fungi is known to be one of the most prevalent aeroallergens. On two-dimensional immunoblotting using patients' sera containing IgE specific for Asp f 13, an allergen with a molecular mass of 33 kDa and a pI of 6.2 was identified. This allergen was also present in *A. fumigatus* culture filtrates. Furthermore, the sequence of the Asp f 13 cDNA was identical to that for alkaline protease isolated from *A. fumigatus* and showed 42–49 % identity of amino acids with two proteases from *P. cyclopium* and *T. album* and with the Pen c 1 allergen from *P. citrinum*. Asp f 13 coding sequences were expressed in *Escherichia coli* as a $[His]_6$-tagged fusion protein which was purified by Ni^{2+}-chelate affinity chromatography. Recombinant Asp f 13 was recognized by rabbit polyclonal antibodies against Asp f 13 and by IgE antibodies from subject allergic to *A. fumigatus*. To identify and characterize the linear epitopes of this allergen, a combination of chemical and enzymatic cleavage and immunoblotting techniques, with subsequent N-terminal sequencing and mass spectrometry, were performed. At least 13 different linear epitopes reacting with the rabbit anti-Asp f 13 antiserum were identified, located throughout the entire molecule. In contrast, IgE from *A. fumigatus*-sensitive patients bound to three immunodominant epitopes at the C-terminal of the protein.

Key words: *Aspergillus fumigatus*, epitope analysis, mould allergens, two-dimensional immunoblotting.

INTRODUCTION

IgE-mediated allergy is a serious problem for patients with symptoms such as allergic rhinitis, conjunctivitis, dermatitis or asthma. It has long been recognized that inhalation of mould spores or mycelial fragments can produce allergic symptoms in susceptible individuals. The Genera Fungi Imperfecti are the major source of allergens, such as *Aspergillus*, *Penicillium*, *Alternaria* and *Cladosporium* species [1]. *Aspergillus* sp. is abundant in the home and is thought to be an important inducer of respiratory allergic diseases [2]. *A. fumigatus* is of major clinical importance and predominantly affects the respiratory system [3]. Inhalation of high concentrations of the spores of this fungus causes a variety of human diseases, including hypersensitivity pneumonitis, allergic rhinitis, IgE-mediated asthma, allergic bronchopulmonary aspergillosis (ABPA), as well as severe invasive infection in immunocompromised persons [4].

Crude preparations of the species *A. fumigatus* are frequently used in clinical allergy laboratories for skin testing and immunotherapy [5]. Over the past few years, a number of IgE binding components have been identified. These include Asp f 1, an 18 kDa secreted allergen characterized as a member of the ribotoxin family [6], Asp f 2, a 34 kDa protein of unknown biological function with a pI range of 5.5–6.6 [7], and Asp f 3, which shows significant sequence similarity with the peroxisomal membrane protein of *Candida boidinii* [8]. These three proteins can be regarded as major allergens of *A. fumigatus*, with a prevalence of $> 80\,\%$ [6–8]. Two other allergens, Asp f 5 (a metalloprotease) and Asp 10 (an aspartic protease), belong to the family of secreted proteases characterized as allergens using serum IgE from *A. fumigatus*-sensitized individuals [9]. An intracellular Mn superoxide dismutase (Asp f 6) induces proliferation of peripheral blood mononuclear cells and elicits skin reactions in ABPA patients [10]. In addition to peptidyl-prolyl isomerase (Asp f 11) and heat shock protein (Asp f 12) [11], three other allergens (Asp f 4, Asp f 7 and Asp f 9) have been identified, but their biological functions are unknown [9]. The catalase subunit [12] and gp 55 [13] allergens have been purified to homogeneity from water-soluble extracts of *A. fumigatus*.

Antigen-specific IgE plays a critical role in allergic disease [14]. The identification and characterization of the epitopes of fungal allergens that bind IgE is essential in order to understand the immunopathogenic mechanisms involved in hypersensitivity reactions. Many allergenic proteins have already been cloned and expressed, but the characterization of their allergenic determinants has been slow and little is known about IgE-binding epitopes on allergens from *A. fumigatus*. The C-terminal end of the Asp f 1 allergen has been shown to bind IgE [15] and nine IgE-binding regions have been identified in the hydrophilic region of Asp f 2 [16].

In a preliminary Western blot study of the binding of IgE from the serum of *Aspergillus*-sensitized patients, we found allergenic reactivity in the mycelial extract and culture filtrate from *A. fumigatus*. On the basis of these observations, we set out to identify candidate *A. fumigatus* allergens. In order to identify the IgE-reactive components from *A. fumigatus*, we performed two-dimensional PAGE of the mycelial protein, followed by immunological characterization. A novel allergen was identified and cloned. This allergen was formally named Asp f 13, according to the WHO/IUIS Allergen Nomenclature Subcommittee recom-

Abbreviations used: ABPA, allergic brochopulmonary aspergillosis; 2-DE, two-dimensional electrophoresis; IPTG, isopropyl-1-thio-β-D-galactopyranoside; pAb, polyclonal antibodies; RACE, rapid amplification of cDNA ends; rAsp f 13, recombinant Asp f 13; HRP, horseradish peroxidase; Lys-C, lysyl endopeptidase C.

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mendation. Recombinant Asp f 13 (rAsp f 13) was expressed in *Escherichia coli*, purified to homogeneity, and its immunogenic properties analysed using anti-Asp f 13 polyclonal antibodies (pAb) and sera from patients with allergic disorders. Furthermore, we extended our study by mapping the antigenic epitopes of the pure antigen. This information is required in order to understand the mechanism, and improve the diagnosis and therapy, of *A. fumigatus*-induced allergic disorders.

MATERIALS AND METHODS

Materials

Pharmalyte 3-10 and oligo(dT)-cellulose were purchased from Pharmacia Biotech (Uppsala, Sweden). PVDF membranes were purchased from Millipore (Bedford, MA, U.S.A.). Restriction enzymes and T4 DNA ligase were obtained from New England Biolabs. The marathon cDNA kit was purchased from Clontech (Palo Alto, CA, U.S.A.). Glu-C endopeptidase was obtained from Sigma, and lysyl-C endopeptidase from Wako (Osaka, Japan). *Taq* DNA polymerase and the ampliTag FS prism ready reaction cycle sequencing kit were from Applied Biosystems (Foster City, CA, U.S.A.). Alkaline phosphatase-conjugated anti-human IgE was obtained from Pharmingen (San Diego, CA, U.S.A.) and peroxidase coupled anti-rabbit IgG from Jackson (West Grove, PA, U.S.A.). All other chemicals were of analytical grade.

A. fumigatus extracts and patient sera

Sera from patients allergic to *Aspergillus* species, collected in the Cathy General Hospital, Taipei, were stored in aliquots at −70 °C. The allergic phenotype was confirmed by the clinical history, diagnosis and a high level of IgE reactivity. Control sera were collected from healthy adults.

Preparation of a crude extract of *A. fumigatus* and two-dimensional electrophoresis (2-DE)

A. fumigatus strain was isolated from the Tainan area in Taiwan and verified by the Food Industry Research and Development Institute, Hsinchu, Taiwan. Fugnal matts of *A. fumigatus*, containing both spores and mycelia, were collected from Czapek–Dox broth medium (Difco). About 1 g of lyophilized matts was ground in liquid nitrogen and the mixture homogenized in cold phosphate extraction buffer (50 mM sodium phosphate buffer, pH 7.0, containing 0.2 mM dithiothreitol and 2 mM PMSF) at 4 °C for 1 h with constant stirring. After centrifugation at 12000 ***g*** for 10 min at 4 °C, the supernatant was brought to a final concentration of 5% trichloracetic acid and the resultant precipitate washed with cold diethyl ether, air dried, and stored at −70 °C. Dried powder (2 mg) was suspended in 100 μl of sample solution (2% pharmalyte, 9.0 M urea, 0.5% Triton X-100, 60 mM dithiothreitol, and 0.003% bromphenol blue) and 2-DE performed as previously described [17]. The first dimension consisted of isoelectric focussing (IEF) with an immobilized pH gradient of 3–10 in a Multiphor II horizontal electrophoresis system (Pharmacia, Uppsala, Sweden), while the second dimension was SDS/PAGE.

Culture filtrate preparation

Fungal culture of *A. fumigatus* was performed as previously described [18]. After 3 days of cultivation, the culture broth was filtered through cheesecloth and the resulting clear filtrate precipitated overnight at 4 °C using ammonium sulphate at a final concentration of 85% saturation. The precipitate was collected by centrifugation and dialysed against water. The protein concentration was determined using a BCA protein assay reagent kit (Pierce), with bovine serum albumin (BSA) as the standard. The antigens in the filtrate were used to evaluate patients' sera for *Aspergillus*-specific IgE.

SDS/PAGE and IgE-specific immunodetection

Briefly, the above crude allergenic extracts were separated by SDS/PAGE essentially as previously described [19] using a 12.5% polyacrylamide separation gel. For immunodetection of IgE binding proteins, the gel was electroblotted onto a PVDF membrane, which was then blocked with skimmed milk, then incubated with the diluted pooled patients' sera at 4 °C for 16 h. Bound IgE antibodies were detected using alkaline phosphatase-labelled goat anti-human IgE antibodies (Pharmingen) as previously described [18].

N-terminal microsequencing

The blotted proteins were visualized by Coomassie Blue staining. The protein spots containing the presumed allergen were cut out and microsequenced by Edman degradation using a Procise 494 sequencer with on-line PTH amino acid analyser (Applied Biosystems, Foster City, CA, U.S.A.). The partial sequences were compared for homologies to known proteins using the Basic Local Alignment Search Tool (BLAST) algorithm.

Preparation of mRNA from *A. fumigatus*, PCR amplification and cloning

Poly(A)$^+$-enriched mRNA was isolated from *A. fumigatus* using the Quick Prep MicroRNA Purification Kit (Pharmacia). The rapid amplification of cDNA ends (RACE) method was employed to produce cDNA fragments coding for Asp f 13 using a Marathon cDNA amplification kit (Clontech), as described previously [20]. After N-terminal sequencing, two oligonucleotides were synthesized based on the published sequence of alkaline protease cDNA [21]. The sense primer used was 5′-GCCTTGACCACTCAAAAGGGC-3′, coding for the seven N-terminal amino acids, and the antisense primer was 5′-TGAGGGGTGGCCATGGAAGTA-3′ for the active site region. To obtain the 5′ and 3′ portion of the Asp f 13 cDNA, the RACE PCR protocol was used as described previously [17]. The coding sequence of the Asp f 13 gene was thus amplified by PCR and the amplified product analysed by electrophoresis and subcloned into the pGEM-T vector (Promega). The sequence of the amplified product was confirmed using an AmpliTaq Cycle Sequencing Kit (Applied Biosystems).

Expression and purification of rAsp f 13

The recombinant allergen was expressed in *E. coli* as previously described [18]. The full-length sequence encoding mature Asp f 13 was obtained from *A. fumigatus* cDNA by PCR amplification. PCR primers were designed to include a *Bam*HI site at the 5′ and 3′ ends of the cDNA. The primers used were 5′-GCGGATCCGATGACGATGACAAAGCCTTGACCACTCAAAAG-3′ (sense primer) and 5′-GCGGATCCTTAAGCATTGCCATGTAGGCAAG-3′ (antisense primer). In addition, the 5′ primer provided the recombinant protein with a new enterokinase cleavage site. The purified PCR products were ligated into a *Bam*HI restriction site of the pQE-30 expression vector containing a sequence coding for a 6 × His-affinity tag (QIAGEN). The ligation mixture was then subcloned into *E. coli* M15 strain cells. After induction with isopropyl-1-thio-β-D-thiogalactopyranoside

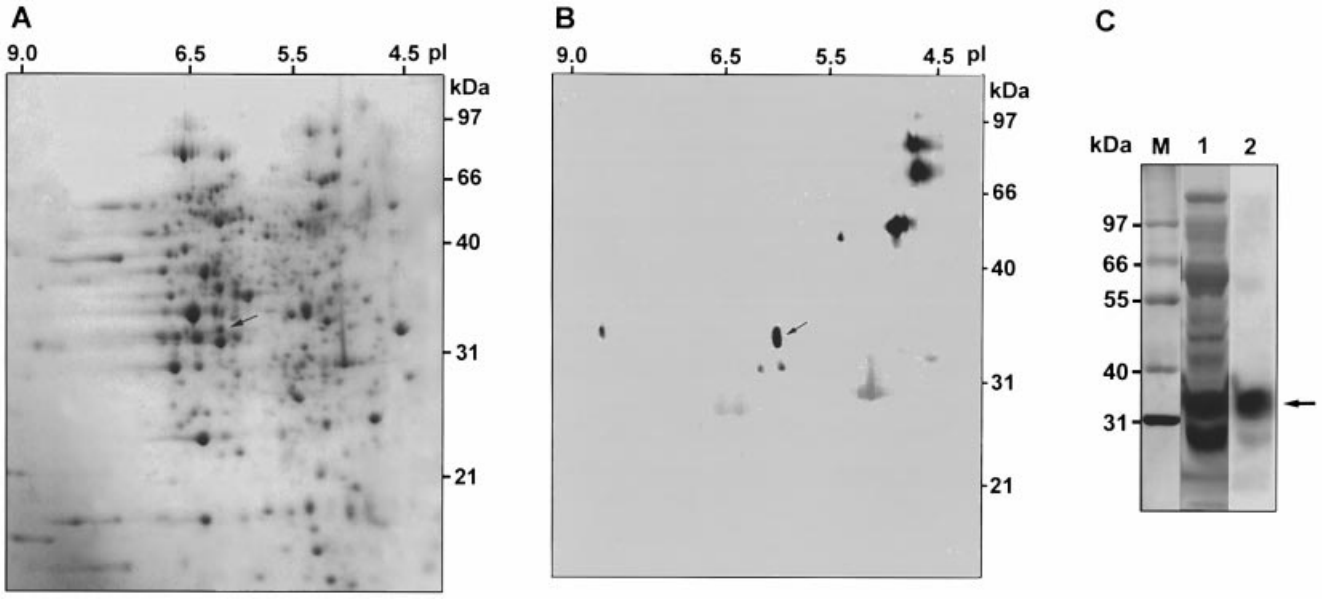

Figure 1 SDS/PAGE and immunoblot analysis of *A. fumigatus* allergens

(**A**) Two-dimensional electrophoresis blot of crude *A. fumigatus* mycelial extract stained with Coomassie Brilliant Blue. (**B**) Electrophoresis and transfer as in A, but tested for binding of serum IgE from *Aspergillus*-sensitized patients. (**C**) SDS gel immunoblotting using patients' sera and an *A. fumigatus* culture filtrate extract: M, molecular mass markers; lane 1, culture filtrate stained with Coomassie Brilliant Blue; lane 2, culture filtrate probed with pooled positive sera. The arrow indicates reactivity of IgE antibodies with a component corresponding to Asp f 13 allergen.

(IPTG), the cells were cultured at 37 °C for 2 h, then harvested by centrifugation and the recombinant protein extracted by sonication. The recombinant His_6-fusion proteins, deposited in inclusion bodies, were purified under denaturing conditions using Ni^{2+}-chelate affinity chromatography as described previously [18]. Immunological detection was performed using pooled sera from five *A. fumigatus*-sensitized patients.

pAb preparation and IgG-specific immunodetection

New Zealand white rabbits were injected subcutaneously with 500 μg of purified Asp f 13 in 1.0 ml of PBS emulsified with an equal volume of Freund's complete adjuvant. After 4 weeks, a booster dose of 500 μg of Asp f 13 emulsified in Freund's incomplete adjuvant was given by intradermal injection. This was followed by one more injection of 500 μg of antigen in the next month. The production of specific antibodies was monitored by enzyme-linked immunosorbent assay.

Western blot analysis using anti-Asp f 13 antiserum was carried out as described above. Bound antibodies were detected using horseradish peroxidase (HRP)-labelled goat anti-rabbit IgG (Jackson) as secondary antibody and development performed using a substrate solution of acetate buffer containing 3-amino-9-ethyl-carbazole and hydrogen peroxide.

Enzymatic and chemical cleavage

Purified rAsp f 13 was digested overnight at 30 °C with enterokinase to remove the N-terminal His-tag. After proteolysis, the digest was dialysed to remove small peptides and lyophilized. For proteolytic epitope mapping, approx. 100 μg of purified non-His-tagged rAsp f 13 was dissolved in 50 μl of 0.1 M pyridine/acetate/collidine, pH 8.2, or 0.1 M pyridine/acetate/collidine buffer, pH 6.0, before overnight digestion at 37 °C with 1 μg of lysyl endopeptidase C (Lys-C) (pH 8.2 sample) or Glu-C endopeptidase (pH 6.0 sample). The resultant peptides were separated by chromatography on a Beckman ODS column (Beckman, Fullerton, CA, U.S.A.) using a linear gradient of acetonitrile (5–60 % in 60 min) in 0.06 % trifluoroacetic acid at a flow rate of 1 ml/min, peptide elution being monitored at 220 nm. Cyanogen bromide cleavage was performed at 37 °C for 24 h in 70 % formic acid with a 100-fold molar excess of CNBr over methionine residues. After cleavage, the fragments were separated by HPLC on a Vydac C4 column (Separations Group, Hesperia, CA, U.S.A.) using the same elution system as for the Beckman column. All fractions were collected and analysed by dot–blot to identify the antigenic components. Each antigenic peptide was subjected to N-terminal sequencing (ABI 494 Procise Sequencer) and mass spectrometric analysis (Finnigan LCQ™ ESI-MS mass spectrometer, Finnigan, San Jose, CA, U.S.A.).

Dot–blot analysis

Approximately 30 pmol of each of the CNBr, Lys-C, or Glu-C peptides from rAsp f 13 was spotted onto a PVDF membrane. After treatment with anti-Asp f 13 pAb (1:10000), antigen–antibody complexes were detected using HRP-linked anti-rabbit IgG antibody (Jackson). Bound IgG was visualized using the enhanced chemiluminescence system (ECL Western blotting analysis system, Amersham) according to the manufacturer's instructions. Positive antigenic peptides were probed using a serum pool from five *Aspergillus*-sensitive patients and serum from one normal subject, bound IgE antibodies being detected using alkaline phosphatase-conjugated goat anti-human IgE antibodies (Pharmingen) and the blots being developed as described above.

Molecular modelling of rAsp f 13

A 3-D model of Asp f 13 was built using the X-ray crystallographic structures of *P. cyclopium* protease (4TST) [22] and proteinase K (2PRK) [23] as templates. Modelling was performed

on a Silicon Graphics workstation using the program Insight II (Biosym Technologies, San Diego, CA, U.S.A.). The detailed data processing was described previously [24].

RESULTS

Allergen identification and microsequencing

In order to characterize antigens recognized by allergen-sensitive sera, two-dimensional immunoblotting was carried out on the crude extract of *A. fumigatus*. Figure 1A shows the 2-DE maps of the mycelial proteins. About 250 distinct protein spots were detected by Coomassie Blue staining. To identify spots corresponding to the allergens, IgE-binding spots were visualized by immunoblotting using pooled sera from *Aspergillus*-sensitized patients. After immunoblotting, 12 different spots with molecular masses of 27–90 kDa and pI values ranging from about 4.7 to > 9.0 were demonstrated (Figure 1B). Intense reactivity was seen with four spots, two with molecular masses of 90 kDa and 70 kDa and a pI of 4.7, one with a molecular mass of 55 kDa and a pI of 4.8, and one with a molecular mass of 33 kDa and a pI of 6.2. To identify allergens in the culture filtrate, culture filtrate concentrate was processed in the same manner. One IgE-binding protein with a molecular mass of 33 kDa was present in both the mycelial extract and the culture filtrate. The N-terminal sequence of both the mycelial 33 kDa allergen with a pI of 6.2 and the major allergen in the culture filtrate (indicated by an arrow in Figure 1A–C) was ALTTQKSAPWG. When compared with the GenBank database sequence, this sequence was found to be identical to that of alkaline protease of *A. fumigatus* [21]. No spots were labelled when the negative serum was used (data not shown).

Molecular cloning, sequence analysis of the cDNA, and comparison of the Asp f 13 protein sequence with related proteases

Reverse-transcription PCR of *A. fumigatus* cDNA generated the expected internal fragment, which was cloned into pGEM-T and sequenced. RACE was then performed to obtain the sequence of these PCR-forced regions plus the 5′ and 3′ coding regions. The

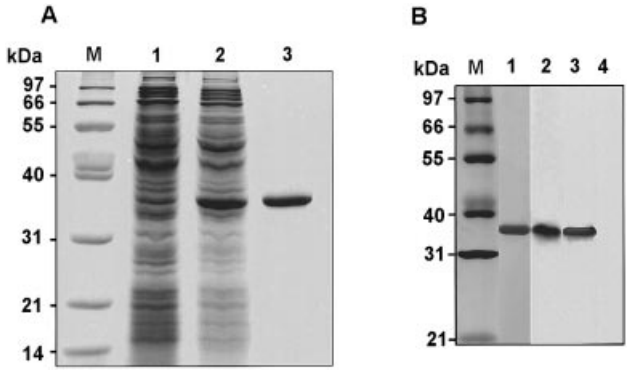

Figure 3 Analysis of purified polyhistidine-tailed rAsp f 13

(**A**) Purification of rAsp f 13 monitored by Coomassie Brilliant Blue-stained SDS/PAGE. Lane M, molecular mass markers; lane 1, non-induced cell lysate; lane 2, IPTG-induced cell lysate; lane 3, affinity chromatography-purified rAsp f 13. (**B**) Immunological characterization of rAsp f 13. Lane 1, Coomassie Blue-stained rAsp f 13; lane 2, reacted with pooled sera from *Aspergillus*-sensitive patients; lane 3, reacted with polyclonal rabbit anti-Asp f 13 antiserum; lane 4, reacted with rabbit preimmune serum. Molecular weight markers (M) are indicated.

determined full-length sequence of Asp f 13 cDNA is identical to the published sequence for alkaline protease [21]. The deduced amino acid sequence is shown in Figure 2. Sequence similarity searches using the BLAST program revealed that Asp f 13 shows the highest similarity with known related proteases and one allergen (Figure 2). The residues of the catalytic triad of Asp f 13 are immediately recognized by sequence comparison as Ser 228, His 72 and Asp 41. Other residues are also highly conserved among these proteases; however, Asp f 13 does not contain the cysteine residues found in proteinase K, *P. cyclopium* protease, and Pen c 1. Asp f 13 shows 49.3% and 44.3% identity with *P. cyclopium* protease [22] and *T. album* proteinase K [23], respectively. A high degree of identity (42.2%) was also found with Pen c 1, which has been described as a major allergen of *P. citrinum* [18], implying that Asp f 13 may show allergenic cross-reactivity with Pen c 1.

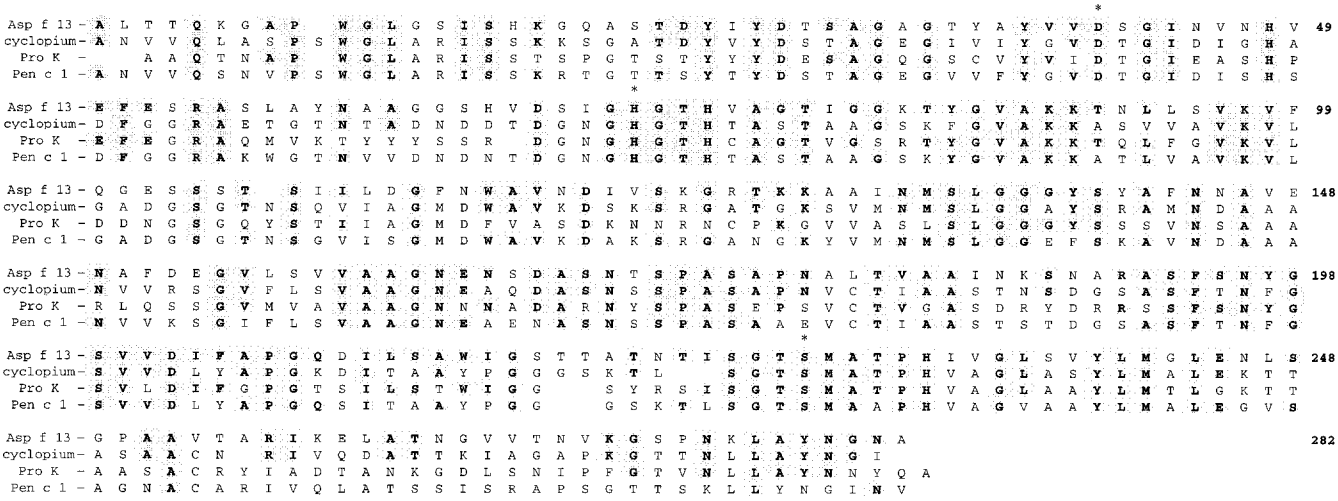

Figure 2 Sequence comparison of Asp f 13 and *P. cyclopium* protease, proteinase K from *T. album* Limber, and Pen c 1 from *P. citrinum*

The numbering system is based on the sequence of Asp f 13 as reference. The gaps are introduced for optimal alignment of, and maximum homology between, all compared sequences. Identical amino acids are shown in shaded boxes. The highly conserved amino acid residues involved in the active site are indicated by asterisks.

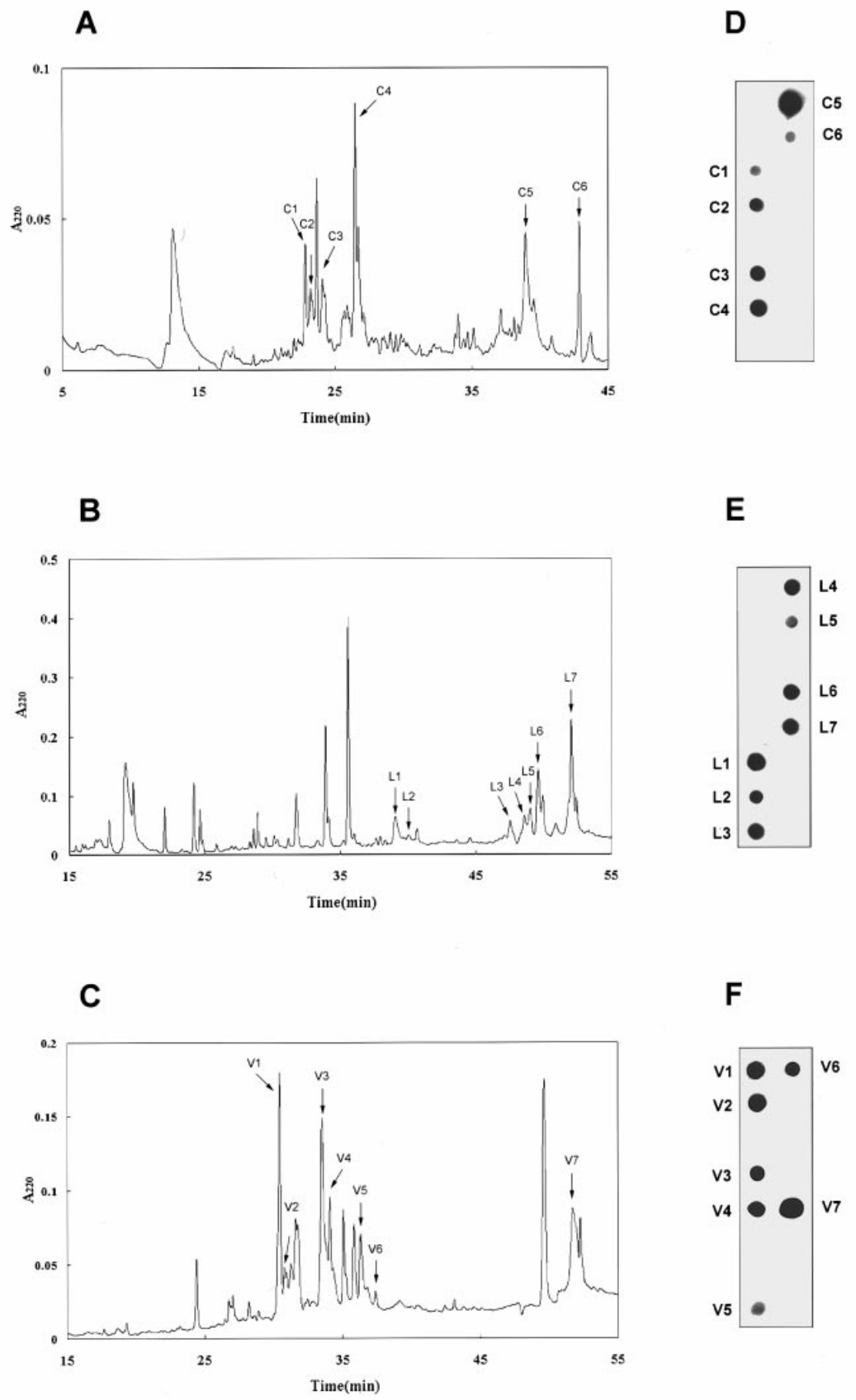

Figure 4 HPLC profile of peptide digests of non-His-tagged rAsp f 13 and screening with anti-Asp f 13 pAb

(**A**) CNBr cleavage, (**B**) Lysyl-C endopeptidase digest, (**C**) Glu-C endopeptidase digest. The immunoreactivity of each fraction was tested by dot–blotting; the fractions containing antigenic epitopes are indicated with arrows. (**D**)–(**F**) The peptides were dotted onto PVDF membrane and probed with anti-Asp f 13 pAb. The details are described in the Materials and methods section.

Table 1 Epitopes derived from cleavage of non-His-tagged rAsp f 13

The N-terminal residue was determined by Edman degradation. Mass was determined by electrospray ionization mass spectrometry. n.d., not determined.

Peptide no.	pAb reactivity	IgE reactivity	N-terminal sequence	Mass (Da) $[M+H]^+$	Possible fragment
C1	+	−	ALTTQ–	n.d.	n.d.
C2	+	−	ALTTQ–	n.d.	n.d.
C3	+	−	ALTTQ–	n.d.	n.d.
C4	+	−	ALTTQ–	n.d.	n.d.
C5	+	+	GLENL–	4009.0	Gly^{243}-Ala^{282}
C6	+	−	ATPHI–	n.d.	n.d.
L1	+	−	GQAST–	6546.0	Gly^{19}-Lys^{83}
L2	+	−	GQAST–	7161.3	Gly^{19}-Lys^{89}
L3	+	−	GQAST–	n.d.	n.d.
L4	+	−	GQAST–	n.d.	n.d.
L5	+	−	VFQGE–	2814.6	Val^{98}-Lys^{123}
L6	+	−	TYGVA–	4318.0	Thr^{84}-Lys^{123}
L7	+	+	SNARA–	7257.0	Ser^{188}-Lys^{258}
V1	+	−	LATNG–	2303.9	Leu^{260}-Ala^{282}
V2	+	−	NLSGP–	1427.0	Asn^{246}-Glu^{259}
V3	+	−	ALTTQ–	2966.0	Ala^{1}-Asp^{28}
V4	+	−	GVLSV–	1015.5	Gly^{154}-Glu^{164}
V5	+	+	ASNTS–	3453.7	Ala^{168}-Asp^{202}
V6	+	+	NSDAS–	3770.0	Asn^{165}-Asp^{202}
V7	+	+	IFAPG–	5831.5	Ile^{203}-Glu^{259}

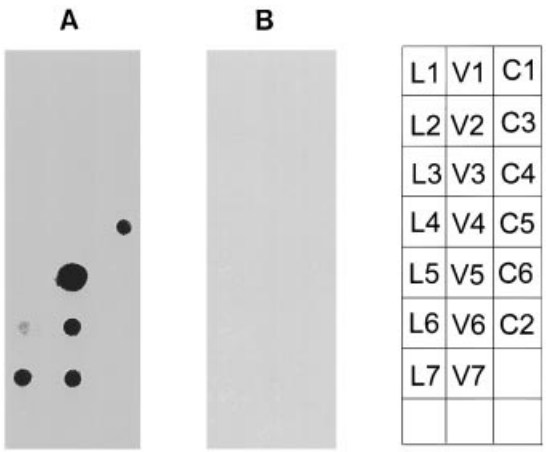

Figure 5 Mapping of IgE-binding epitopes by dot–blotting

All the antigenic peptides determined in Figure 4 were tested for reactivity with serum IgE using a pool of sera from five *Aspergillus*-sensitive patients (**A**) or serum from one normal control (**B**). The diagram on the right shows the pattern of peptides on the blots.

Expression and purification of rALP

The recombinant protein was produced in a bacterial system, after cloning of the corresponding Asp f 13 cDNA sequence into a PQE 30 plasmid, which allows expression of a recombinant protein with a polypeptide tail containing a polyhistidine tag at the N-terminal end. To obtain the recombinant protein without any additional amino acids for use in epitope mapping, an enterokinase proteolytic site (DDDDK↓) was inserted between the tail and the recombinant protein. Optimal production was obtained after induction by IPTG. On SDS/PAGE, rAsp f 13 was found to represent about 20% of the total bacterial protein (Figure 3A, lane 2). The fusion protein was extracted from inclusion bodies under denaturing conditions and purified using Ni^{2+}-chelate affinity chromatography by elution with increasing imidazole concentrations. On SDS/PAGE, the purified allergen appeared as a single band with a molecular mass of approx. 37 kDa (Figure 3A, lane 3), the slightly larger size being due to the fusion tail.

Immunogenicity of His-tagged rAsp f 13

When a pool of selected Asp f 13-reactive sera from individuals allergic to *A. fumigatus* was tested for reactivity with His-tagged rAsp f 13 by immunoblot analysis after SDS/PAGE (Figure 3B), it showed high IgE reactivity with the recombinant allergen, indicating the presence of allergenic epitopes in the expressed protein (Figure 3B, lane 2). Sera from nonallergic patients failed to bind to His-tagged rAsp f 13 (data not shown). His-tagged rAsp f 13 was also recognized by a rabbit anti-serum produced against Asp f 13 (Figure 3B, lane 3), whereas rabbit preimmune serum gave no signal (Figure 3B, lane 4). All the above antibodies recognized the denatured antigen, suggesting that the epitopes are of the continuous or sequential type.

Antigenic epitope mapping

In order to locate and characterize the peptide fractions of non-His-tagged rAsp f 13 representing the major epitopes, the peptides, produced by CNBr cleavage or proteolysis, were separated by reverse phase HPLC and tested by dot–blotting using rabbit anti-Asp f 13 pAb. The elution profiles (Figure 4A–C) and pAB binding results (Figure 4D–F) show that the pAb recognized six CNBr peptides (C1–C6) (Figure 4D), seven Lys-C peptides (L1–L7) (Figure 4E), and seven Glu-C peptides (V1–V7) (Figure 4F). The detailed sequencing data and mass spectrum analyses are summarized in Table 1.

Of the six antigenic CNBr peptides, the strongest binding was seen with C5, located near the C-terminus. Peptides C1 and C6 showed weak and C2, C3, and C4 moderate binding activity (Figure 4D). Of the seven antigenic Lys-C peptides, L5 showed moderate reactivity, while the other six showed strong reactivity. L1 and L2 were different lengths, but had the same N-terminal sequence located near the N-terminus of Asp f 13. L3 and L4 also contained this N-terminal sequence, but no mass data were obtained. Peptides L5 and L6 are located in the central region of the molecule, while peptide L7 is located near the C-terminus (Figure 4E). Of the seven antigenic V8 peptides, V3, near the N-terminus, showed weak reactivity, while the remaining six, all from the C-terminus of the protein, showed pronounced reactivity (Figure 4F). The results suggest that the antibodies recognize 13 linear epitopes covering almost the entire sequence of Asp f 13.

Identification of linear IgE-binding epitopes of Asp f 13

To assess the binding of specific IgE to these epitopes, all the peptides were probed by dot–blot using a pool of sera from five patients known to have *Aspergillus* hypersensitivity reactions and a serum sample from a normal control with no mould allergy (Figure 5A and 5B). The IgE-binding results identified several regions of strong reactivity. Clearly positive results were obtained with peptides V5, V6, V7, L7 and C5; the very weak reaction with peptide L6 was classed as negative. All 5 strongly reactive peptides were from the C-terminal region of Asp f 13. The control serum sample showed no IgE reactivity. The epitopes mapping results are shown in Figure 6.

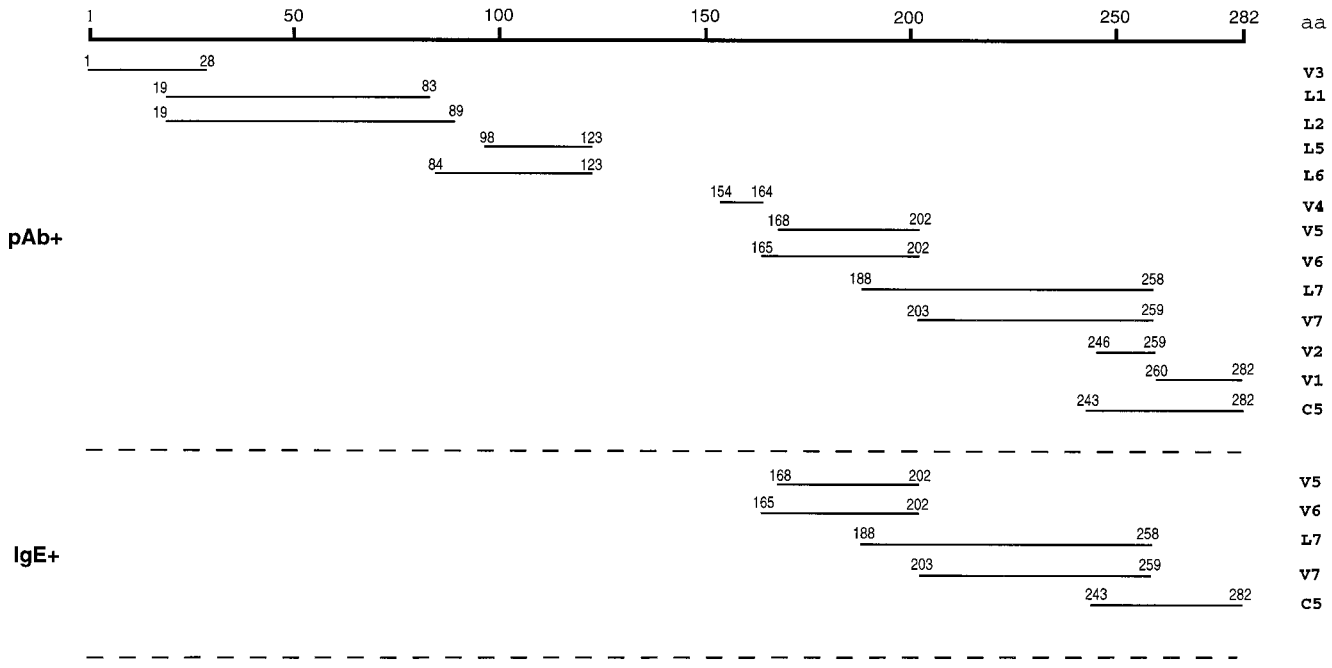

Figure 6 Schematic map of the location of epitope fragments on the complete Asp f 13 molecule

The numbers refer to the locations of the residues in Asp f 13. The corresponding peptides are shown on the right. pAb+, positive reaction with rabbit polyclonal antibodies; IgE+, positive reaction with human IgE antibodies.

Location of the IgE binding epitopes on the molecular model of Asp f 13

The Asp f 13 sequence was modelled using the coordinates of *P. cyclopium* protease and proteinase K because of the high sequence identity (49% and 44%). Figure 7A shows that the α-carbon backbones of Asp f 13 and proteinase K can be superimposed on the backbone of *P. cyclopium* protease. PROCHECK and Profile 3-D programs were run to check for correctness of side-chain conformations. As seen in Figure 7B and 7C, the major secondary structural elements of the proteins were virtually superimposable, consisting, in essence, of an internal core of parallel β-sheets and buried helices, surrounded by amphipathic helices and antiparallel β-sheets. If the pairs of peptides, V5/V6 and V7/L7, are considered to contain the same allergenic sites, the five IgE-binding epitopes would correspond to three allergenic sites, although there is a possibility that other allergenic sites exist. Figure 7D shows that the V5 epitope consists of β-turns and β-sheets, exposed to solvent and heavily hydrated. Figure 7E shows that the largest part of the second epitope, V7, consists of three β-sheets and two α-helices, with one antiparallel β-sheet, formed by residues 209–224, being exposed to solvent, and one α-helix, formed by residues 226–246, being partly buried in the bulk of the molecule. Figure 7F shows that the third epitope, C5, consists of one antiparallel sheet and part of the amphipathic helix.

DISCUSSION

Aspergillus has been described as one of the most important aeroallergens. Several apparently different antigens with IgG- and/or IgE-binding capacities have been extensively studied. In the present study, the allergenic profiles of *A. fumigatus* were analysed by 2-DE immunoblot using pooled sera from *Aspergillus*-sensitized patients. One of the major IgE-binding components, a 33 kDa protein with a pI of 6.2, was also found in the culture filtrate. This novel 33 kDa allergen protein, designated as Asp f 13 and showing 100% sequence identity to a previously reported alkaline serine protease [21], was cloned, expressed in *Escherichia coli*, and its immunological properties tested. Epitope mapping of the non-fusion recombinant protein, non-His-tagged rAsp f 13, was performed by dot–blotting of fractionated digests of the protin.

In previous studies, an extracellular alkaline protease from *A. fumigatus* with a molecular mass of 33 kDa and a pI of 8.2 has been demonstrated by several laboratories [22–27]. Furthermore, this enzyme was able to degrade collagen, elastin and fibrinogen, and to induce epithelial cells to detach from the basement membrane [26–29]. It was suggested that such an extracellular protease may be involved in the pathogenesis of aspergillosis [30]. Recently, it has been shown that recombinant alkaline protease, expressed in *E. coli* failed to exhibit any significant allergenicity or antigenicity when tested using sera from patients with ABPA [31]. These observations suggest that the secreted *A. fumigatus* alkaline protease is not an allergenic protein in patients with ABPA. Serological analyses and skin test studies have shown that *Aspergillus* allergens can be subdivided into two types, secreted allergens, recognized by *A. fumigatus*-sensitized patients, and cytoplasmic proteins, recognized exclusively by ABPA patients [9]. In our study, secreted Asp f 13 was shown to be allergenic in *Aspergillus*-sensitive patients. The results strongly suggest that there may be a difference between *Aspergillus*-sensitive patients and ABPA patients in the recognition of *A. fumigatus* allergens.

Some allergens from *A. fumigatus* appear to have enzymatic functions, including acid protease [9] and metalloprotease activity [9]. In addition, some dust mite allergens have proteolytic

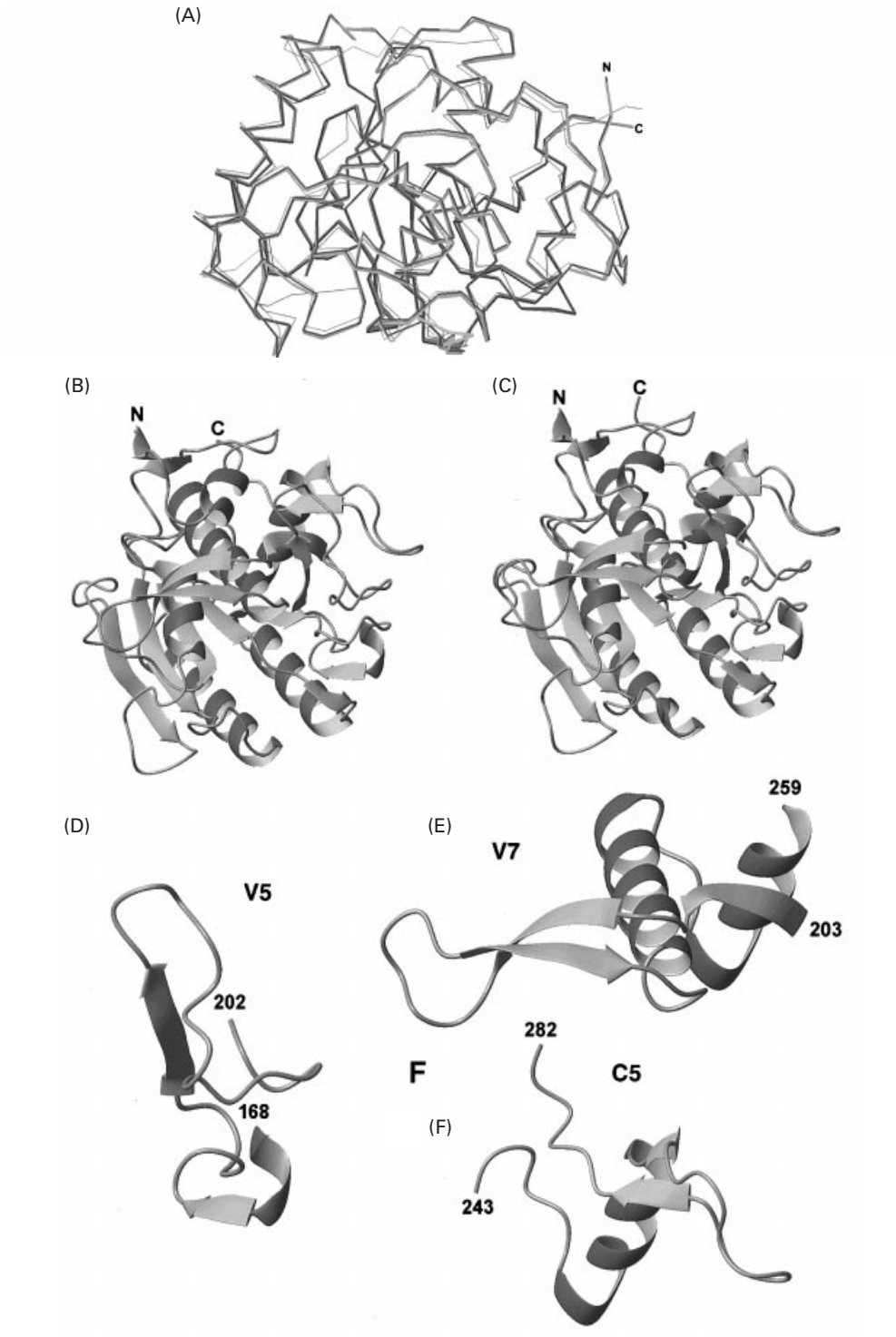

Figure 7 Molecular models and IgE-binding epitopes of Asp f 13

(**A**) Superposition of the three-dimensional models for Asp f 13 (thick line) and its closest structurally known homologues, *P. cyclopium* protease (medium line) and proteinase K (thin line). (**B**) Stereodiagram showing a ribbon representation of *P. cyclopium* protease. (**C**) Stereodiagram showing a ribbon representation of Asp f 13. (**D**) Stereodiagram showing epitope V5 of Asp f 13. (**E**) Stereodiagram showing epitope V7 of Asp f 13. (**F**) Stereodiagram showing epitope C5 of Asp f 13.

activity, e.g. Der p 1, which is a cysteine proteinase [32], and Der p 3, Der p 6, and Der p 9, which are serine proteases [33–35]. Moreover, we previously reported that the major allergens Pen c 1 and Pen c 2 in *P. citrinum* are also serine proteases [17,18]. The epithelium-detaching protease activity of allergens is currently under investigation. Der p 1, a cysteine protease allergen, has been shown to cause some epithelial cell detachment at high concentrations [36]. Dust mite serine protease allergens have

been shown to have a direct effect on epithelial integrity and permeability [36], to cause mast cell degranulation in a non-IgE-dependent manner [37], and to induce cytokine release from the respiratory epithelium [38]. Culture filtrates from *A. fumigatus* cause cell detachment of pulmonary epithelial cell lines and induce the production of proinflammatory cytokines [39]. Allergen-associated proteases may contribute to allergenicity by causing detachment of the epithelium, resulting in chronic damage to the epithelial surface layers, thus allowing penetration of antigens into the tissue and stimulating the release of pro-inflammatory cytokines from respiratory epithelium cells. Studies are under way to determine if this extracellular allergen, Asp f 13, plays such a role.

Recently, the B-cell epitopes of *Aspergillus* allergens have been studied [15,16], but information is still incomplete. In the present study, a number of peptides were found to be capable of binding to IgG and IgE antibodies, suggesting strong antigenicity and allergenicity of the molecule, as illustrated schematically in Figure 6. Human IgE antibodies recognized epitopes predominantly from the C-terminal of the Asp f 13 molecule, while rabbit IgG antibody-binding determinants were distributed throughout the protein. The immunodominant IgE-binding epitopes are found on the surface of the molecule and contain solvent-exposed residues. In epitope V5 (residues 168–202), the exposed regions contain structurally-conserved β-sheets and turns (Figure 7D). Epitope V7 (residues 203–259) contains one antiparallel β-sheet exposed to the solvent (Figure 7E). Epitope C5 (residues 243–282) consists of the exposed amphipathic helix, the hydrophilic residues of which point towards the solvent (Figure 7F). These regions are potential candidates for harbouring cross-reactive antibody-binding allergen epitopes. It is possible that they represent flexible or exposed regions which constitute the best recognition sites for an antibody molecule. A more detailed characterization of these surface regions will help in understanding the structural basis of the cross-reactivity of specific serum IgE from mould-allergic patients. Additional studies using a large number of individual allergic sera will be required to obtain more precise information about the distribution and range of IgE binding sites on the Asp f 13 molecule.

This report presents the detailed study of IgE-reactive epitopes. This information will allow further immunotherapy studies, since IgE-binding haptens, which do not cross-link the effector molecules, could be used to induce blocking IgG antibodies that interfere with the allergen-IgE interaction [40]. Such hapten therapy is a novel approach which has the potential to be applied to the specific therapy of allergic disease.

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REFERENCES

1 Burge, H. A. (1985) Clin. Rev. Allergy **3**, 319–329
2 Beaumont, F., Kauffman, H. F., Sluiter, H. J. and De Vries, K. (1985) Ann. Allergy **55**, 740–746
3 Kauffman, H. F., Tomee, J. F., van der Werf, T. S., de Monchy, J. G. and Koeter, G. K. (1995) Am. J. Respir. Crit. Care. Med. **151**, 2109–2116
4 Bardana, Jr., E. J. (1981) Crit. Rev. Clin. Lab. Sci. **13**, 21–83
5 Kurup, V. P. and Kumar, A. (1991) Clin. Microbiol. Rev. **4**, 439–456
6 Arruda, L. K., Platts-Mills, T. A., Fox, J. W. and Chapman, M. D. (1990) J. Exp. Med. **172**, 1529–1532
7 Banerjee, B., Kurup, V. P., Phadnis, S., Greenberger, P. A. and Fink, J. N. (1996) J. Lab. Clin. Med. **127**, 253–262
8 Hemmann, S., Blaser, K. and Crameri, R. (1997) Am. J. Respir. Crit. Care. Med. **156**, 1956–1962
9 Crameri, R. (1998) Int. Arch. Allergy Immunol. **115**, 99–114
10 Crameri, R., Faith, A., Hemmann, S., Jaussi, R., Ismail, C., Menz, G. and Blaser, K. (1996) J. Exp. Med. **184**, 265–270
11 Banerjee, B. and Kurup, V. P. (1998) Immunol. Allergy Clin. North. Am. **18**, 610–618
12 López-Medrano, R., Ovejero, M. C., Calera, J. A., Puente, P. and Leal, F. (1995) Infect. Immun. **63**, 4774–4780
13 Teshima, R., Ikebuchi, H., Sawada, J., Miyachi, S., Kitani, S., Iwama, M., Irie, M., Ichinoe, M. and Terao, T. (1996) J. Allergy Clin. Immunol. **92**, 698–706
14 Sutton, B. J. and Gould, H. J. (1993) Nature (London) **366**, 421–428
15 Kurup, V. P., Banerjee, B., Murali, P. S., Greenberger, P. A., Krishnan, M., Hari, V. and Fink, J. N. (1998) Peptides **19**, 1469–1477
16 Banerjee, B., Greenberger, P. A., Fink, J. N. and Kurup, V. P. (1999) Infect. Immun. **67**, 2284–2291
17 Chow, L. P., Su, N. Y., Yu, C. J., Chiang, B. L. and Shen, H. D. (1999) Biochem. J. **341**, 51–59
18 Su, N. Y., Yu, C. J., Shen, H. D., Pan, F. M. and Chow, L. P. (1999) Eur. J. Biochem. **261**, 115–123
19 Laemmli, U. K. (1970) Nature (London) **227**, 680–685
20 Frohman, M. A., Dush, M. K. and Martin, G. R. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 8998–9002
21 Jaton-Ogay, K., Suter, M., Crameri, R., Falchetto, R., Fatif, A. and Monod, M. (1992) FEMS Microbiol. Lett. **71**, 163–168
22 Koszelak, S., Ng, J. D., Day, J., Ko, T. P., Greenwood, A. and McPherson, A. (1997) Biochemistry **36**, 6597–6604
23 Betzel, C., Pal, G. P. and Saenger, W. (1988) Eur. J. Biochem. **178**, 155–171
24 Chow, L. P., Chou, M. H., Ho, C. Y., Chuang, C. C., Pan, F. M., Wu, S. H. and Lin, J. Y. (1999) Biochem. J. **338**, 211–219
25 Reichard, U., Buttner, S., Eiffert, H., Staib, F. and Ruchel, R. (1990) J. Med. Microbiol. **33**, 243–251
26 Monod, M., Togni, G., Rahalison, L. and Frenk, E. (1991) J. Med. Microbiol. **35**, 23–28
27 Frosco, M., Chase, T. and Macmillan, J. D. (1992) Infect. Immun. **60**, 728–734
28 Larcher, G., Bouchara, J. P., Annaix, V., Symones, F., Chabasse, D. and Tronchin, G. (1992) FEBS Lett. **308**, 65–69
29 Robinson, B. W., Venaille, T. J., Mendis, A. H. and McAleer, R. (1990) J. Allergy Clin. Immunol. **86**, 726–731
30 Kothary, M. H., Chase, Jr., T. and Macmillan, J. D. (1984) Infect. Immun. **43**, 320–325
31 Moser, M., Menz, G., Blaser, K. and Crameri, R. (1994) Infect. Immun. **62**, 936–946
32 Chua, K. Y., Stewart, G. A., Thomas, W. R., Simpson, R. J., Dilworth, R. J., Plozza, T. M. and Turner, K. J. (1988) J. Exp. Med. **167**, 175–182
33 Stewart, G. A., Ward, L. D., Simpson, R. J. and Thompson, P. J. (1992) Immunology **75**, 29–35
34 Yasueda, H., Mita, H., Akiyama, K., Shida, T., Ando, T., Sugiyama, S. and Yamakawa, H. (1993) Clin. Exp. Allergy **23**, 384–390
35 King, C. M., Simpson, R. J., Mortiz, R. L., Reed, G. E., Thompson, P. J. and Stewart, G. A. (1996) J. Allergy Clin. Immunol. **4**, 739–747
36 Herbert, C. A., King, C. M., Ring, P. C., Holgate, S. T., Stewart, G. A., Thompson, P. J. and Robinson, C. (1995) Am. J. Resp. Cell. Mol. Biol. **12**, 369–378
37 Stewart, G. A., Boyd, S. M., Bird, C. H., Krska, K. D., Kollinger, M. R. and Thompson, P. J. (1994) Am. J. Ind. Med. **25**, 105–107
38 King, C. M., Brennan, S., Thompson, P. J. and Stewart, G. A. (1998) J. Immunol. **161**, 3645–3651
39 Tomee, J. F., Wierenga, A. T., Hiemstra, P. S. and Kauffman, H. F. (1997) J. Infect. Dis. **176**, 300–303
40 Ball, T., Vrtala, S., Sperr, W. R., Valent, P., Susani, M., Kraft, D. and Valenta, R. (1994) J. Biol. Chem. **269**, 28323–28328

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