

## Immunological Characterization of Asp f 2, a Major Allergen from *Aspergillus fumigatus* Associated with Allergic Bronchopulmonary Aspergillosis

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**The 37-kDa recombinant protein Asp f 2, encoding an allergen of *Aspergillus fumigatus*, was expressed in a prokaryotic expression system and immunologically evaluated for its functional and structural properties. The open reading frame for a 310-amino-acid-long protein was shown to encode a signal peptide of 31 amino acids. A native 37-kDa culture filtrate protein and a 55-kDa mycelial glycoprotein (gp55) exhibited complete N-terminal sequence homology to Asp f 2. A GenBank search for homologous proteins revealed 60 and 44% sequence homologies to the cytosolic protein ASPND1 from *Aspergillus nidulans* and fibrinogen binding protein from *Candida albicans*, respectively. The glycosylation sites and cysteine molecules are conserved in all the three proteins. The extracellular matrix protein laminin showed a dose-dependent interaction with Asp f 2. This protein, expressed as a major cell-associated protein within 24 h of in vitro fungal culture, comprises 20 to 40% of total fungal protein. Furthermore, both native and recombinant Asp f 2 exhibited specific immunoglobulin (IgE) binding with allergic bronchopulmonary aspergillosis (ABPA) and cystic fibrosis-ABPA patients, whereas *A. fumigatus*-sensitized allergic asthma and normal control subjects failed to show IgE binding with Asp f 2. These results indicate that Asp f 2 is a major allergen of *A. fumigatus* exhibiting IgE antibody binding with sera from patients with ABPA. The antigen should be explored further for its potential role in the differential diagnosis of *A. fumigatus*-associated allergic diseases.**

One of the major *Aspergillus* species responsible worldwide for fungal respiratory disorders is the opportunistic pathogen *Aspergillus fumigatus*. It is associated with a wide spectrum of diseases in humans and animals such as hypersensitivity pneumonitis, allergic asthma (AA), allergic bronchopulmonary aspergillosis (ABPA), and aspergilloma as well as invasive aspergillosis in immunocompromised and immunodeficient patients (5, 6, 29, 51). ABPA is the most severe *A. fumigatus*-induced respiratory disease and is usually found in atopic individuals (19–21). Although previously considered a rare disease, ABPA is currently reported to occur in about 1% of *A. fumigatus*-sensitized asthmatics, while in patients with cystic fibrosis (CF) the incidence varies from 10 to 35% (7, 21, 32, 40). The major diagnostic criteria for ABPA are a history of asthma, immediate wheal and flare, immunoglobulin E (IgE) mast cell-mediated cutaneous reactivity to *A. fumigatus*, elevated total serum IgE (usually >1,000 ng/ml), elevated serum *A. fumigatus*-specific IgE and IgG, pulmonary infiltrates, and central bronchiectasis (CB) (19–21, 48). However, these diagnostic criteria are not always present at the same time even in patients with classic ABPA. In addition, CB and pulmonary infiltrates, the hallmarks of ABPA, also occur in CF patients without ABPA. Therefore, the serological detection of *A. fumigatus*-specific antibodies in patients is considered to be of diagnostic importance in the differential diagnosis of *Aspergillus*-induced asthma from ABPA and indistinguishable ABPA in CF patients.

The antigens and allergens of *A. fumigatus* comprise a het-

erogeneous mixture of proteins, carbohydrates, and glycoproteins (8, 22). Although several *A. fumigatus* antigenic components have been purified and characterized by various methods, there is still a paucity of purified and characterized antigens for reliable immunodiagnosis of *A. fumigatus*-induced diseases (2, 29–31, 33, 44, 45, 47). In a recent study, cytoplasmic *A. fumigatus* allergens were reported to be recognized exclusively by serum IgE of ABPA patients, whereas secretory proteins were recognized by IgE antibodies in *A. fumigatus*-sensitized asthmatics as well as ABPA patients (13). Hence, purification and characterization of various cell-associated and metabolic *A. fumigatus* proteins may aid in the differential diagnosis of *A. fumigatus*-induced diseases. With the advent of DNA technologies and biotechnological procedures, cloning and production of large amounts of pure *A. fumigatus* allergens are now possible (1, 4, 26, 37). Recently, phage surface display technologies for cloning cDNA have been used for isolation and characterization of a number of major and minor *A. fumigatus* allergens (13–16, 23).

Recently we have reported the partial nucleotide sequence of a cDNA clone representing the C-terminal region of a major *A. fumigatus* allergen, Asp f 2 (4). Here we present the complete nucleotide sequence of Asp f 2 and expression of the mature recombinant protein. The expression kinetics and immunochemical properties of both the native and recombinant allergen were studied. The results indicate that Asp f 2 is a major allergen of *A. fumigatus*, recognizing specific IgE antibodies in ABPA patients.

### MATERIALS AND METHODS

The construction of a cDNA library in the  $\lambda$  ZAPII vector was carried out as described previously (26). As the cDNA clone was found to be incomplete at the N-terminal region, a genomic library of *A. fumigatus* carrying inserts of 9 to 22 kb in  $\lambda$  Fix II vector (Stratagene, La Jolla, Calif.) was screened by using a

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[<sup>32</sup>P]dATP-labeled cDNA clone, and a positive plaque was identified. The Asp f 2 gene was amplified by PCR using  $\lambda$  left-arm sequences as the sense primer 5'ATTGATTACAATTTGTCCCACTC 3'; the antisense primer 5'CTAAGTGCAATGAAGCTGTCCACC 3' was designed from the C-terminal-end sequences of Asp f 2 and using  $\lambda$  DNA isolated from the amplified plaque as the template. Long-range PCR was carried out with an XL PCR kit as specified by the manufacturer (Perkin-Elmer, Foster City, Calif.). The resulting 3,000-bp product carrying the complete Asp f 2 gene was then cloned in a TA vector by using a PCR 2.1 cloning kit (Invitrogen, San Diego, Calif.) and sequenced by the chain termination method (49).

**Megaprimer PCR amplification of the Asp f 2 gene.** The Asp f 2 gene for overexpression was obtained by megaprimer PCR amplification. A 618-bp PCR product was amplified from the partial cDNA clone of Asp f 2 by using sense primer 5'GTCGGTGCCTACGATGCATC 3' and the C-terminal 5'AGTGC AATGAAGCTGTCCACCTTC3' sequence of Asp f 2 as the antisense primer. This PCR product was used further as the antisense primer along with the Asp f 2 N-terminal sequence 5'GACGCTGGCGGGTACCTCGT 3' as the sense primer for PCR amplification of the complete Asp f 2 gene. The PCR reaction was performed with 300 ng of megaprimer and 80 ng of sense primer; the template was 20 ng of TA cloned Asp f 2 DNA obtained from genomic library of *A. fumigatus*. The amplification conditions were 30 cycles of 30 s at 94°C and 11 min at 65°C. An amplified PCR product of 804 bp was purified from the gel, subcloned into PCR 2.1 vector (Invitrogen), and sequenced by the dideoxy-chain termination method (49).

**Expression of Asp f 2 protein.** To express mature Asp f 2 in *Escherichia coli*, the amplified PCR product with a *Bam*HI site at the N-terminal end and *Xho*I site at the C-terminal end was cloned and expressed in pET 23b(+) vector as previously described (4). Recombinant Asp f 1 (rAsp f 1) and rAsp f 12 were expressed in the same way in the pET expression system.

**Purification of Asp f 2.** rAsp f 2 was expressed in the pET system along with a six-histidine tag at its C-terminal end; a Ni<sup>2+</sup>-nitrilotriacetic acid agarose column (Qiagen, Santa Clarita, Calif.) was used to purify the proteins. Other *A. fumigatus* antigens such as native Asp f 2 (nAsp f 2) and culture filtrate antigens (AF102 and AF104) were obtained as described earlier (29). The cytosolic fraction complex (CFC) from *A. fumigatus* and ASPND1 protein from a mycelial extract of *Aspergillus nidulans* were gifts from F. Leal (University of Salamanca, Salamanca, Spain).

**Production of polyclonal antibodies.** Three purified *A. fumigatus* proteins, rAsp f 2 and two nAsp f 2 proteins isolated from separate strains of *A. fumigatus*, were used to immunize BALB/c mice. In brief, 50  $\mu$ g of purified protein was emulsified in an equal volume of complete Freund's adjuvant and injected subcutaneously. Three consecutive injections were given at weekly intervals with antigen mixed in incomplete Freund's adjuvant. The animals were bled, and anti-Asp f 2 antibodies were measured by enzyme-linked immunosorbent assay (ELISA). Animals were bled by cardiac puncture, and the serum was stored at -70°C. The rabbit polyclonal serum against the p40 component of *A. fumigatus* was obtained from F. Leal. All animal studies were approved by the institutional animal studies committee.

**Human serum samples.** Serum samples from three groups of patients were used: 10 patients with CF who also had the diagnostic criteria of ABPA (CF/ABPA group), 10 patients with asthma and with ABPA (ABPA group) and 10 patients with asthma with immediate wheal and flare skin reactivity to *A. fumigatus* and without sufficient features of ABPA (AA group). Sera from the CF/ABPA patients satisfying the criteria for ABPA as reported previously were obtained from the regional CF center at the Medical College of Wisconsin (39). ABPA and AA patients were seen at the Division of Allergy-Immunology of the Northwestern University Medical School or the Medical College of Wisconsin. Serum samples from 10 control (healthy) subjects also were selected. The human study committees of the Medical College of Wisconsin and Northwestern University Medical School approved this investigation.

**Aspergillus mycelial extract and culture filtrate antigen preparation.** *Aspergillus conidia* (10<sup>7</sup>/ml) were grown in Czapek-Dox-*AOAC* (1:1) liquid medium at 37°C under continuous shaking to isolate mycelial antigens. The cultures were harvested at 24 h, 48 h, 72 h, 96 h, 5 days, 7 days, 14 days, and 21 days, and the mycelia and culture filtrates were collected separately. The mycelia were washed and disrupted in a French press at a pressure of 16,000 lb/in<sup>2</sup> and centrifuged. The supernatants were dialyzed against distilled water, and the retentate was freeze-dried (12). The culture filtrates were dialyzed and lyophilized in the same way as mycelial extracts. The protein concentrations of the extracts were determined by BCA (bicinchoninic acid) assay (BCA kit; Pierce Chemicals, Rockford, Ill.).

**Kinetics of Asp f 2 production.** An ELISA was carried out to measure Asp f 2 harvested at different time intervals from culture filtrates and mycelial extracts. The amount of Asp f 2 in the preparations was detected by treating antigen (1  $\mu$ g/ml)-coated microtiter plates with mouse anti-rAsp f 2 antibodies (1:500) for 3 h (27). The subsequent steps, including the addition of biotinylated anti-mouse IgG, enzyme, and substrate, were as described previously (27). Asp f 2 concentrations at different stages of *A. fumigatus* growth were calculated from a standard curve plotted for concentration of Asp f 2 ranging from 10 to 500 ng/ml.

**Antigen-specific ELISA.** The purified rAsp f 2 was evaluated for IgE antibody binding with sera from the various subject groups by ELISA as previously described (10).

**Inhibition ELISA.** Monospecific mouse sera raised against recombinant and native Asp f 2 were diluted 1:500 and incubated with various concentrations of rAsp f 2 (1 ng to 10  $\mu$ g) overnight at 4°C. The serum samples were centrifuged, and the supernatants were transferred to the washed and blocked nAsp f 2-coated plates. Further steps in the assay were carried out with reagents as described before (3, 4). The dose-dependent inhibition of antibody titers was determined from the ELISA (absorbance optical density [OD] values) compared with uninhibited serum.

**Asp f 2 production in *A. fumigatus* cultures.** Antigenic fractions from mycelia and culture filtrates collected at various stages of growth (5  $\mu$ g per sample) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12% gel and transferred onto a nitrocellulose membrane by using a trans-blot apparatus as described previously (4). After blocking and washing, the membranes were treated with anti-rAsp f 2 antibodies (1:250) and reactivity was analyzed by Western blotting (4).

**Western blotting of various *Aspergillus* antigens with anti-Asp f 2 and anti-p40 antibodies.** The antigenic similarities between Asp f 2, ASPND1 from *A. nidulans*, and other *A. fumigatus* antigenic components were analyzed by Western blotting using mouse antisera against rAsp f 2 and rabbit antisera against p40 protein from the cytosolic fraction of *A. fumigatus* culture. The culture filtrate proteins from strain AF102, the CFC of *A. fumigatus* (consisting mainly of proteins p90, p60, p40, and p37), rAsp f 2, and protein ASPND1 purified from mycelial extracts of *A. nidulans* were separated by SDS-PAGE (12% gel) at a concentration of 5  $\mu$ g sample per lane and transferred onto a nitrocellulose membrane, and reactivities of the resolved proteins were determined by Western blot assay as described previously (28, 29).

**IgE binding of Asp f 2 and ASPND1.** An antigen-specific ELISA was carried out to compare the IgE binding of ASPND1 to that of Asp f 2, using sera from three groups of subjects. Ten individual serum samples from the ABPA, AA, and control groups were used, and the ELISA was carried out as described above (3, 27).

**Laminin-Asp f 2 interaction.** Wells of microtiter plates were coated overnight with rAsp f 1, rAsp f 2, rAsp f 12, nAsp f 2, and *A. fumigatus* culture filtrate antigens at 100  $\mu$ g/ml and incubated first with 10  $\mu$ g of laminin (Boehringer Mannheim) per ml for 3 h at room temperature and then with mouse antilaminin antibody (Sigma, St. Louis, Mo.) at a 1:1,000 dilution for 1 h. The subsequent incubations were with biotinylated goat anti-mouse IgG (1:1,000) and streptavidin peroxidase (1:10,000) for 1 h each. The color reaction was developed, and the intensity of the reaction was measured as OD as described previously (3, 4).

The binding of Asp f 2 to laminin was evaluated by ELISA where the wells of the microtiter plate were coated with Asp f 2 (4  $\mu$ g/ml) followed by addition of laminin (1 ng to 10  $\mu$ g). In another set of experiments, laminin (1 ng to 10  $\mu$ g) was added directly to the wells. Further steps include addition of mouse antilaminin antibody, biotinylated goat anti-mouse IgG, streptavidin peroxidase, and chromogen substrate as described earlier (3, 4). The reactivities in the two plates were compared.

**Statistical analysis.** The mean ( $\pm$  standard deviation [SD]) OD values of antigen-antibody reactions by ELISA using serum samples from the four groups of subjects were compared with those of the control group by using Student's *t* test (two-tailed independent samples) with the Statworks program (Crick Software, Inc., Philadelphia, Pa.). Values for ABPA, CF/ABPA, AA, and control groups were compared, and *P* < 0.05 was taken as significant.

**Nucleotide sequence accession number.** The GenBank accession no. for Asp f 2 is U56938.

## RESULTS

**Nucleotide and deduced amino acid sequences of Asp f 2.** We recently reported the expression of a truncated protein from a cDNA clone of *A. fumigatus* having strong allergenic activity (4). In the present study, in order to isolate the complete Asp f 2 gene, this cDNA clone was labeled with [<sup>32</sup>P]dATP and used as a probe to screen the *A. fumigatus* genomic library. Eleven positive plaques were identified by screening 50,000 plaques. A PCR-amplified product of 939 bp was detected in all plaque lysates, indicating the presence of the correct inserts. One of the 11 plaques was selected for further cloning and sequencing. With isolated  $\lambda$  DNA as the template, the  $\lambda$  left-arm primer as the sense primer, and the C-terminal-end nucleotide sequences from the cDNA clone as the antisense primer, a 3,000-bp PCR product was obtained. The PCR product was cloned into PCR 2.1 vector, and the plasmid DNA was sequenced. The complete nucleotide sequence of the Asp f 2 gene is shown in Fig. 1. Comparison of the cDNA and genomic sequences shows that the Asp f 2 gene consists of three exons encoding a 310-amino-acid-long protein interrupted by two introns of 83 and 52 bp. Both introns have 5'GT and 3'AG dinucleotides at the intron-exon junctions

CATAGCTTTCGGAATCATCCGACTCGGAGAACCGCTTGCCTACCAAGGTGTCCTTGG 60  
 CATCTCTGTAGAAACCCACTAGAAATACGAACAAACGAACCATGCATCTTGTGGCTTGGCG 120  
 CCGTACCTACTGTTGGGGTGGGCGGTGGCGTGTCTACCCGTATGGCCCAATCCAACAA 180  
 TGAGTACCCTCTGGCACCGCGAAAGGAGGCCATCCCTCGAGGTATATAAGGATGTCTTAC 240  
 CTCTGGCTACAATGCAAGAGTACGCGACCATCCAACCTTGAAGAAAGAAAATGTACTCAT 300  
 AAATGGCTGCTCTCCCTCCGCTTGCCTTCTCTCCCTCCGCGCCCTTGTGGCCA 360  
 MetAlaAlaLeuLeuArgLeuAlaValLeuLeuProLeuAlaAlaProLeuValAlaT  
 CCCTCCCTACCTCCCGCTCCCATCGCGCGCGAGCAACCCCGCAGAACCCGCTCTTCT 420  
 hrLeuProThrSerProValProIleAlaAlaArgAlaThrProHisGluProValPheP  
 TCTCTGGGACGCTGGCGCGGTGACCTCGTTCCCATCCACTCCAGCTGCAATCGGACCC 480  
 heSerTrpAspAlaGlyAlaValThrSerPheProIleHisSerSerCysAsnAlaThrG  
 AGCGCCGGCAGATCGAGGCGCGCTGAACGAGCGGCTCGAGCTCGCCGGCAGCCCAAG 540  
 InArgArgGlnIleGluAlaGlyLeuAsnGluAlaValGluLeuAlaArgHisAlaLysA  
 CCCACATCTCCGCTGGGCAACAGAGCGAGACTTACCGGAAGTACTTTGGCAACCGGC 600  
 laHisIleLeuArgTrpGlyAsnGluSerGluIleTyrArgLysTyrPheGlyAsnArgP  
 CCACCATGGAGGCGCTCGCTGCTACGATGTCATCGTGAACGGGCAAGGCCAACGTGC 660  
 roThrMetGluAlaValGlyAlaTyrAspValIleValAsnGlyAspLysAlaAsnValL  
 TCTTCCGGTGTGACAACCCGACGGCACTGTGCTTTTGAAGGtaaggatccccctcca 720  
 euPheArgCysAspAsnProAspGlyAsnCysAlaLeuGluG  
 ttctccgaagtgactaagggactactgggtagacctgctgctgctgctgctgctgctgctg 780  
 ttcagGCTGGGCGGCCACTGGCGCGCGCAACGCCACTCCGAAACCGTCACTGTGTA 840  
 lyTrpGlyGlyHisTrpArgGlyAlaAsnAlaThrSerGluThrValIleCysAs  
 TCGAGCTACACCACCCGCGCTGGCTTGTCTCCATGTGCTCCCGAGGCTACACCGTCGC 900  
 pArgSerTyrThrThrArgArgTrpLeuValSerMetCysSerGlnGlyTyrThrValAl  
 CGGCTCCGAGACAAACACCTTCTGGGCTTCGGACCTGATGCACCGTCTGTACCATGTGC 960  
 aGlySerGluThrAsnThrPheTrpAlaSerAspLeuMetHisArgLeuTyrHisValPr  
 TGCTGTGGTCAAGGCTGGGTCGACCACTTCGCGCAGCGCTACGACGAGGTGATTGCCCT 1020  
 oAlaValGlyGlnGlyTrpValAspHisPheAlaAspGlyTyrAspGluValIleAlaLe  
 GGCAAGACCAACCGCACCGAGTCCACGATGACTCGGAGCGGTCGAGTATTTCCGCCCT 1080  
 uAlaLysSerAsnGlyThrGluSerThrHisAspSerGluAlaPheGluTyrPheAlaLe  
 TGAGGCGTATGCGTTGATATGTCTGCTCCCGGTGTCGGATGTGCTGGCGAGAGTCCAG 1140  
 uGluAlaTyrAlaPheAspIleAlaAlaProGlyValGlyCysAlaGlyGluSerHisGl  
 CCCTGACCGAGACATGACACCGGCTGCTGCGCGCTGCTGCTACCTCCACCTCTAG 1200  
 yProAspGlnGlyHisAspThrGlySerAlaSerAlaProAlaSerThrSerThrSerSe  
 CTCAGCTCGGGCTCGGGCTCGGGCGCCACGACTACCCCGACGGATTCTCCAGTGCCAC 1260  
 rSerSerSerGlySerGlySerGlyAlaThrThrThrProThrAspSerProSerAlaTh  
 TATTGATGTGCGCTCGtaaggacagctccggatctcgtaagatggatggaatgactgac 1320  
 rIleAspValProSer  
 gttgtagAAGTCCATACCCATGAAGGTGGACAGCTTCATTGCACTTAGCGGAGGACAC 1380  
 AsnCysHisThrHisGluGlyGlyGlnLeuHisCysThrEnd  
 GATATTTTATGACCGTATGCTTACGCGGTATGTTAACCGGTATACTCCCGGTGGACC 1440  
 GGCCCTGAGTAATGGGATTAATATCTATTGTTTAAAAGGAAGTGTATGAGACCGCGT 1500  
 TACGTAATGGATGCTTGCCTGAATATACATGCTATATAGCAATGTAACCAATAGAGAC 1560  
 GTTCTTTATAAA 1572

FIG. 1. Complete nucleotide sequence and the deduced amino acid sequence of the Asp f 2 gene of *A. fumigatus*. The intron sequences are represented in lowercase. The 20 N-terminal amino acids of the purified native mycelial protein gp55 demonstrate sequence homology from amino acid 32 onward, as represented in italics. The *A. fumigatus* culture filtrate protein with N-terminal sequence homology from Asp 43 onward are underlined; glycosylation sites are in boldface.

involved in the splicing process, and four possible glycosylation sites are located at Asn 57, 87, 143, and 216 (Fig. 1, boldface). The deduced amino acid sequence of this gene exhibits complete homology with the amino acid sequence of the cDNA clone starting from Glu 65 to the C-terminal-end Thr 310. However, the N-terminal amino acid residues of the nAsp f 2

	1				50
Asp f 2	MAALLRLAVL	LPLAAPLWPT	LPTSPVPIAA	RATPHEPVFF	SWDAGAVTSF
ASPND1		MQLL	AL-ALLCAS I	AAL-TQQTPL	PLEDPI KSP
<i>C. alb</i> p		FFFA	FSVAAPVTVT	-FVDAS-TGY	D-RADW-KG-
	51				100
Asp f 2	PIHSSCNATQ	RRQIEAGLNE	AVELARHAKA	HILRWGNESE	IYRKYFGNRR
ASPND1	-----E	Q--LATA-Q-	T-T-E---D	-----E	-----D-
<i>C. alb</i> p	--D-----	YN-LST-Q-	-QL-E--RD	-T-F-SK-P	FF-----ET
	101				150
Asp f 2	PTMEAVGAYD	VIVNGDKANV	LFRCDNPDGN	CALEGWGGHW	RGANATSETV
ASPND1	-S LT- I - - - -	IT - - - NPD - I	-----	-----	--E--SD--
<i>C. alb</i> p	ASA-V - - HFE	NV-DA- - SSI	-L--DL-DK	-KND--A-Y-	--S-HS DQ-I
	151				200
Asp f 2	ICDRSYTTRR	WLVMSCSQGY	TVAGSETNTF	WASDLMHRLY	HVPVAVGGWV
ASPND1	--EL-----	S-ST-----	-ISEW-----	-PG--L-----	-M--I--L-
<i>C. alb</i> p	--L-FV---	Y-SQL--G--	--SK-K--I-	--G--L--FW	-LKSI--LVI
	201				250
Asp f 2	DHFADGYDEV	IALAKNSGTE	STHDSEAFEY	FALEYAFDI	AAPGVGTCAG
ASPND1	--Y---EG-	LE--EGNR--	AV---TLQ-	---V--Y-V	-V--I--V-
<i>C. alb</i> p	--Y--T-EE-	LE--QENS-Y	AVRN-NSLI	Y--DV--Y-V	TI--E--N-D
	251				300
Asp f 2	ESHGPDQGH	TGSASAPAST	ST S SSSG SG	SGA TTTPT DS	PSATIDVPSN
ASPND1	GEEEN--QG	EEQTEE--QD	DQDQEXXXXX	XXXXXXXXXX	AAEEEL-E-
<i>C. alb</i> p	GTSYKKSDFS	SFED-DSG-D	-GA- TAS- S	HQHTDSN PSA	TTDDD-AN-H
	301	312			
Asp f 2	CHTHEGGLH	CT			
ASPND1	-----E--	--			
<i>C. alb</i> p	-----AD-EV--	--			

FIG. 2. Conserved sequences of ASPND1 from *A. nidulans*, a fibrinogen binding protein from *C. albicans* (*C. alb* p), and Asp f 2. The cysteine molecules and glycosylation sites are conserved in all the three proteins.

are located within the open reading frame starting from Asp 43 (Fig. 1, underlined). The N-terminal 20-amino-acid sequence of a previously reported *A. fumigatus* glycoprotein (gp55) exhibits complete homology to Asp f 2 from Ala 32 to Pro 51 (Fig. 1, italics). This sequence is preceded by a 31-amino-acid-long probable signal peptide with a stretch of hydrophobic amino acids.

**Sequence homology of Asp f 2 with cell wall-associated proteins.** A search of GenBank by using the BLAST program revealed a high degree of sequence homology to ASPND1, a recently described protein from *A. nidulans*, as well as to cell wall-associated proteins from *Candida albicans* (12). Figure 2 shows the sequence alignment of Asp f 2 from Ala 32 to Pro 51 exhibited 60% sequence identity and 75% similarity, whereas Asp f 2 had about 44% identity and 65% similarity with fibrinogen binding protein and a similar degree of identity with pH-regulated antigen 1 (PRA1) from *C. albicans* (12, 50). The four possible glycosylation sites and eight cysteine molecules present in Asp f 2 also are conserved in ASPND1 and *C. albicans* proteins.

**Recombinant and native Asp f 2 are similar in structural conformation.** To evaluate the specificity of anti-rAsp f 2 antibodies, an ELISA inhibition was carried out with mouse antibodies raised against rAsp f 2, nAsp f 2 (AF104), and nAsp f 2 (AF102). The antibodies against these three proteins showed binding to solid-phase coated nAsp f 2 (AF104). This binding was inhibited by increasing amounts of recombinant Asp f 2 (Fig. 3). We were able to inhibit more than 80% binding of these antibodies to solid-phase coated nAsp f 2. For anti-nAsp f 2 (AF104), 550 ng of rAsp f 2 was required to obtain 50% inhibition, whereas anti-rAsp f 2 and anti-nAsp f 2 (AF102) required about 75 ng of rAsp f 2 to exhibit 50% inhibition of binding to solid-phase coated native protein.

**High expression of Asp f 2 in mycelial extract.** The time kinetics of Asp f 2 production in *A. fumigatus* culture (AF104) were analyzed by comparing Asp f 2 levels in culture filtrates

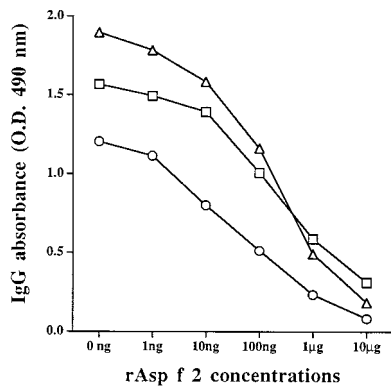


FIG. 3. Inhibition of IgG binding to solid-phase coated nAsp f 2. Polyclonal mouse sera raised against nAsp f 2 AF102 ( $\Delta$ ), nAsp f 2 AF104 ( $\square$ ), and rAsp f 2 ( $\circ$ ) were preincubated overnight with different amounts of rAsp f 2 as indicated on the x axis. Preincubated serum samples were transferred to nAsp f 2 (AF104)-coated wells, and antibody binding was measured.

and mycelial extracts over 21 days of shake culture. Asp f 2 could be detected in the mycelial growth within 24 h of culture. This protein represented about 20% of total mycelial proteins at 96 h and 40% by day 7. This increase was followed by a gradual decrease and stabilization at 30% during weeks 2 and 3 of culture (Table 1). The culture filtrates collected at the same intervals of growth after harvesting of the mycelium demonstrated significantly less secreted Asp f 2. The amount of Asp f 2 varied from 1.6% at 24 h to 0.5% at the 3 weeks of culture. In Western blot analysis using mouse sera against rAsp f 2, both mycelial and culture filtrate preparations at various time intervals exhibited IgG binding in the molecular mass range of 35 to 37 kDa. More than one protein band in this narrow range exhibited distinct antibody binding (Fig. 4). Although the intensity of the antibody binding was different, the mycelial extract showed a very high level of Asp f 2 even at 24 h, whereas the 24-h, 48-h, and 5-day culture filtrates exhibited binding with anti-Asp f 2 in the molecular mass range of 35 to 37 kDa. The reason for complete absence of secretory Asp f 2 at 72 h, 96 h, and 7 days of culture was not clear; it may be due to the involvement of different secretory pathways and regulatory mechanisms at different stages of fungal growth or due to the metabolic degradation of the protein. On the other hand, the stationary culture filtrates collected at various intervals of time exhibited a gradual increase in Asp f 2 production

TABLE 1. Asp f 2 concentration and pH of *A. fumigatus* culture (AF104) at various time intervals

Incubation time	Mycelial wt (g)	pH of culture medium	Asp f 2 concn (1,000 ng/ml of crude extract) <sup>a</sup>	
			Culture filtrate	Mycelia
0 h		7.25		
12 h	0.276	7.2	10	
24 h	0.723	6.9	16	11
48 h	3.3	6.4	8	69
72 h	3.7	5.7	12	185
96 h	5.8	5.9	9	200
5 days	6.32	5.7	7	281
7 days	8.4	5.65	9	403
14 days	4.9	4.5	9	284
21 days	3.7	4.35	5	291

<sup>a</sup> Calculated from the Asp f 2 standard curve by ELISA, using concentrations of 10 to 500 ng of Asp f 2/ml versus anti-Asp f 2 antibody.

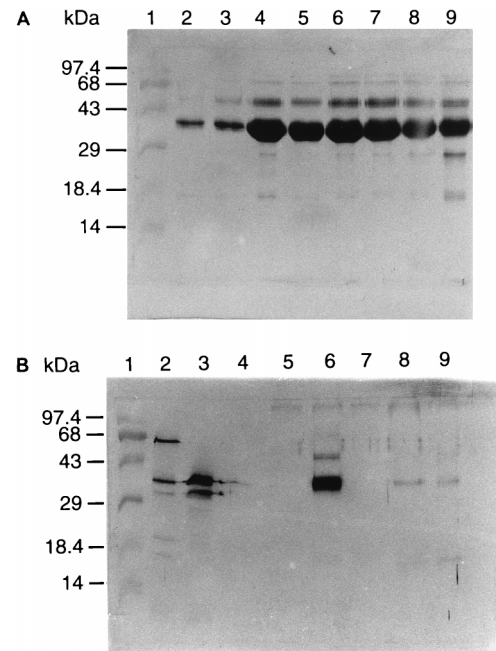


FIG. 4. Time kinetics of expression of Asp f 2 in *A. fumigatus* mycelial extracts. Mycelial proteins (5  $\mu$ g/ml) at various intervals of growth were separated by SDS-PAGE on a 12% gel and transferred onto nitrocellulose membranes. Separated proteins were evaluated for antibody binding by using polyclonal mouse sera against rAsp f 2. Lane 1, molecular weight markers. Different time interval cultures: lane 2, 24 h; lane 3, 48 h; lane 4, 72 h; lane 5, 96 h; lane 6, 5 days; lane 7, 7 days; lane 8, 14 days; lane 9, 21 days. (B) Time kinetics of expression of Asp f 2 in *A. fumigatus* culture filtrates. The culture filtrate proteins (5  $\mu$ g/ml) were separated by SDS-PAGE on a 12% gel and transferred onto nitrocellulose membranes. Separated proteins in lanes 2 to 9 are culture filtrates at the same time intervals as for mycelial extracts (A) and were evaluated for antibody binding by using polyclonal mouse sera against rAsp f 2.

from day 2 onward, reaching the highest concentration at 2 weeks (data not shown). The variation in antigenic profiles of culture filtrates from stationary and shake cultures of *A. fumigatus* could be due to the complex nature of the fungal morphology and metabolic pathways. For the culture filtrates, a number of proteins appeared to have weak antibody binding with anti-Asp f 2 in the wide range of 18 to 100 kDa (Fig. 4B).

**ASPND1 and Asp f 2 share common epitopes.** ASPND1, the protein present in the water-soluble extract from *A. nidulans*, exhibited significant sequence homology with Asp f 2. To ascertain the presence of common epitopes in these proteins, antibody binding of ASPND1 was evaluated by using polyclonal sera against rAsp f 2 and the p40 component of CFC isolated from *A. fumigatus* culture. In Western blots, rAsp f 2 and ASPND1 exhibited strong IgG binding with sera against rAsp f 2 (Fig. 5, lanes d and e, respectively). Similarly, anti-p40 antibody exhibited strong IgG binding with rAsp f 2 as well as ASPND1 (Fig. 5, lanes h and i, respectively). However, ASPND1 when treated with anti-p40 antibodies demonstrated two additional IgG binding proteins at lower molecular masses. At the same time, the CFC component of *A. fumigatus* (composed of p90, p60, p40, and p37 antigens) showed similar antibody binding with anti-rAsp f 2 and anti-p40 antibodies, with a doublet type of reaction at 35 to 40 kDa (Fig. 5, lanes c and g). Crude *A. fumigatus* culture filtrate (AF102) showed multiple anti-Asp f 2 as well as anti-p40 antibody binding proteins in the molecular mass range of 18 to 70 kDa (Fig. 5, lanes b and f). These results strongly suggest the presence of common antigenic epitopes in Asp f 2, ASPND1, and p40 *A. fumigatus* antigen.

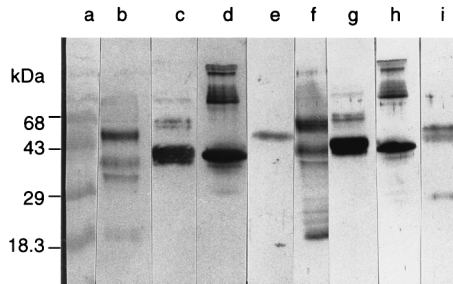


FIG. 5. Western blot analysis of various *Aspergillus* proteins, using mouse polyclonal sera against rAsp f 2 and rabbit sera against the p40 cytosolic component of *A. fumigatus*. Lanes: a, molecular weight markers; b to e, treated with anti-rAsp f 2 sera; f to i, treated with anti-p40 sera; b and f, *A. fumigatus* culture filtrate antigens (AF102); c and g, CFC fraction from *A. fumigatus*; d and h, rAsp f 2; e and i, ASPND1 from *A. nidulans*.

Purified ASPND1 and Asp f 2 were evaluated by using an indirect ELISA for specific IgE antibody binding in sera from ABPA ( $n = 10$ ), AA ( $n = 10$ ), and control ( $n = 10$ ) groups (Fig. 6). In ELISAs, the mean IgE absorbance value (OD at 490 nm [OD<sub>490</sub>]) for ASPND1 with sera from ABPA patients was 0.25, compared to 0.05 for controls ( $P < 0.001$ ). However, the mean IgE absorbance value for Asp f 2 in sera from ABPA patients was almost threefold higher (OD<sub>490</sub> of 0.708) than that with ASPND1 (OD<sub>490</sub> of 0.248).

**Specific binding of Asp f 2 to extracellular matrix protein laminin.** Both recombinant and native Asp f 2 demonstrated high binding affinity to laminin in a direct solid-phase ELISA. Laminin binding of Asp f 2 was more than twofold higher than that with rAsp f 1 and rAsp f 12. However, native Asp f 2 exhibited higher affinity than rAsp f 2 (Fig. 7A). For dose-dependent binding of laminin to Asp f 2 in wells coated with a constant concentration of Asp f 2, gradual increases in laminin concentration resulted in increased binding with antilaminin antibodies (Fig. 7B), indicating the specific interaction between laminin and Asp f 2. On the other hand, high antilaminin antibody binding was observed in wells directly coated with 100 ng of laminin or more. With 1  $\mu$ g of laminin, direct laminin-antilaminin interaction demonstrated fourfold-higher ELISA absorbance (OD<sub>490</sub> of 1.365) than with Asp f 2-coated plates (OD<sub>490</sub> of 0.295).

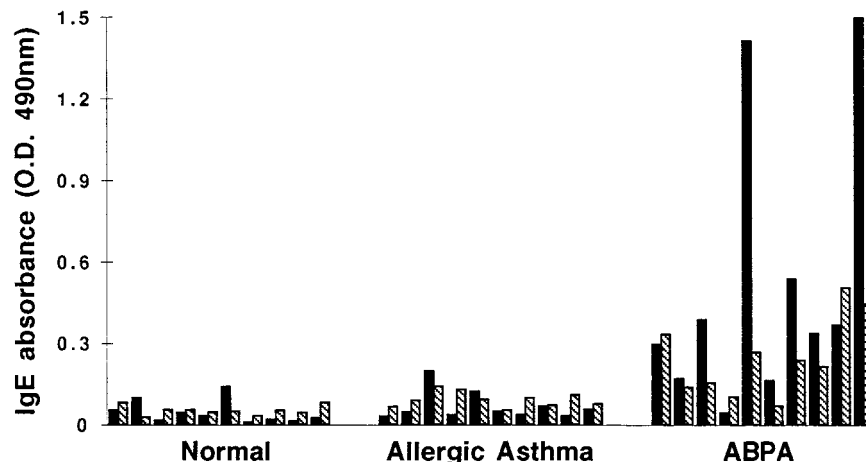


FIG. 6. IgE binding of purified rAsp f 2 and ASPND1, using 10 serum samples from each of the indicated groups. Solid bars, ELISA absorbance values at 490 nm for rAsp f 2; shaded bars, absorbance values for ASPND1.

**Specific IgE binding of Asp f 2 in ABPA sera.** The biologically active rAsp f 2, nAsp f 2, and crude culture filtrate AF104 were evaluated for IgE antibody with 10 serum samples from each of the CF/ABPA, ABPA, and AA groups of patients along with 10 healthy controls (Fig. 8). Serum IgE antibody against all of the antigens was elevated in CF/ABPA and ABPA patients in comparison with AA patients and controls. The mean IgE absorbance in CF/ABPA samples for both native and recombinant Asp f 2 was higher than in ABPA samples. The difference between rAsp f 2-specific IgE in CF/ABPA (mean 0.606, SD  $\pm$  0.438) and control (mean 0.048, SD  $\pm$  0.042) groups was statistically significant ( $P < 0.01$ ). A significant difference was observed also for rAsp f 2-specific IgE antibody in ABPA (mean 0.408, SD  $\pm$  0.380) and control groups ( $P < 0.01$ ).

## DISCUSSION

Recently we reported the nucleotide sequence of a cDNA clone from *A. fumigatus* representing the C-terminal region of Asp f 2 (4). The recombinant protein expressed in *E. coli* without the N-terminal amino acid residues also exhibited distinct IgE binding with sera from ABPA patients and appeared to be a part of a major allergen from *A. fumigatus*. These results prompted us to isolate and characterize the gene carrying the complete nucleotide sequence for Asp f 2 and also to clone and overproduce the recombinant allergen.

The Asp f 2 sequence encodes a protein with a predicted molecular size of 29 kDa. However, on SDS-PAGE analysis, this protein exhibited a band at 37 kDa. Although the reasons for such differences are not clear, they could be attributed to the amino acids at the C-terminal of the protein. The involvement of C-terminal amino acids in slow migration of Asp f 2 in SDS-PAGE is evident from the differences in the molecular sizes of two recombinant polypeptides from Asp f 2, representing 200 amino acids from either the N- or C-terminal region of Asp f 2 (data not shown). The polypeptide encoding the C-terminal *A. fumigatus* region migrated during SDS-PAGE to a position about 5 to 6 kDa greater than its predicted size. Similar differences between the predicted molecular size of the native protein and the apparent size on SDS-PAGE have been reported for serine proteases from *A. fumigatus*, mycelial antigen from *A. nidulans*, and cell wall antigen from *C. albicans*

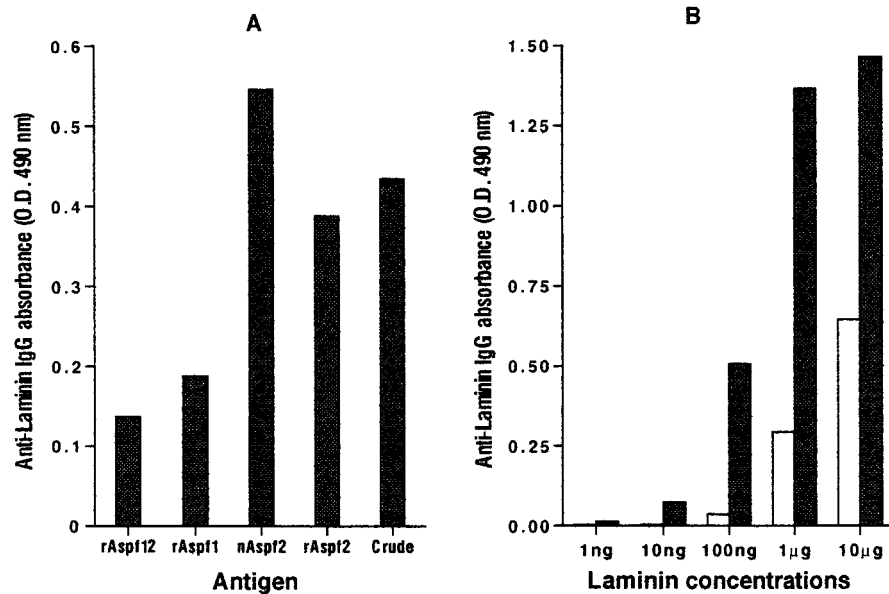


FIG. 7. (A) Specific binding of laminin to Asp f 2, rAsp f 1, rAsp f 12, rAsp f 2, nAsp f 2, and crude *A. fumigatus* culture filtrates (100  $\mu$ g/well) were incubated with 10  $\mu$ g of laminin per ml. The subsequent incubation was with a 1:1,000 dilution of mouse antilaminin antibody. The ELISA IgG absorbance was measured at 490 nm (for details, see Materials and Methods). (B) Dose-dependent binding of laminin (1 ng to 10  $\mu$ g) to solid-phase coated Asp f 2. Open bars, IgG absorbance of laminin-antilaminin interaction on Asp f 2-coated wells; closed bars, absorbance values for direct laminin-antilaminin binding on laminin-coated wells.

(12, 24, 25, 36, 46, 50). For a  $\text{Ca}^{2+}$ -dependent serine protease from *Saccharomyces cerevisiae*, the deletion experiments precisely demonstrated that the high negative change of the C-terminal region of the protein is responsible for its slow migration in gel (18).

Comparison of amino acid sequences of the product of the entire Asp f 2 gene with the N-terminal amino acid sequences of other purified proteins exhibited complete homology with a recently purified and characterized 37-kDa concanavalin A-nonbinding protein from an *A. fumigatus* AF104 culture filtrate and with another previously reported glycoprotein, gp55, characterized after isolation from a water-soluble extract from mycelium of strain NHL5759 (3, 52). Both native proteins demonstrated complete N-terminal sequence homology to the *A. fumigatus* gene characterized in this study, starting from different N-terminal regions on the same gene. This result indi-

cates that the posttranslational modifications such as phosphorylation, glycosylation, and enzymatic cleavages may be responsible for the differences in the molecular weight of the same core protein under different culture conditions and in different strains of *A. fumigatus*. The 37-kDa culture filtrate protein as well as deglycosylated gp55 showed high IgE binding, indicating that the core protein without glycosylation in both molecules is involved in IgE binding. Another 58-kDa glycoprotein antigen exhibiting high IgG binding with sera from patients with aspergillosis appeared to be different from Asp f 2 and gp55, as the antibody binding of this protein was totally destroyed by treatment with sodium metaperiodate and partially destroyed by protease treatment (17, 52).

As the correct three-dimensional structure of the allergen is essential for IgE antibody binding, the Asp f 2 allergen overproduced in the prokaryotic expression system is functionally

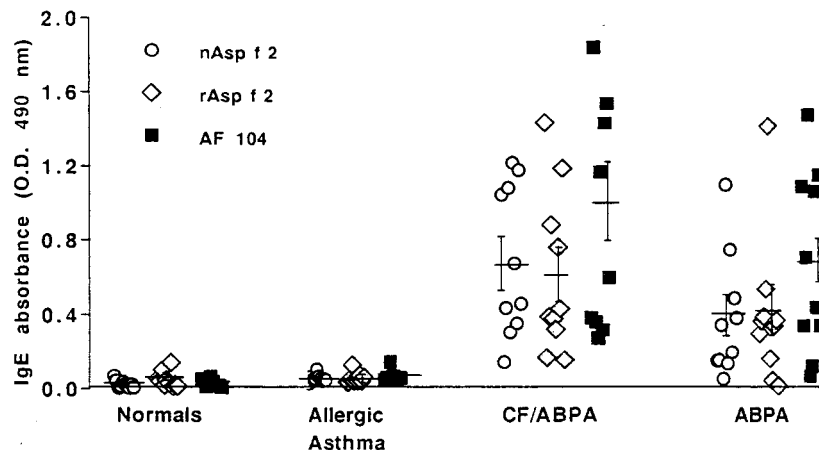


FIG. 8. IgE binding of nAsp f 2, rAsp f 2, and *A. fumigatus* culture filtrate (AF104) with 10 serum samples from each of the indicated groups.

comparable with its native counterpart. In an ELISA inhibition study, mouse antisera against native Asp f 2 preincubated with rAsp f 2 exhibited more than 80% inhibition in binding to solid-phase coated native protein, indicating conformational similarities between these two proteins.

We have evaluated further the IgE antibody binding of recombinant and native Asp f 2 as well as crude culture filtrate in four groups of subjects. None of the AA patients, all of whom showed immediate cutaneous reactivity to *A. fumigatus*, exhibited IgE binding with Asp f 2 proteins, whereas all patients from the ABPA and CF/ABPA groups showed IgE binding. In a recent study, the polypeptide representing truncated Asp f 2 demonstrated distinct IgE antibody binding with ABPA-CB patients, whereas IgE antibodies in ABPA-S (seropositive for ABPA without CB) patients failed to show significant binding to this protein (4). These observations indicated the possible involvement of Asp f 2 in the acute phase of the disease and bronchial wall injury in patients. The typical immune response of Asp f 2 in ABPA-CB patients emphasized its use in differential diagnosis of ABPA and other *A. fumigatus*-sensitized patients as well as ABPA-S and ABPA-CB patients. Two recently reported recombinant *A. fumigatus* allergens, rAsp f 4, and rAsp f 6, also demonstrated distinct IgE binding with ABPA patient sera, and serodiagnosis with these two allergens together showed 100% specificity and greater than 90% sensitivity (13, 15, 23, 34). In the present study, we found 100% specificity in CF/ABPA patients and 90% in ABPA patients, while none of the controls showed significant levels of Asp f 2-specific IgE. The other recombinant *A. fumigatus* allergens also demonstrated distinct IgE binding in ABPA patients. However, they also reacted with IgE antibodies of *A. fumigatus*-sensitized non-ABPA patients, and this overlap in IgE binding lowers the specificity when these antigens are used together (13, 37, 38).

The levels of high IgE antibodies against Asp f 2 in CF/ABPA patients indicates the allergen's usefulness in the diagnosis of patients with CF complicated by ABPA. In a recent study, intracellular rather than secretory *A. fumigatus* proteins were reported to show distinct IgE antibody binding in CF patients with ABPA but not in CF patients without ABPA (13, 41). Colonization of the fungus in the bronchi and the release of intracellular proteins may be responsible for sensitizing ABPA patients against nonsecretory fungal proteins, whereas the nonavailability of intracellular *A. fumigatus* protein has been attributed to the lack of specific IgE response against these allergens in *A. fumigatus*-sensitized allergic asthmatics.

Asp f 2, with its extensive sequence homology to fibrinogen binding protein of *C. albicans* as well as its specific binding to laminin, appears to be involved in fungal adherence to the extracellular matrix (9, 42, 43). Laminin and fibrinogen are the major structural proteins of the basement membrane and are mainly involved in mediating adherence of conidia to the extracellular matrix. To our knowledge, this is the first *A. fumigatus* allergen reported to show distinct binding to major extracellular matrix proteins. Thus, one of the initial steps in host colonization may be the recognition in patients of basement membrane laminin by Asp f 2. Recently, a 72-kDa cell wall surface component of *A. fumigatus* with receptors for laminin was reported (53). The recently reported *C. albicans* antigen PRA1 demonstrated pH-sensitive expression, with maximum expression at neutral pH and no expression below pH 6.0 (50). Although Asp f 2 exhibited high sequence homology and conserved glycosylation sites with PRA1, the mycelial expression of Asp f 2 appears to be unaffected by the pH of the medium. In shake culture, however, the secretion of Asp f 2 into the medium gradually decreases as the pH changes from neutral to

acidic. We cannot explain the high expression of Asp f 2 only at day 5 when the culture is acidic (Fig. 4B), with continuous decreases in concentration in late cultures. The involvement of PRA1 in temperature-dependent hypha formation suggests a possible role of this protein family in fungal morphogenesis.

The extensive sequence homology between Asp f 2 and the mycelial protein ASPND1 from *A. nidulans* as well as the presence of conserved glycosylation sites and cysteine molecules in these proteins raises the possibilities of common antigenic and allergenic epitopes in the two proteins (11, 12). Indeed, ASPND1 strongly reacted with polyclonal anti-rAsp f 2 antibodies and also exhibited specific IgE binding with sera from patients with ABPA, indicating the presence of common B-cell epitopes in these two proteins. However, the threefold less IgE binding with ASPND1 than with Asp f 2 may be due to the differences in posttranslational modification and three-dimensional structure of these two proteins. The peptide mapping studies of various cytosolic *A. fumigatus* components demonstrated that p60, p40, and p37 cytosolic proteins are the differentially modified forms of a common peptide core (12). The antisera against the p40 component used in this study reacted strongly with rAsp f 2 as well as ASPND1, indicating the presence of common core protein in all of these antigens.

To obtain well-characterized and reproducible allergen preparations, it is essential to identify the *A. fumigatus* proteins which are produced by most of the strains and exhibit specific binding with patient sera. Antisera raised against the recombinant allergens expressed both in prokaryotic and eukaryotic systems should be used to screen mycelial and culture filtrate preparations from various strains of *A. fumigatus* as well as proteins from other *Aspergillus* species in order to identify the commonly expressed allergens. These allergens can be used for routine diagnosis and analyzed for their involvement in the immunopathogenesis of *A. fumigatus*-induced respiratory diseases.

In conclusion, the recombinant and native forms of the allergen Asp f 2 evaluated in this study are immunologically comparable, and the distinct IgE binding ability of this major allergen with sera from ABPA patients, particularly those with CB, may be of value for specific diagnosis. Further analysis of this protein in fungal colonization studies may shed new light in the role of *A. fumigatus* allergens in the immunopathogenesis of ABPA.

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