

Characterization of the *Aspergillus nidulans* *aspnD1* Gene Demonstrates that the ASPND1 Antigen, Which It Encodes, and Several *Aspergillus fumigatus* Immunodominant Antigens Belong to the Same Family

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For the first time, an immunodominant *Aspergillus nidulans* antigen (ASPND1) consistently reactive with serum samples from aspergilloma patients has been purified and characterized, and its coding gene (*aspnD1*) has been cloned and sequenced. ASPND1 is a glycoprotein with four N-glycosidically-bound sugar chains (around 2.1 kDa each) which are not necessary for reactivity with immune human sera. The polypeptide part is synthesized as a 277-amino-acid precursor of 30.6 kDa that after cleavage of a putative signal peptide of 16 amino acids, affords a mature protein of 261 amino acids with a molecular mass of 29 kDa and a pI of 4.24 (as deduced from the sequence). The ASPND1 protein is 53.1% identical to the Asp fII allergen from *Aspergillus fumigatus* and 48% identical to an unpublished *Candida albicans* antigen. All of the cysteine residues and most of the glycosylation sites are perfectly conserved in the three proteins, suggesting a similar but yet unknown function. Analysis of the primary structure of the ASPND1 coding gene (*aspnD1*) has allowed the establishment of a clear relationship between several previously reported *A. fumigatus* and *A. nidulans* immunodominant antigens.

The increasing incidence of *Aspergillus*-related diseases (43) has prompted many investigators to search for fungal molecules relevant either for the immunodiagnosis of or for understanding the pathology of the different forms of aspergillosis (ranging from pulmonary aspergilloma to invasive aspergillosis, passing through allergic situations). The role of several putative virulence factors has been investigated without too much success (for a review, see reference 3). In contrast, several antigens of demonstrated value as immunodiagnostic reagents for different *Aspergillus* infections have been purified, and some of their coding genes have been cloned. The Asp fI ribotoxin (27), the Asp fII allergen (2), and heat shock protein 1 (HSP-1) (16) from *Aspergillus fumigatus* have been obtained as recombinant antigens. The CAT1 (the subunit of a catalase) (22) and superoxide dismutase (13) antigens have been purified to homogeneity from *A. fumigatus* water-soluble extracts. All of these antigens have been shown to be consistently reactive with sera from patients with different forms of aspergillosis but not with sera from control or healthy individuals (2, 10, 24, 26). Certain other purified molecules, such as the 58-kDa (6) and gp55 (40) antigens, or semipurified fractions, such as the CS2 complex (30) or the cytosolic fraction complex (CFC) (24) from *A. fumigatus*, have been claimed to be useful reagents for immunodiagnosis. In this paper, we attempt to simplify the entangled antigenic array of *A. fumigatus* antigens by using data obtained from the purification and immunochemical characterization of a previously reported *Aspergillus nidulans* antigen (4) (now designated ASPND1), cloning of its coding gene, and analysis of its primary structure. The conclusion of this study is that several previously reported antigens or antigenic *Aspergillus* preparations contain a polypeptide core of common origin, which is the part of the molecule mainly responsible for the immunogenic ability. Different glycosylation levels or light proteolytic digestions could account for the variations in mo-

lecular mass, pI, or reactivity with a particular group of patient sera.

Consequently, the design of a standard reagent containing all of the antigens of demonstrated immunodiagnostic value, preferably from recombinant origin, is feasible. This should help in accurate diagnosis of the different forms of aspergillosis, mainly allergic bronchopulmonary aspergillosis (ABPA) and pulmonary aspergilloma. The universality of the strong response of the immune system to the family of Asp fII and ASPND1 antigens suggests a significant role for these molecules in the pathogenesis of infections caused by *Aspergillus* spp.

MATERIALS AND METHODS

Organisms and growth conditions. *A. nidulans* G1059wt (*adF17 pabaA1 yA2*) was obtained from A. J. Clutterbuck, Glasgow, Scotland.

The organism was maintained on solid YED medium (1% [wt/vol] D-glucose, 1% [wt/vol] Difco yeast extract, 2% [wt/vol] agar). To obtain high yields of conidia, the fungus was grown on solid *Aspergillus* complete minimal medium (AMM) containing 0.1% glucose, 0.6% NaNO₃, 0.052% MgSO₄, 0.052% KCl, 0.15% KH₂PO₄, traces of FeSO₄ and ZnSO₄, and 1.5% (wt/vol) agar (pH 6.5). Plates were incubated at 28°C for at least 4 days.

For liquid growth cultures, Bacto Czapek-Dox broth–Bacto synthetic broth AOAC (1:1) (CDA) medium was used (obtained from Difco Laboratories, Detroit, Mich.).

AMM and CDA media were supplemented with 10 mg of *p*-aminobenzoic acid and 200 mg of adenine per liter.

Aspergillus cells were grown by inoculation of 10⁵ conidia per ml in 1-liter Erlenmeyer flasks containing 300 ml of the corresponding liquid medium followed by incubation at 37°C in an Adolph Kühner orbital shaker at 280 rpm. Mycelia were harvested from liquid medium cultures by filtration through Whatman GF/C paper and were washed thoroughly with double-distilled H₂O. The wet cake was immediately frozen and kept at –70°C until used. *Escherichia coli* NM538 was used for propagation of bacteriophage λEMBL4. All plasmid subcloning experiments were performed with *E. coli* DH5α. To obtain single-stranded DNA for sequencing, *E. coli* MV1190 was used. All bacterial strains were grown at 37°C in Luria broth medium (35).

Preparation of cell extracts. Frozen mycelia were thawed and mixed with lysing buffer (100 mM Tris-HCl [pH 7.5], containing 1 mM EDTA, 5 mM dithiothreitol [DTT], 1 mM freshly added phenylmethylsulfonyl fluoride [Sigma Chemical Co.], 5 μg of aprotinin per ml, and 5 μg of pepstatin A per ml [both obtained from Boehringer Mannheim]) to give a dense suspension. Samples were then disrupted in a French press (SLM Aminco) with the 20,000-lb/in² cell, previously refrigerated at –20°C, at a pressure of 16,000 lb/in². Complete breakage was monitored by microscopical observation. Broken mycelia were centri-

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fused at $150,000 \times g$ for 1 h to pellet cell walls and total membranes, and the resulting supernatant was considered as the water-soluble fraction (23). To obtain total mycelial extracts, 2% (final concentration) sodium dodecyl sulfate (SDS) was added to the broken mycelia, and the lysate was incubated for 10 min at 100°C and centrifuged at $20,000 \times g$ for 30 min. Both water-soluble fractions and total mycelial extracts were divided into aliquots and stored at -70°C .

Protein was quantitated by a modification of the Lowry method (29).

SDS-PAGE. SDS-polyacrylamide gel electrophoresis (PAGE) was carried out on a Protean II or Mini-Protean apparatus (Bio-Rad Laboratories) on isotropic 10, 14, or 16% (wt/vol) acrylamide slab gels (16 by 18 by 0.1 cm or 8 by 6 by 0.1 cm) with the discontinuous buffer system of Laemmli (19). Bio-Rad low-molecular-mass standards and Bethesda Research Laboratories high- and low-molecular-mass or Sigma prestained markers were used as molecular mass protein standards.

When samples were electrophoresed under native conditions, no boiling step was included; SDS and reducing agents were omitted from all of the buffers (including the lysing buffer), and polyacrylamide percentages of 4, 6, 8, and 10% were used. A nondenatured protein molecular mass marker kit (Sigma) was used to construct the standard curve for apparent molecular mass determinations.

Proteins in gels were detected by a sensitive silver stain (25) or by a 30-min staining with 0.5% Coomassie brilliant blue R-250 in acetic acid-isopropanol-water (1:3:6) and destaining in acetic acid-methanol-water (10:5:85).

Electroelution of proteins. After SDS-polyacrylamide gel electrophoresis (PAGE) and Coomassie blue staining, the protein of interest was electroeluted and electroeluted from the corresponding polyacrylamide sections in a model 422 Electro-Eluter (Bio-Rad) according to the manufacturer's instructions.

Treatments with EndoH. After acetone precipitation, a dry sample (10 μg) of electroeluted protein was solubilized in a suitable buffer supplemented with β -mercaptoethanol (0.1 M final concentration) and a 1.2-fold excess (wt/wt) of SDS (relative to total protein concentration) and heated to 100°C for 5 min. After cooling, the sample was diluted sixfold with 50 mM sodium citrate (pH 5.5) to reduce the SDS concentration, and various amounts (0.0004 to 0.4 U/mg of electroeluted protein) of endo- β -N-acetylglucosaminidase H from *Streptomyces plicatus* (Endo-H; Boehringer Mannheim; catalog no. 1088734) were added. Phenylmethylsulfonyl fluoride (0.5 mM final concentration) was added to prevent proteolytic degradation. The mixture was then incubated at 37°C for 24 h.

N-terminal amino acid sequence analysis of ASPND1. *A. nidulans* cell extracts prepared as described above were separated by SDS-PAGE and electroblotted onto a polyvinylidene difluoride (PVDF) membrane (Immobilon P; Millipore) (21). After staining with Ponceau S red, the ASPND1 polypeptide band was cut out and used for N-terminal amino acid sequencing. Twenty nanomoles of ASPND1 was subjected to automated Edman degradation at EUROSEQUENCE (Gröningen, The Netherlands). The sequence determined was LP TQOTPLPLEDPI.

Immunogens and immunization procedures. Electroeluted SDS-containing protein preparations were acetone precipitated; the dried pellets were resuspended at 1 mg of protein per ml in phosphate-buffered saline (10 mM K_2HPO_4 [pH 7.5], 150 mM NaCl) and used as immunogens. Antibodies were raised in 4 New Zealand rabbits by injection of 1 mg of electroeluted protein, emulsified with complete Freund's adjuvant, and intracutaneously distributed over 10 sites (100 μl /site). Booster injections of 0.5 mg of the same protein emulsified with incomplete Freund's adjuvant were given subcutaneously every 2 weeks. Sample bleedings were taken on days -6 (preimmune serum), 30, 45, 60, and 90. The animals were bled on day 148.

Electrophoretic blotting procedures and immunological detection of proteins. Proteins from extracts were first subjected to electrophoresis (SDS-PAGE or two-dimensional gel electrophoresis) and then transferred to nitrocellulose sheets (0.45 μm pore diameter; Schleicher & Schuell) in a Trans-Blot Cell (Bio-Rad) as previously described (4). The blots were soaked for 15 min in 10 mM Tris-HCl-150 mM NaCl (pH 7.5) (TNa) and then were soaked in 5% nonfat dry milk in 20 mM Tris-HCl (pH 7.5)-500 mM NaCl (TBS) for 1 h at room temperature to saturate additional protein-binding sites. They were then incubated for 2 h with antiserum appropriately diluted in TNa at the concentrations and with the species indicated in the legends to the figures. The sheets were washed in TNa (for three 10-min periods) and incubated with horseradish peroxidase-conjugated goat immunoglobulin G (IgG) anti-human IgG preparations (Bio-Rad Laboratories) at a 1/1,500 dilution in TNa. The blots were incubated for 2 h at room temperature and washed in TNa (four 10-min periods). For the detection of antibody-antigen binding, the blots were soaked in a solution of 0.5 mg of 4-chloronaphthol (Sigma) per ml of 0.025% H_2O_2 in 50 mM Tris-HCl (pH 7.7)-150 mM NaCl. This was prepared freshly from stock solutions of 3 mg of 4-chloronaphthol per ml in methanol. After color development, usually 5 to 10 min, the reaction was stopped by washing with water. The blots were dried between filter paper, photographed, and stored protected from light.

Immunoaffinity purification of monospecific antibodies. A 1:1,000 dilution of the anti-ASPND1 serum was incubated with a piece of PVDF containing 2 mg of electroblotted ASPND1 protein. Immunoaffinity purification was carried out as described by Sambrook et al. (35).

Human serum samples. A total of 89 human serum samples was provided by microbiology laboratories of different Spanish hospitals. Twenty-one of these serum samples were from aspergilloma patients. Aspergilloma was demonstrated

by chest X-ray changes showing a discrete lesion with a halo and positive cultures for *A. fumigatus* obtained repeatedly either from sputum cultures or by bronchoscopy. The serologic response against *Aspergillus* antigens was demonstrated by immunodiffusion tests with commercial antigens, showing at least three precipitating arcs against *A. fumigatus* mycelial or culture filtrate antigens. Sixty-eight serum samples were used as controls: 49 were from healthy individuals (blood donor bank), 4 were from patients with fungal sepsis due to *Candida albicans*, 3 were from patients with bacteremia, 8 were from patients with pulmonary tuberculosis, 2 were from patients with leukemia, 1 was from a patient with positive C-reactive protein, and 1 was from a patient with positive rheumatoid factor. No antibodies specific for *Aspergillus* antigens were detected in these sera with the same immunodiffusion tests described above.

ASPND1 apparent molecular mass determination. The nondenatured apparent molecular mass of ASPND1 was estimated by the polyacrylamide gel method of Hedrick and Smith (11). Polyacrylamide gels were prepared as described above with resolving gels of 4, 6, 8, and 10% acrylamide and a stacking gel of 3.5% acrylamide. The corresponding ASPND1 electrophoretic mobility (R_f) values were calculated and plotted (on a semilogarithmic scale) against acrylamide concentrations. The slope of a best-fit line was determined for these points by least-squares regression ($R = 0.98$), and this slope was used to estimate molecular mass from a standard curve generated from commercially available protein markers (Sigma).

Nucleic acid manipulations. Normal DNA manipulations were carried out as indicated by Sambrook et al. (35). Plasmid DNA was extracted from *E. coli* DH5 α according to the instructions of the Wizard DNA Purification System (Promega). Restriction enzymes and T4 DNA ligase were obtained from Boehringer and used under the recommended conditions.

Restriction fragments of genomic DNA carrying the *aspid1* gene were subcloned into pBluescript SK+ and KS+ vectors (Stratagene). Single-stranded DNAs from overlapping deletions generated according to a double-stranded nested deletion kit (Pharmacia Biotech) were sequenced by the dideoxy chain termination method (36) with the Pharmacia T7 polymerase sequencing kit according to the supplier's instructions, with ^{35}S -dATP (Amersham) as labelled deoxynucleoside triphosphate.

Construction and screening of the cDNA expression library. Total fungal RNA was isolated from *A. nidulans* mycelia grown in CDA liquid culture for 24 h at 37°C . Hyphae were harvested and pulverized to a fine powder in the presence of liquid nitrogen in a Waring blender. Total RNA was isolated from the mycelia by the hot phenol purification method (42). Poly(A) $^+$ RNA was isolated from the mycelia with the mRNA Separator kit (Clontech). Double-stranded cDNA was synthesized from 5 μg of poly(A) $^+$ RNA with the cDNA Synthesis System Plus kit (code RPN 1256 Y/Z; Amersham). After addition of *Eco*RI adapters according to the cDNA Rapid Adapter Ligation Module (code RPN 1712; Amersham), the cDNA was ligated with λ gt11 arms, digested with *Eco*RI, dephosphorylated (cDNA Rapid Cloning Module- λ gt11, code RPN 1714; Amersham), and packaged in vitro according to the manufacturer's instructions (λ -DNA in vitro Packaging Module, code RPN 1717; Amersham), yielding a library with a packaging efficiency of 4.6×10^6 PFU/ μg of cDNA. The immunological screening of the expression library was performed with the immunopurified monospecific anti-ASPND1 antibody as described in standard protocols (35). Positive plaques were identified and recovered from agar plates.

The positive phages were purified by the glycerol step gradient method as described by Sambrook et al. (35). DNA inserts were excised with *Eco*RI and subcloned into pBluescript SK+ and KS+ by standard procedures (35).

Southern experiments and screening of the λ EMBL4 DNA genomic library. Genomic DNA was obtained by phenol extraction (33). Ten micrograms of DNA per lane was typically loaded for Southern blotting. The DNA was transferred to positively charged nylon membranes by standard protocols (35) and hybridized with an *aspid1* gene cDNA digoxigenin (DIG) labelled by random priming (Boehringer Mannheim kit and protocol). Hybridization was carried out at 65°C ; blots were washed under high-stringency conditions (65°C , $0.1 \times \text{SSC}$ [$1 \times \text{SSC}$ is 0.15 M NaCl plus 0.015 M sodium citrate] and 0.1% SDS) according to the recommendations of the membrane manufacturer (Boehringer) as described by Sambrook et al. (35).

A λ EMBL4 *A. nidulans* genomic library (provided by M. Peñalva) was screened by a standard protocol (35) to search for the *aspid1* gene. The corresponding DIG-labelled *aspid1* cDNA was used as a probe.

Nucleotide sequence accession number. The accession number for the *aspid1* gene sequence described in this paper is Z50175, as filed in the EMBL/GenBank/DBJ database.

RESULTS

Identification of *A. nidulans* antigens consistently recognized by serum samples from aspergilloma patients. As shown in our previous studies (4), which describe the effect of the culture media used on the presence of *Aspergillus* antigens specifically reactive to aspergilloma sera, CDA medium induced a strong biosynthesis of immunodominant antigens. The proteins present in total mycelial extracts from *A. nidulans*

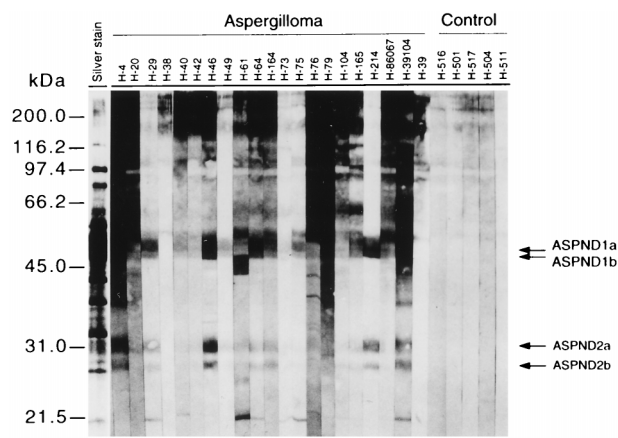


FIG. 1. SDS-PAGE (16% [wt/vol] polyacrylamide) of total mycelial proteins extracted from *A. nidulans*. Gels were either silver stained (15 μ g of protein per lane) or immunoblotted (60 μ g of protein per lane) with 21 serum samples from patients with pulmonary aspergilloma and 5 control serum samples as the first antibody (1:200 dilution).

grown at 37°C in CDA medium for 8 days were separated by SDS-PAGE (16% polyacrylamide) and silver stained or electrotransferred to nitrocellulose papers. As previously described (4), at least 30 polypeptide bands were detected after silver staining (Fig. 1, silver stain lane). Their apparent molecular masses ranged from 14 to 200 kDa, with those from 25 to 97 kDa being more abundant. To study which of these polypeptides were immunoreactive, blots were scissored into numerous strips, and each of these was reacted with a different human serum sample from a collection of sera containing 21 serum samples from aspergilloma patients and 68 control serum samples. Figure 1 shows the results obtained after developing the blots with peroxidase reagents. Two quite evident antigenic zones appeared with the aspergilloma sera. One of them (coinciding with a strongly silver-stained region) corresponded to an apparent molecular mass of 47 to 54 kDa and was designated ASPND1 (with at least two main components, ASPND1a and ASPND1b, and, depending on the preparations, a third one, ASPND1c). The other smaller double antigenic zone of around 28 to 31 kDa was designated ASPND2 (also with at least two components, ASPND2a and ASPND2b). None of these zones was reactive to any of the 68 control serum samples (only 5 representative serum samples are shown in the figure). Both zones appeared not as sharp bands but rather as broad zones and were thus considered as unique zones because of their simultaneous reactivity with the different sera. Only two aspergilloma serum samples (H-38 and H-39) did not react at all to the ASPND zones. A further two serum samples (H-49 and H-73) gave only a slight reaction, barely visible in Fig. 1. The relatively high dilution of the sera (1/200) could be responsible for diminished reactivity in cases of milder *Aspergillus* infections.

The high-molecular-mass zone also contained highly reactive antigens, although many of them were also reactive to control sera (not shown in the figure) or were very complex membrane-associated polypeptides difficult to isolate, characterize, and manipulate for routine use.

Since the ASPND1 zone was one of the most consistently reactive zones, and since, due to its apparent molecular mass and hydrosolubility, it was easier to manipulate and purify, we concentrated our efforts on the characterization of this antigen.

Determination of the N-terminal amino acid sequence of ASPND1 and generation of specific antibodies. The upper, most abundant component of the ASPND1 antigen (ASPND1a) was purified by electroelution from preparative SDS-PAGE separations. Water-soluble fractions of *A. nidulans* cells grown in CDA medium for 8 days at 37°C, instead of total mycelial extracts, were used to enrich the preparations in ASPND1. Figure 2 shows the presence of a single 49.2-kDa band in the electroeluted preparation (lane E) compared with the complex pattern of the original water-soluble fraction (lane T). Only a thin upper portion of the ASPND1-stained zone was cut out from the gel for electroelution. Two-dimensional electrophoretic analysis demonstrated (data not shown) that ASPND1 was the only polypeptide present in the purified band, thus ruling out the possibility of contamination with other polypeptides.

Electroeluted ASPND1 was blotted onto PVDF membrane and subjected to N-terminal sequencing by automated Edman degradation, revealing a sequence of 14 amino acid (aa) residues (with at least 75% yield per cycle) L-P-T-Q-Q-T-P-L-P-L-E-D-P-I.

Electroeluted ASPND1 was also used to immunize two New Zealand White rabbits. The specific antibodies in sera were diluted 1:1,000 for Western blot analysis of the *A. nidulans* water-soluble fraction. After blot development with horseradish peroxidase reagents, only one complex-stained zone was obtained (Fig. 2, lane W); this corresponded to the same ASPND1 zone reactive to the aspergilloma sera (Fig. 1). Anti-ASPND1 specific IgGs were purified from rabbit sera with pure ASPND1 antigen bound to PVDF paper as an affinity matrix, as indicated in Materials and Methods.

The ASPND1 antigenic zone contains at least three differently N-glycosylated forms of the same polypeptide core. The heterogeneous electrophoretic behavior of the ASPND1 antigenic zone was reminiscent of that of some previously characterized *A. fumigatus* glycoprotein antigens. This observation suggested that that zone was probably composed of several reactive glycopeptide bands. To confirm this hypothesis, we carefully excised the ASPND1 zone from Coomassie blue-stained gels into three different horizontal pieces (ASPND1a, ASPND1b, and ASPND1c) and extracted the polypeptide content of each piece by electroelution. The polypeptides thus separated were subjected to digestion with progressively higher amounts of End-H to analyze the existence and number of N-linked sugar chains. Digestion products were separated by SDS-PAGE (10% polyacrylamide) and either silver-stained or electroblotted and probed with anti-ASPND1 rabbit antibody

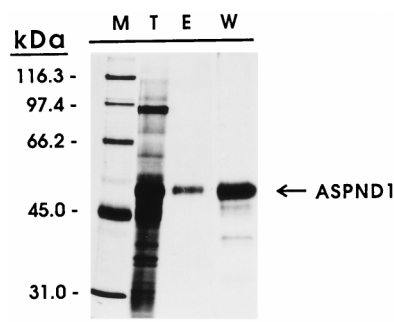


FIG. 2. SDS-PAGE (16% [wt/vol] polyacrylamide) of molecular mass markers (lane M), *A. nidulans* total mycelial extract (lanes T and W [10 μ g of protein]), and electroeluted ASPND1 (lane E [0.5 μ g of protein]). Mini-gels were either silver stained (lanes M, T, and E) or immunoblotted with anti-ASPND1 monospecific rabbit serum (lane W [1:1,000 dilution]).

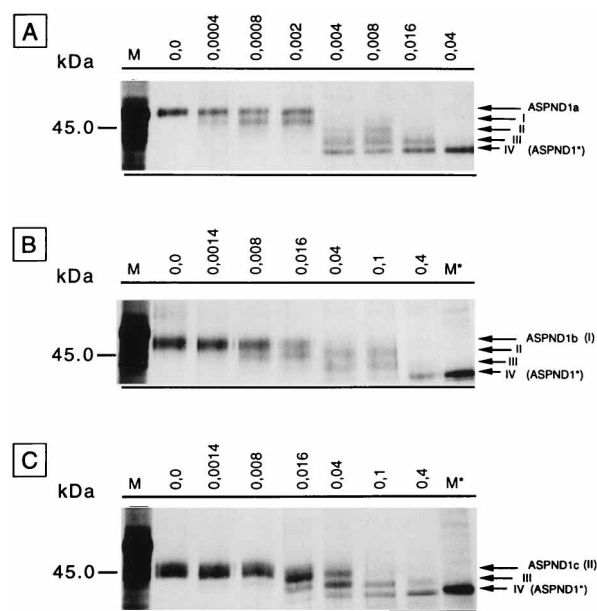


FIG. 3. SDS-PAGE (10% [wt/vol] polyacrylamide) of electroeluted ASPND1a (A), ASPND1b (B), and ASPND1c (C) antigenic components from *A. nidulans* after treatment with the indicated amounts (units per milligram of protein) of Endo-H. The original total mycelial extract (lane M) and the extract treated with Endo-H (lane M* [1 U per mg of protein]) were included as controls. Mini-gels were silver-stained. I, ASPND1a first deglycosylation product; II, ASPND1a second deglycosylation product; III, ASPND1a third deglycosylation product; IV, ASPND1* (ASPND1a fourth deglycosylation product).

or with several aspergilloma serum samples. Figure 3 shows the results obtained after silver staining of the digestion products. Four deglycosylated products—I (with identical electrophoretic mobility to ASPND1b), II, III, and IV—were observed for the ASPND1a band (Fig. 3A). Three deglycosylated products—II (with identical electrophoretic mobility to ASPND1c), III, and IV—were detected for the ASPND1b band (Fig. 3B), and two deglycosylated products—III and IV—were detected for the ASPND1c band (Fig. 3C). The apparent molecular mass of the final deglycosylation product (IV; from this point designated ASPND1*) was identical in the three cases (40.7 kDa). A complete Endo-H digestion of total mycelial extract (Fig. 3B and C, lanes M*) was included to demonstrate that no partial deglycosylation products remained after digestion with the highest Endo-H concentration. Immunoblots with aspergilloma serum samples or with anti-ASPND1 rabbit sera demonstrated that both the partially and completely deglycosylated products obtained in all three cases remained reactive to the specific IgGs (results not shown), suggesting that the epitopes recognized are located mainly in the polypeptide region of ASPND1 and not exclusively in the sugar moieties.

It may be concluded that ASPND1 is a polypeptide with at least four N-linked sugar moieties.

Monospecific antibodies help to establish a close relationship between *A. nidulans* and *A. fumigatus* immunodominant antigens. After screening the collection of sera from control or aspergilloma patients, we realized that all of the human sera from aspergilloma patients reactive to ASPND1 antigen (Fig. 4B, Aspergilloma panel, lane N) were also consistently reactive to the previously reported (23) antigenic CFC (composed of p90, p60, p40, and p37 antigens) from *A. fumigatus* (Fig. 4B, Aspergilloma panel, lane F). To ascertain whether this cross-

reactivity was due to the presence of some highly conserved epitopes in both types of antigens, we used the anti-ASPND1-specific antibody as a specific probe in Western blot experiments with *A. nidulans* and *A. fumigatus* water-soluble proteins. The anti-ASPND1 antibody recognized the above-described broad band of around 49.2 kDa corresponding to the ASPND1 antigen in the case of *A. nidulans* water-soluble extracts (Fig. 4B, anti-ASPND1 panel, lane N); the typical p60, p40, and p37 antigenic CFC pattern (with the exception of the p90 catalase band) was recognized in the corresponding *A. fumigatus* extracts (Fig. 4B, anti-ASPND1 panel, lane F). The strongly stained band observed with the anti-ASPND1 antibody towards the lower part of lane N was observed only in some preparations and probably corresponded to a partially degraded form of ASPND1 not recognized by the pool of aspergilloma sera.

When parallel blots were developed with an antibody specifically directed against deglycosylated p40 from *A. fumigatus* (anti-AF p40*), identical results were obtained, although with a less intense reaction (Fig. 4B, anti-AF p40* panel, lanes N and F). These results strongly suggest that all of these antigens share common epitopes. Further evidence in support of this idea came from blocking experiments exploiting the different sources of specific antibodies. Blots (containing *A. nidulans* and *A. fumigatus* water-soluble fraction proteins) identical to those just described were blocked with anti-ASPND1, with anti-AF p40* rabbit serum, or with an aspergilloma cocktail serum (Fig. 4C, BLOCKING row). After preincubation, the blots were probed (as indicated in Fig. 4C, INCUBATION row) with an aspergilloma cocktail serum, with anti-ASPND1, or with anti-AF p40* sera. The blots were developed using the appropriate horseradish peroxidase-conjugated anti-human or anti-rabbit IgG. As shown in Fig. 4C (Anti-ASPND1/Aspergilloma and Anti-AF p40*/Aspergilloma panels, lanes N and F), both rabbit sera blocked part of the epitopes present in *A.*

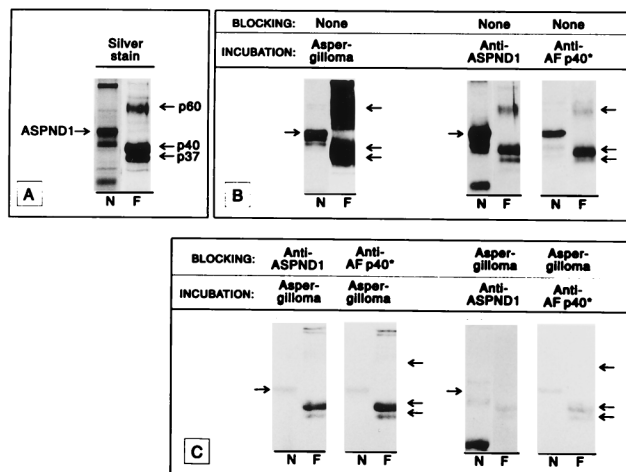


FIG. 4. SDS-PAGE (16% [wt/vol] polyacrylamide) of *A. nidulans* (lanes N) and *A. fumigatus* (lanes F) water-soluble extracts. Mini-gels were either silver stained (A [4 μ g of protein per lane]) or electrotransferred to nitrocellulose paper (B and C [20 μ g of protein per lane]). (B) Blots were probed with the following sera: a pool of 10 different aspergilloma serum samples at a 1/250 dilution (Aspergilloma), a monospecific anti-ASPND1 rabbit serum at a 1/1,000 dilution (Anti-ASPND1), and a monospecific rabbit serum specific for fully deglycosylated *A. fumigatus* p40 at a 1/400 dilution (Anti-AF p40*). (C) Blots were probed as in panel B after a previous 2-h blocking step (as indicated) with the anti-ASPND1 or anti-AF p40* sera at a 1/200 dilution or with the pool of aspergilloma sera at a 1/250 dilution. Arrows indicate the position of the ASPND1 antigen from *A. nidulans* and of the antigenic components p60, p40, and p37 from *A. fumigatus*.

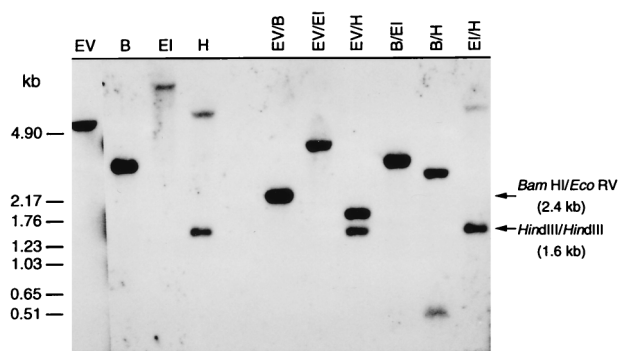


FIG. 5. Southern hybridization of *A. nidulans* genomic DNA (10 μ g per lane) digested with restriction enzymes *EcoRV* (lane EV), *Bam*HI (lane B), *Eco*RI (lane EI), and *Hind*III (lane H) or combinations of *EcoRV*/*Bam*HI (lane EV/B), *EcoRV*/*Eco*RI (lane EV/EI), *Bam*HI/*Eco*RI (lane B/EI), *Bam*HI/*Hind*III (lane B/H) and *Eco*RI/*Hind*III (lane EI/H). DIG-labelled *A. nidulans* ASPND1 cDNA was used as a probe. Experimental details are provided in the text.

nidulans and *A. fumigatus* antigens reactive to human aspergilloma serum (compare to Fig. 4B, Aspergilloma panel). Similarly (Fig. 4C, Aspergilloma/Anti-ASPND1 and Aspergilloma/Anti-AF p40* panels, lanes N and F), the human aspergilloma cocktail serum blocked part of the epitopes reactive to both types of rabbit serum (compare to Fig. 4B, anti-ASPND1 and anti-AF p40 panels). In all cases, reactivity diminished noticeably after preincubation with the heterologous antibodies, reinforcing the notion of the existence of common epitopes among all of these antigens.

More direct demonstration of this hypothesis could only be provided by comparison of the amino acid sequences of these proteins, and we therefore decided to clone the corresponding coding genes.

Cloning of the *aspn1* gene. Affinity-purified anti-ASPND1 antibodies were employed for identification of the *aspn1*-specific cDNA sequences contained within a λ gt11 expression library, and six positive clones (F1 to F6) were identified by immunoblotting with the purified antibody. Sequencing of the six cDNA clones demonstrated that all of them came from the same mRNA. Differences in size were due to the presence of different 5' ends, probably caused by arrest of the reverse transcriptase at different positions in the mRNA template. The longest (1-kb) clone (F3) contained a nearly full-length cDNA fragment (lacking only the first 43 nucleotides of the coding sequence). Nucleotide sequence analysis of this clone confirmed that it encoded the ASPND1 antigen, since the first 14 residues of the predicted amino acid sequence of the translation product coincided exactly with the corresponding residues of the previously determined N-terminal amino acid sequence of purified ASPND1. The cDNA clones F1, F2, F4, and F6 were shorter and lacked the 5' coding region corresponding to the predicted amino acid sequence; however, the expressed protein was still recognized by the polyclonal anti-ASPND1 rabbit serum. The cDNA segment of clone F3 was used as a probe to screen an *A. nidulans* λ EMBL4 genomic DNA library. Screening of 4×10^4 colonies yielded 4 positive clones designated G1, G2, G3, and G4. Restriction analysis of the isolated phage DNAs with *EcoRV*, *Bam*HI, *Eco*RI, and *Hind*III or *Bam*HI/*EcoRV* double digestion revealed that G1, G2, and G3 carried common fragments. All of these fragments hybridized with the cDNA probe in Southern blot analysis (results not shown). Also, exactly the same hybridization bands were observed in a Southern blot of *A. nidulans* genomic DNA (Fig. 5), strongly suggesting that the fragment would represent the au-

thentic ASPND1 antigen gene. Consistent with the presence of a single copy of the gene, only one hybridization band was detected with all the restriction enzymes that had no cleavage sites within the gene except *Hind*III, which provided two different fragments (Fig. 5, lane H). Since no *Hind*III target was present in the cDNA sequence, the only explanation for this result was that the *Hind*III-specific sequence would be located in an intron (see below). The G4 clone contained only one part of the gene.

The *Bam*HI/*EcoRV* 2.4-kb fragment (the shortest one putatively containing the *aspn1* gene) from clone G1 was subcloned into pBluescript SK+ and KS+ and sequenced. The sequenced *Bam*HI/*EcoRV* fragment had 2,405 bp and contained the whole coding sequence but only 36 bp of the 5' end. The sequence of 435 bp upstream of the ATG was obtained after subcloning of the 1.6-kb *Hind*III/*Hind*III fragment hybridizing with the cDNA probe in pBluescript SK+. (A 16-mer synthetic oligonucleotide was designed complementary to bases -14 to -29 [both included] from the ATG.) For brevity's sake, only the relevant part of the sequence is shown here (Fig. 6).

Characterization of the *aspn1* gene. The nucleotide sequence of the *aspn1* gene is shown in Fig. 6. A single open reading frame was found, encoding a 277-aa protein. The predicted molecular mass of the encoded ASPND1 antigen is 30.6 kDa. The N-terminal amino acid sequence of the purified ASPND1 antigen, previously determined by protein sequencing, was located within the open reading frame starting at Leu-17 and perfectly matching the amino acid sequence deduced from the nucleotide sequence of the gene (Fig. 6, underlined). This result suggests that the ASPND1 antigen is first translated as a precursor form consisting of 277 aa. At the N-terminal region, a series of uncharged amino acid residues with a high content of hydrophobic amino acids was found. Analysis of the hydropathy profile of the protein suggested that this region would correspond to a signal peptide of 16 aa preceding the mature product. If no additional processing occurs, the mature antigen would be composed of 261 aa, with a calculated molecular mass of 29.0 kDa and a pI of 4.24. There are four potential N-linked glycosylation sites (Asn-X-Ser/Thr [8]) in the deduced amino acid sequence of ASPND1 (double underlined in Fig. 6). All four of these sites were in fact glycosylated, as demonstrated by the detection of four different deglycosylated products in controlled deglycosylation experiments with increasing concentrations of Endo-H (Fig. 3).

Comparison of the cDNA and genomic sequences revealed that the *aspn1* coding region is interrupted by two introns of 48 and 49 bp, respectively (Fig. 6, lowercase). Both introns have splice donor, acceptor, and lariat branch acceptor sites (Fig. 6, lowercase and boldface) that show homology to the consensus fungal splice signals (9), and the first intron contains the predicted *Hind*III site. The small size of the introns is also consistent with that of the introns sequenced from other filamentous fungi (9). The 5' untranslated region of *aspn1* does not contain any perfect consensus TATA or CCAAT boxes, but an AT-rich region (ATAAATA) and a CCAAG sequence are located 86 and 148 bp, respectively, upstream of the putative translation start site (Fig. 6, boldface). These locations are in agreement with those described for certain genes from other *Aspergillus* spp. (5, 38).

At least five possible PacC binding (boldface underlined) sites containing the consensus sequence 5'-GCCARG-3' (41) are contained within the 5' noncoding region, suggesting the existence of a possible regulatory effect of ambient pH on the expression of the *aspn1* gene.

1 CACAGAAGAATCTTCCCACGCATCATCGGTGAGTGTTCAGTTTCCATCCTGCTAACCAAGGTACACACGCCAAGCGTTCTGCCAAGGGACTTGATT
 101 GTGCCAAGTGCCACAGCAGGGCGCGACAGATCTGACCTCTGACAGTGGAGAAATGCCCGGGCAGTTTCTTTCTTGCAAGTTAGCCCGTCGTCCTCA
 201 GGGTCTCTGTGGCTTGGCTACTTGGCAAGACGTGGGATGCAAAGTGATGCCATACGCAAGGCATCAGAAGATACGAGGGACCAAGGATTCATCTC
 301 TTGGGGATCCCAAGGGTACTCGAGAGTTGAGGAAGAGGAAAGGCATAAATACCGTCAGCATGGCGGCCCTGCATGTCCCTGCAGATTGACAGCGGTGAGC
 BamHI
 401 TTGAAACCCAGAGCAGACGCAGCTTCAGCTTTCAATATGACAGCTCCTCGCTCTCACTTTGGCCCTCTGCGCCTCAATCGCGCTCTTCCCACTCAGCAA
 1 M Q L L A L T A L L C A S I A A L P T Q Q T
 501 CCCCCTTGCCGTGGAGGACCCGATCAAGTCTCCTTTCCCATCCACCAGCTGCAATGCCACCGAACAGCGCCAGCTAGCCACCGCTCTCCAGGAGAC
 23 P L P L E D P I K S P F P I H H S C N A T E Q R Q L A T A L Q E T
 601 CGTGACCTTGTGTAGCAGCGCCAAAGACCATATCCTCCGTGGGGCAATGAGAGCGCCATCTATCGCAAGTACTTTGGCGACAGGCCCTCTTAACCGCC
 56 V T L A E H A K D H I L R W G N E S A I Y R K Y F G D R P S L T A
 701 ATCGGAGCATACGACATCATCGTCAACGGAAACCCAGATAACATCCTCTTCCGCTGCGATAACCCGACGGCAACTGTGCTCTTGGAGgttaagcttagag
 89 I G A Y D I I V N G N P D N I L F R C D N P D G N C A L E G..HindIII...
 801 acccctgtctgtgtgtgttcgaagctaaccattcacagGCTGGGGTGGCCATTGGCGCGCGAGAACGCCAGCGATGAGACTGTCTATCTGCGAACTCAGCTA
 119 Intron 1..... W G G H W R G E N A S D E T V I C E L S Y
 901 CACCACTCGTCGGTCCCTGAGCACAATGTGCTCGCAGGGATACACCATCTCTGAGTGGGAGACGAACACCTTCTGGGCTGGTGAATTGCTGCACCGACTG
 140 T T R R S L S T M C S Q G Y T I S E W E T N T F W P G D L L H R L
 1001 TACCACATGCCTGCTATAGGACAGGGCTTGGTTGAGCACTACCGGATGGATACGAGGGCGTTTGGAGCTGGCGGAGGGGAACCGGACAGAAGCTGTGC
 173 Y H M P A I G Q G L V E H Y A D G Y E G V L E L A E G N R T E A V H
 1101 ATGACTCGGAGACCTTTCAGTACTTTGCGCTTGAGGTGTATGCATATGATGTTGCGGTCCAGGTATTTGGGTGTGTTGGTGGAGAGGAAGAGAATGATGG
 207 D S E T L Q Y F A L E V Y A Y D V A V P G I G C V G G E E E N D G
 1201 CCAGGTTGAAGAACAGACTGAGGAGCCTGCTCAGGATGATCAGCAGGATGAGGCGGCTGAGGAGGAGATTCTTGAGgtgagcgaatgtgctgtttggtga
 240 Q G E E Q T E E P A Q D D Q Q D E A A E E E I P E
 1301 cagctctggagctaacaacccttagAATTGCCATACTCATGAAGGTGGAGAGCTGCACTGCACCTAAGTTATTCACCTTCAGACGATTCGTGCCATGACA
 265 Intron 2.....N C H T H E G G E L H C T *
 1401 CATTACCTATTTCTGCGACTGCTCCCGTCTTCACATCCCTTGAGAAAGAGAAAAGAACTTTATACGATCTATGATGCAGACTTGGATGACCACCTCGTT
 1501 TAACCCACCTATAATGCATTAAATATATGTCTATCAGTCATTTCTTATATCAATAATCACGCAGAAGCACAAATGCCCTCCATCGACCTCAGATCCGCCC

FIG. 6. Nucleotide sequence of the *A. nidulans aspdn1* gene and flanking regions. In the nontranslated 5' region, five possible PacC binding sites containing the GCCARG consensus in either orientation are marked in boldface and underlined, and sequences resembling CCAAT and TATA boxes are in boldface. In the coding region, the two introns derived from cDNA sequencing are indicated by lowercase letters, and their consensus splice signals are in boldface. In the 3' noncoding region, polyadenylation signals are shown in boldface, and the arrow indicates the precise site of polyadenylation (C). The predicted amino acid sequence of the largest ORF is shown in the one-letter amino acid code. Italics denote a potential signal peptide. The N-terminal sequence found by analysis of native ASPND1 protein is underlined. The four putative N-glycosylation sites are double underlined, and the asterisk denotes the stop codon.

The conserved environment (A and C at positions -3 and -4, respectively) of the initiation codon of *Aspergillus* spp. (9) was also found.

Determination of ASPND1 apparent molecular mass under native conditions. The SDS-PAGE-determined molecular mass of the totally deglycosylated ASPND1 was 40.7 kDa (Fig. 3). Consequently, a considerable difference (11.7 kDa) exists between the molecular mass deduced from the sequence and that determined experimentally. This difference probably could be due to the interference of the strong electric charge of the C-terminal region of the ASPND1 protein with quantitative SDS binding. There are several pieces of evidence pointing to the abnormal behavior of ASPND1 in SDS-PAGE. When the boiling step was omitted in the process of sample preparation (Fig. 7, Silver stain panel, lane +DTT/nb), the silver-stained pattern of the *A. nidulans* water-soluble proteins changed dramatically with respect to that of the normal boiled sample (Fig. 7, lane +DTT/b). When the gels were immunoblotted with anti-ASPND1 antibodies (anti-ASPND1 panel), the ASPND1 antigen showed a spectacular shift in the apparent molecular mass from the previous 49.2 kDa (lane +DTT/b) to the region of around 31 kDa (lane +DTT/nb). The opposite and more normal effect was observed for a high-molecular-mass band containing one of the catalases of *A. nidulans* (CatB, silver stain panel). CatB is a protein composed of subunits which separate upon heating at 100°C and constitute an internal control of denaturing. A different molecular mass shift was

observed when the reducing agent (DTT) was omitted from the sample buffer: surprisingly, the ASPND1 antigen (Fig. 7, anti-ASPND1 panel) moved faster in the absence of DTT (independently of the presence or not of a boiling step), to-

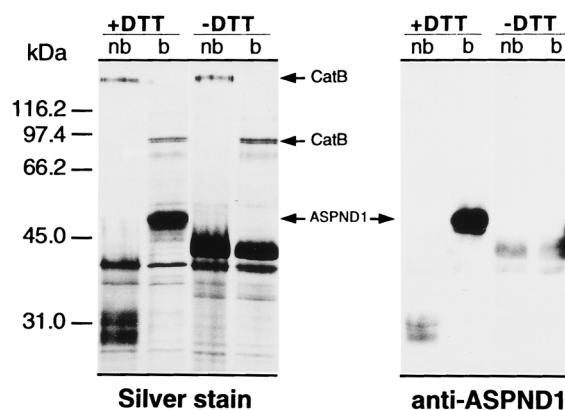


FIG. 7. SDS-PAGE (16% [wt/vol] polyacrylamide) of *A. nidulans* water-soluble proteins obtained under nondenaturing conditions and electrophoresed in the absence or presence of 5 mM DTT, including (b) or not including (nb) a 5-min boiling step in the process of sample preparation. Mini-gels were either silver stained (4 µg of protein per lane) or immunoblotted with anti-ASPND1 serum at a 1/1,000 dilution (20 µg of protein per lane). CatB, *A. nidulans* catalase B.

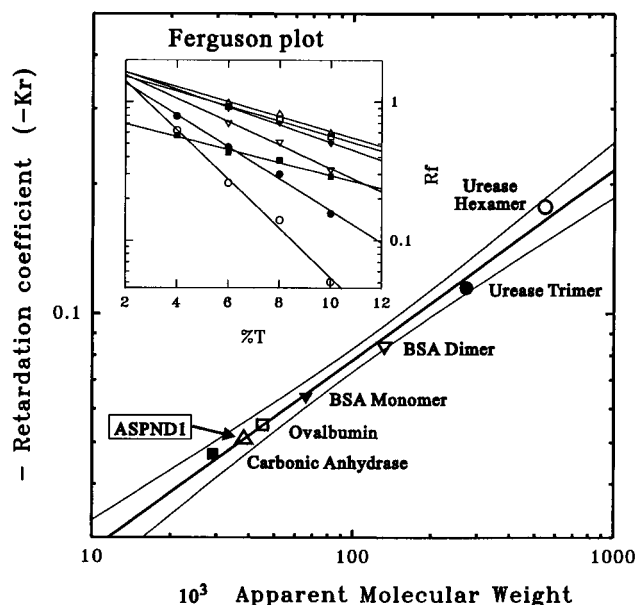


FIG. 8. Determination of the apparent molecular weight of the *A. nidulans* ASPND1 antigen. ASPND1 and molecular weight markers were electrophoresed, under native conditions, on a set of mini-gels containing 4, 6, 8, and 10% polyacrylamide concentrations. In each gel, the electrophoretic mobility of each protein relative to the tracking dye (R_f) was calculated and plotted on a semi-logarithmic scale against the percent gel concentration (Ferguson plots). The slopes for such plots are the retardation coefficients (K_r s) for ASPND1 and standard proteins. A standard curve was obtained by plotting the minus K_r value against the apparent molecular weight of the standards (double logarithmic scale). The ASPND1 apparent molecular weight was calculated from this standard curve after its corresponding K_r had been determined. The protein standards (molecular weight in parentheses) were carbonic anhydrase (29,000), chicken egg albumin (45,000), bovine serum albumin monomer (66,000), bovine serum albumin dimer (132,000), jack bean urease trimer (272,000), and jack bean urease hexamer (545,000).

wards the 42-kDa-molecular-mass region (lanes -DTT/nb and -DTT/b). The CatB protein, which does not contain disulfide bridges, was not affected by the presence of DTT. In all cases, the reactivity of anti-ASPND1 serum with ASPND1 was stronger with the totally denatured protein. These results indicated that SDS-PAGE was not the method of choice for reliable determination of the apparent molecular mass of the ASPND1 antigen.

To avoid the problems associated with the proposed abnormal SDS binding, we determined the apparent molecular mass of ASPND1 under native conditions. *A. nidulans* water-soluble fraction proteins and molecular mass standards were separated on native gels containing four different percentages of acrylamide (4, 6, 8, and 10%). Gels were silver stained or electrotransferred to nitrocellulose and probed with anti-ASPND1 and anti-AF p40* rabbit sera. In all of the gels, the reactivity with both antibodies was restricted to a zone corresponding in electrophoretic mobility and protein content to a heavily stained band detected by silver staining. The data from 4, 6, 8, and 10% acrylamide gels (Fig. 8) were used to calculate (according to the method of Hedrick and Smith [11]) a native molecular mass of around 38.2 kDa for the band reactive to the specific antibodies. This value was very close to the 37.5-kDa molecular mass predicted for ASPND1 from the sequence analysis (29.0 kDa) plus the corresponding mass of the sugar moiety (8.5 kDa).

Similarity of ASPND1 to other proteins. A search of non-redundant protein databases with the BLAST algorithm (1)

<i>Asp f</i> II	001	-----SARDEA
ASPND1	001	MQLLALTALCASIAALPTQQTPLPLEDP-----IKSPFFIHHSNAT
<i>Asp f</i> II	007	GLNEAVELARHAKAHILRWGNESEIYRKYPGNRPTMEAVGAYDIVVNGDK
ASPND1	051	ALQETVTLAERHAKDHILRWGNESEIYRKYPGNRPTSLTAIGAYDIVVNGDK
<i>Asp f</i> II	057	ANVLFRCDNPDGNCALGEGWGHWRGNATSETVICDRSYTTRRLVSMCS
ASPND1	101	DNILFRCDNPDGNCALGEGWGHWRGNATSETVICELSYTTRRLVSMCS
<i>Asp f</i> II	107	QGYTVAGSETNTFWASDLHRLHYHVPVAVGQWVDHFDAGYDEVIALAKSN
ASPND1	151	QGYTISEWETNTFWAGDLLHRLHYHMPAIGQGLVEHYADGYEGVLELAEGN
<i>Asp f</i> II	157	GTESTHDEAFEFYFALEAYAFDIAAPGVGCAGESHGPDQGHDTGSASAPA
ASPND1	201	RTEAVHDSSETLQYFALEAYAVDVAVPGVGVGEEEND-GQGEETREPA
<i>Asp f</i> II	207	STSTSSSSSGSGGATTPTDPSATIDVPSNCHTHEGGQLHCT
ASPND1	250	QDDQQ-----DE-AAEEIIPENCHTHEGGELHCT

FIG. 9. Comparison of *A. nidulans* ASPND1 antigen with *A. fumigatus* AspII allergen (EMBL/GenBank U20722 [2]). Sequences were aligned by the CLUSTAL program (12). Asterisks represent identical or conserved amino acids. Conserved cysteines and putative glycosylation sequences are underlined.

indicated a high degree of sequence similarity to AspII, a recently described allergen of *A. fumigatus* (2). Figure 9 shows a CLUSTAL (12) alignment of both proteins. The similarity of ASPND1 (277 aa) to AspII (250 aa) is 53.1% (147 of 277 aa), and it is 65% when amino acid substitutions are included (180 of 277 aa). The highest similarity is concentrated between ASPND1 aa 52 to 232 and 265 to 277. When these two zones are considered together apart from the rest of the molecule, the similarity reaches 72.2%, or 85.6% when amino acid substitutions are included. At least three of the four potential N-glycosylation sites and seven of the eight cysteine residues present in ASPND1 are conserved in AspII (Fig. 9). Another protein (299 aa) from *C. albicans* cell walls (37) has a more restricted similarity to ASPND1. In this case, the similarity is 48% (133 of 277 aa), and it is 63.2% when amino acid substitutions are included. The most conserved zones are between ASPND1 aa 34 and 232 and 265 and 276. When these two zones are considered together apart from the rest of the molecule, the similarity reaches 57.3%, and it reaches 71.6% when amino acid substitutions are included. Four potential N-glycosylation sites and all eight cysteine residues present in ASPND1 are conserved in *C. albicans* protein. When the three proteins were considered simultaneously, an identity of 31.4% (87 of 277 aa) or a similarity of 48% (133 of 277 aa), including amino acid substitutions, were found. The cysteine residues, potential glycosylation sites, and the aforementioned blocks are perfectly conserved in all three of the proteins. When we analyzed whether the published N-terminal amino acid sequence of a gp55 *A. fumigatus* antigen (40) was similar to that of any of the aforementioned antigens of different origins, we found that we could align the gp55 20-aa stretch with the N terminus of the ASPND1 mature protein and also with that of the *C. albicans* antigen (Fig. 10). These results suggest the existence of some degree of relatedness among all of these antigens and should help to simplify the com-

ASPND1	MQLLALTALCASIAALPTQQTPLPLEDP-----IKSPFFIHHSNAT
pCalb.	MNYLLFCLFFAFSV-AAPVTVTRFVNASPTGYDWRADWVGKFPIDLSCNAT
gp55	ATP-----HEPVFFSWDAGAVTSFP

FIG. 10. Comparison of the N-terminal sequences of *A. nidulans* ASPND1 antigen, *C. albicans* protein (pCalb.) (37), and *A. fumigatus* gp55 (40) allergen. Sequences were aligned by the CLUSTAL program (12). Asterisks represent identical or conserved amino acids.

plex array of similar *Aspergillus* antigens reported in recent years.

DISCUSSION

The fully glycosylated ASPND1 immunodominant antigen purified from *A. nidulans* displays an apparent molecular mass of 49.2 kDa by SDS-PAGE, while the calculated molecular mass for the mature protein from the deduced amino acid sequence is 37.5 kDa (29.0 kDa of the peptide part plus 8.5 kDa of the four N-linked sugar chains). Such a large difference raises concern that the cloned gene might not encode the purified protein. However, the difference (which does not depend on the presence of glycosylation) is also seen with other proteins, as in the cases of several serine proteinases from *Aspergillus* species (14, 15, 34, 39), although the reason is not clear. The same situation was found for the ASPND1-like *C. albicans* cell wall antigen (37). In the case of the *Saccharomyces cerevisiae* KEX2 gene product (a Ca^{2+} -dependent serine protease) (7), progressive deletion experiments have demonstrated that the high negative charge (-15) of the carboxy terminus of the protein contributes decisively to a similar discrepancy between the SDS-PAGE-determined molecular mass and the actual molecular mass of the native Kex2 protein. Something similar could account for the consistently confirmed anomalous behavior of ASPND1 in SDS-PAGE. Proteins can be examined by electrophoresis in nondenaturing systems (in the absence of SDS and reducing agents) to determine the molecular masses of native molecules, thus avoiding the problems of nonquantitative SDS binding (11). The ASPND1 molecular mass determined with a nondenaturing system was 38.2 kDa, in fact very close to the predicted 37.5 kDa.

On the other hand, after immunological screening of the cDNA library, we found that the six different proteins expressed by the six independently isolated overlapping *A. nidulans* cDNA clones (F1 to F6, with different 5' ends and consequently expressing proteins with different N termini) were recognized by anti-ASPND1 antibodies. This observation rules out the possibility that the antibody to the purified protein used to clone the cDNA of ASPND1 might recognize the N-terminal ends of both the purified protein and a hypothetical different protein encoded by the *aspn1* gene. All of these data confirm that the *A. nidulans aspn1* gene really does encode the purified ASPND1 antigen.

Since 1983, several apparently different (but with some coinciding properties) *A. fumigatus* antigens with IgG and/or IgE binding capacities have been reported. Some of them were almost crude fractions, such as that designated as CS2 (30), or CFC (23). Others were partially purified preparations, such as glycoprotein antigens from culture filtrates (17) or from mycelium (18). The rest were pure antigens, such as the 58-kDa antigen (6), the gp55 allergen (40), and the AspII allergen (2). In some of our earlier papers, we suggested the possibility that some of these antigens share common epitopes (23, 24) and highlighted the usefulness of *A. nidulans* as an alternative source of molecules of interest with which to study the development of the different forms of aspergillosis and their early diagnosis (4, 32). In the present work, we offer additional data to further support these ideas.

Chemical (BrCN) and enzymatic (trypsin or V8 digestions) peptide mapping has demonstrated that the p60, p40, and p37 components of the *A. fumigatus* CFC are differentially glycosylated forms of a common peptide core (28). Something similar happens in the case of *A. nidulans* with the differential glycosylation of ASPND1.

The antibodies raised against original or fully deglycosylated

ASPND1 recognize the p60, p40, and p37 components (and their deglycosylated products) of *A. fumigatus* CFC, and antibodies raised against p40 or its fully deglycosylated form are highly reactive against ASPND1 (with or without its sugar moiety). Furthermore, ASPND1 and the above-mentioned CFC components display identical patterns of reactivity against the same group of sera from aspergilloma patients or control individuals. It follows that all of these molecules must have common peptide epitopes responsible for their cross-reactivity.

Their similar immunochemical characteristics and their electrophoretic behavior in two-dimensional SDS-PAGE led us to consider that the p60, p40, and p37 components of the CFC might belong to the same family as the 53- and 41-kDa components of CS2 (23, 24). ASPND1 should be included in that family.

Now we have cloned and sequenced the *aspn1* gene; analysis of its primary structure has provided strong support for our previous hypothesis. The similarity between ASPND1 and AspII proteins demonstrates that they have a common origin, that they probably share several epitopes, and that they should elicit the formation of IgG or IgE antibodies able to recognize both antigens similarly. The immunochemical characteristics of AspII and its electrophoretic behavior in two-dimensional SDS-PAGE (2) are reminiscent of those of some of the CS2 (31) and CFC components (23) and of a partially purified *A. fumigatus* glycoprotein antigen (17). Consequently, we propose that all of these *A. fumigatus* antigens would have a common peptide part and that the slight differences observed in molecular mass, characteristics, or reactivity with aspergilloma or ABPA sera would be due to differential glycosylation or proteolytic processing. ASPND1 must therefore be a close relative of that family.

The fact that the N-terminal sequence of *A. fumigatus* gp55 antigen (40) could be aligned with the corresponding N terminus of the ASPND1 antigen or the *C. albicans* antigen suggests that AspII and gp55 may also be related. Unfortunately, we do not have the sequence of the AspII N terminus to confirm such a hypothesis.

The 58-kDa antigen of *A. fumigatus* (6) has an amino acid composition very similar to those of ASPND1 and AspII (deduced from the incomplete published sequence). Like gp55, the 58-kDa antigen is N glycosylated and phosphorylated and is therefore probably another member of the family.

Digestions with different endoglycosidases have shown that ASPND1, gp55, the 58-kDa antigen, and the p60, p40, and p37 components of the CFC are all N glycosylated (6, 23, 40). According to the immunochemical and sequencing data, there are four N-glycosidic residues in ASPND1 (and the same occurs in the *C. albicans* antigen). The published AspII sequence has three potential glycosylation sites in positions identical to those of the corresponding ones of ASPND1; the fourth one should be in the missing N-terminal sequence of AspII. p60 and p40 CFC components also have four N-linked sugar chains. With the exception of the 58-kDa antigen, the N-glycosidic moieties are not required for recognition by the different aspergilloma or ABPA serum samples tested (23, 40).

Finally, all the cysteines of the primary structure are perfectly conserved in very close positions in ASPND1, AspII, and the *C. albicans* antigen, suggesting a common tertiary structure and probably a similar function. Work is currently being carried out at our laboratory with the purpose of defining the biological role of these proteins in the fungal life cycle.

We believe that our interpretation offers a much simpler view of the former complex array of reported *Aspergillus*

antigens. We propose that several of the well-characterized antigens should be considered together, or in appropriate combinations, for the manufacture of defined reagents for a consistent diagnosis of the different forms of aspergillosis, mainly aspergilloma and ABPA. The proposed antigens, several of them already obtained in recombinant form, are CAT1 (22), AspII (2), AspI (20, 26, 27), and the superoxide dismutase antigen (10) from *A. fumigatus* and ASPND1 from *A. nidulans*. Because of the differences in molecular mass and their higher reactivity in denatured form, all of these antigens can readily be separated by SDS-PAGE, and standard Western blot assays would provide much information about the particular pathology analyzed.

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