

Humoral and Cell-mediated Autoimmune Reactions to Human Acidic Ribosomal P₂ Protein in Individuals Sensitized to *Aspergillus fumigatus* P₂ Protein

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Summary

A panel of cDNAs encoding allergenic proteins was isolated from an *Aspergillus fumigatus* cDNA library displayed on the surface of filamentous phage. Solid phase-immobilized serum immunoglobulin E (IgE) from *A. fumigatus*-allergic individuals was used to enrich phage displaying IgE-binding molecules. One of the cDNAs encoded a 11.1-kD protein that was identified as acidic ribosomal phosphoprotein type 2 (P₂ protein). The allergen, formally termed rAsp f 8, shares >62% sequence identity and >84% sequence homology to corresponding eukaryotic P₂ proteins, including human P₂ protein. The sequences encoding human and fungal P₂ protein were subcloned, expressed in *Escherichia coli* as His₆-tagged fusion proteins, and purified by Ni²⁺-chelate affinity chromatography. Both recombinant P₂ proteins were recognized by IgE antibodies from allergic individuals sensitized to the *A. fumigatus* P₂ protein and elicited strong type 1-specific skin reactions in these individuals. Moreover, human and fungal P₂ proteins induced proliferative responses in peripheral blood mononuclear cells of *A. fumigatus*-allergic subjects sensitized to the fungal P₂ protein. These data provide strong evidence for in vitro and in vivo humoral and cell-mediated autoreactivity to human P₂ protein in patients suffering from chronic *A. fumigatus* allergy.

Key words: phage display • cDNA libraries • IgE • allergens • autoimmunity

A *Aspergillus fumigatus*, a ubiquitous mold (1), is considered an opportunistic pathogen responsible for a vast variety of pulmonary complications in humans and animals. The spectrum of diseases associated with the fungus ranges from mild forms, like saprophytic colonization of the lung and allergy, to life-threatening diseases, such as invasive systemic aspergillosis or allergic bronchopulmonary aspergillosis (ABPA) (2). These different clinical presentations indicate that not only the virulence of the fungus itself, but also other underlying conditions, including impaired immune status, play a role in the development of opportunistic mycoses (3). Cloning and sequencing of allergen-encoding cDNAs from *A. fumigatus* (4–6) have permitted characterization of the biochemical function of some allergens by sequence comparison. The 18-kD protein Asp f 1, a member of the ribotoxin family (4, 7), represents a major allergen of the fungus (7, 8) and was found in the urine of patients with invasive aspergillosis (9). Serologic studies with rAsp f 1 and other *A. fumigatus* allergens (10) clearly demonstrated the existence of disease-specific allergens able to elicit IgE

responses exclusively in patients suffering from ABPA (10–12). Although the pathophysiologic mechanisms leading to *Aspergillus*-related pulmonary complications remain largely unknown, the availability of recombinant allergens from the fungus substantially contributed to improved diagnosis of the diseases (11–14). At the molecular level, the cloned allergens can be subdivided into two categories: secreted and cytoplasmatic proteins (10). Interestingly, at least one of the ABPA-specific allergens, manganese-dependent superoxide dismutase (MnSOD), a phylogenetically highly conserved protein (15), also shows cross-reactivity with human MnSOD (16). Phylogenetically conserved proteins are often involved in fungal allergy (17) and also have the potential to be involved in humoral and cell-mediated autoimmune reactions (15, 18). We have cloned a large panel of cDNAs encoding allergenic *A. fumigatus* proteins using phage surface display technology (5, 6, 19). Here, we describe the sequence and properties of one of these allergens identified as an *A. fumigatus* acidic ribosomal phosphoprotein type 2 (P₂ protein) by sequence homology. The acidic

ribosomal phosphoproteins P₀ (38 kD), P₁ (13 kD), and P₂ (13 kD), contained in the 60 S ribosomal subunit, are highly conserved among eukaryotes and are required for the functional activity of the ribosome (20). These proteins have been reported as antigens capable of inducing IgG antibody responses in systemic lupus erythematosus, the prototypic systemic autoimmune disease (21, 22). Additionally, the P₂ proteins from *Alternaria alternata* and *Cladosporium herbarum* have been reported to be minor allergens of these molds (17). The *A. fumigatus* P₂ protein is recognized by IgE antibodies of individuals sensitized to the mold and shows significant humoral cross-reactivity to human P₂ protein. Both human and *A. fumigatus* P₂ proteins induce strong type 1 skin reactions and proliferative responses in PBMCs of individuals sensitized to the fungal P₂ protein.

Materials and Methods

Construction and Screening of an *A. fumigatus* cDNA Library Displayed on Phage Surface. Phage displaying IgE-binding proteins were enriched from an *A. fumigatus* cDNA library constructed in phagemid pJuFo (19) and displayed on the surface of filamentous phage M13 as described (5, 6). Serum IgE from *A. fumigatus*-sensitized individuals was captured in microtiter plates coated with anti-human mAb TN 142 and used as ligand for selective enrichment of allergen-displaying phage (6). Sera used for screening were selected according to case history, skin test reactivity to commercial *A. fumigatus* extracts, and specific IgE to *A. fumigatus* determined by radioallergosorbent test (RAST) (8). The screening procedure yielded a wide variety of phage able to bind specifically to human serum IgE and thus displaying allergenic molecules (5, 6).

Identification of a Clone Encoding *A. fumigatus* P₂ Protein. Inserts carried by phage displaying IgE-binding proteins differing in length were sequenced as described (23) on an ABI prism 373A sequencer using the d-rhodamine terminator cycle sequencing kit (Perkin-Elmer) according to the manufacturer's instructions. Both DNA strands were sequenced using vector-derived primers. Homology searches and sequence comparisons were performed with BLAST and the Genetics Computer Group program FASTA (24). One clone revealed strong homology with nucleotide sequences encoding acidic ribosomal phosphoprotein type 2 (see Fig. 1).

Cloning of the Human P₂ Protein; Production and Characterization of Recombinant Proteins. The cDNA encoding human P₂ protein was amplified by PCR from a commercial human lung cDNA library (Stratagene) using the following primers: 5'-primer, 5'-GCG-GATCCATGCGCTACGTCGCTCCTACC-3'; 3'-primer, 5'-GCTCTAGATTAATCAAAAAGGCCAAATCCC-3'. The complete cDNA coding for the putative *A. fumigatus* P₂ protein was amplified from the original clone by PCR using the following primers: 5'-primer, 5'-GCGGATCCATGAAGTACCTC-GCAGCTTTCC-3'; 3'-primer, 5'-CCCGACTTTAAGTC-GAAGAGACCGAAGCCC-3'. PCR cycling conditions were 30 cycles of 95°C for 60 s, 57°C for 60 s, and 72°C for 60 s, followed by a terminal extension cycle at 72°C for 10 min. The amplification products were purified using a commercial kit (QIAquick; QIAGEN, Inc.), digested with BamHI and XbaI or BamHI and HindIII, respectively, and ligated to appropriately restricted pHis₆-DHFR (dihydrofolate reductase) vector (4). Ligation mixtures were transformed into *Escherichia coli* strain M 15; transformants were grown in liquid to verify the nucleotide sequence (23) and used to produce hexahistidine-tagged recombinant pro-

teins (4, 6). After a single-step purification over Ni²⁺-chelate affinity columns (6), molecular size and purity of the recombinant proteins were analyzed by polyacrylamide gradient gels (4.5–20%) and 1-mg samples lyophilized for long term storage (14).

ELISA and IgE Immunoblots. The specific binding properties of serum IgE from *A. fumigatus*-sensitized individuals to recombinant fungal and human P₂ protein were analyzed by an allergen-specific ELISA (8). Absorbency was measured at 405 nm with a Molecular Devices reader and optical densities converted into arbitrary ELISA units (EU/ml) calibrated against an in-house serum pool arbitrarily defined as 100 EU/ml (8, 11, 12). Values below 1 EU/ml were set as 1 EU/ml for graphic display and nonparametric statistical analysis (Mann-Whitney U test). For IgE immunoblots, proteins were separated on SDS-polyacrylamide gradient gels (4–20%), transferred to nitrocellulose, incubated with patient sera diluted 1:10, and processed as described (16, 25).

Proliferative Responses of PBMCs. PBMCs were isolated from heparinized peripheral venous blood by Ficoll density gradient centrifugation, washed three times, and resuspended in RPMI 1640 supplemented with 1 mM sodium pyruvate, 2 mM l-glutamine, 50 μM 2-ME, 1% MEM nonessential amino acids and vitamins, 100 μg/ml streptomycin, 100 U/ml penicillin (all from Life Technologies), and 10% heat-inactivated FCS (Sera-Lab). Samples of 5 × 10⁵ cells/100 μl were stimulated with different concentrations of recombinant *A. fumigatus* or human P₂ protein or with *A. fumigatus* extract in triplicate for 7 d. Proliferation was measured as incorporation of tritiated thymidine (DuPont-NEN) during the final 16 h of culture. A stimulation index >3 was considered positive.

Skin Tests with Recombinant P₂ Protein. Recombinant proteins were dissolved in 0.9% saline at concentrations ranging from 10⁻⁵ to 1 μg/ml. Intradermal skin tests were performed on the patient's back by injecting 100 μl test solution containing 1 ng recombinant protein. If no positive reaction could be observed after 15 min, testing was continued by injecting a 10-fold higher amount of protein. The test was stopped and considered positive when the wheal surface reached at least half of the histamine wheal size (8, 16) or after injection of 1 μg protein. 0.01% histamine dihydrochloride and 0.9% saline were used as positive and negative controls, respectively (8). An ethical approval for skin testing human subjects with recombinant proteins was obtained from the local ethics committee (Davos, Switzerland). A full explanation of the procedure was given to all participants, and their written consent was obtained before testing.

Results and Discussion

Isolation of cDNA Clones and Sequence Analysis. The cloning technology based on phage surface display of expression products from cDNA libraries (5, 6, 19) is particularly suitable for selective isolation of cDNAs encoding IgE-binding proteins from complex allergenic systems (26). Starting from a phage surface-displayed cDNA library generated from mRNA of *A. fumigatus* (6), we selectively enriched phage able to bind human serum IgE from individuals sensitized to the fungus. cDNAs isolated from single phagemids after four rounds of affinity selection, carrying inserts of different lengths, were sequenced and shown to code for different allergenic proteins (10). Among these, a clone containing an open reading frame spanning 333 bp (sequence data available from EMBL under accession no. AJ224333) revealed strong homology with sequences encoding eukary-

otic type 2 acidic ribosomal phosphoproteins. The deduced amino acid sequence of this cDNA clone was homologous to P₂ proteins, showing a high sequence identity to the human (62%), *C. herbarum* (71%), and *A. alternata* (72%) P₂ proteins (Fig. 1). Acidic ribosomal phosphorylated (P) proteins have been isolated and characterized from a variety of eukaryotic cells and share significant sequence identity and similarity (20).

Production and Characterization of Recombinant *A. fumigatus* and Human P₂ Proteins. Both complete cDNAs encoding the putative *A. fumigatus* P₂ protein and the human P₂ protein were amplified by PCR, subcloned into the high level expression plasmid pHis₆-DHFR, verified by sequencing, and used to produce hexahistidine-tagged P₂ proteins (see Materials and Methods). The constructs yielded, after single-step purification by Ni²⁺-chelate affinity chromatography, 33 and 28 mg/liter *E. coli* culture of virtually pure *A. fumigatus* and human P₂ protein, respectively. Purity was analyzed by reducing, denaturing SDS-PAGE, and Coomassie blue staining. In each preparation, only one band with estimated molecular mass in agreement with the calculated values of ~12.6 kD for the hexahistidine-tagged *A. fumigatus* and human P₂ protein was visible (data not shown).

Allergenic Properties of the P₂ Proteins. As expected from the selection procedure devoted to isolate allergens from a phage surface display library, the *A. fumigatus* P₂ protein was able to bind IgE present in the serum used for screening. However, both *A. fumigatus* and human P₂ proteins were identified as allergens by ELISA with sera from individuals allergic to *A. fumigatus* (Fig. 2) and by IgE immunoblots (data not shown). Inhibition experiments showed that increasing amounts of human recombinant P₂ protein added to the fluid phase were able to inhibit the binding of serum IgE from patients sensitized to *A. fumigatus* P₂ protein (Fig. 3), demonstrating that the proteins share common IgE-binding epitopes. 14/92 patients studied that were suffering from ABPA and 6/75 patients that were allergic to *A. fumigatus* and suffering from severe atopic dermatitis studied showed relevant levels of serum IgE antibodies to the *A. fumigatus* P₂ protein, resulting in an incidence of sensitization in the range of 15 and 8%, respectively. Sensitization to P₂ protein was not observed in *A. fumigatus*-sensitized individuals with mild forms of atopic dermatitis or in patients without ABPA.

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A. fum: MKYLAARLLDAAAGNSPSSSEVRAVLSVSGIDADEERINKLTAELEGKDLQSLTAEQST 60
A. alt: MKHLAAVLLIGGNGSPSADVRAVLSVSGIDAEADSDRLDKLISELEGKDLNILLTAEQST 60
C. her: MKYLAARLLIGGNGSPSADVRAVLSVSGIDADEEPSQLLKELEGKDLNILLTAEQST 60
H. sap: MRRVLSYLLAAGNSPSSAQRKRLKILDSVSGEADDDRNRVISELNGRKHEDVFAQIG 60

A. fum: KLASVPSGGGANANPAAAGAAAGC...NANPAAKEENEEKEESDEDMGFGLEFD 111
A. alt: KLASVPSGGAGGAAASGGAAAAGCSAQAEPAEAA...EEKEESDEDMGFGLEFD 113
C. her: KLASVPSGGACAASGCAAAAGCA...DEAAPEAEAEKEESDDDMGFGLEFD 111
H. sap: KLASVFAAGAVVSAAPPSAAPAAASAP...AEEKKDEKKEESESDDDMGFGLEFD 115

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Figure 1. Alignment of the deduced amino acid sequences *A. fumigatus* (*A. fum*), *A. alternata* (*A. alt*), *C. herbarum* (*C. her*), and human (*H. sap*) P₂ protein sequences. Identical amino acid residues in at least three of the sequences are noted by shading, and gaps are indicated by dots. Sequence identity between *A. fumigatus*, human, *C. herbarum*, and *A. alternata* P₂ proteins are 62.16, 72.07, and 71.17%, respectively. Numbers after the sequences indicate the residue numbers, without gaps, starting at the NH₂-terminal methionine residues.

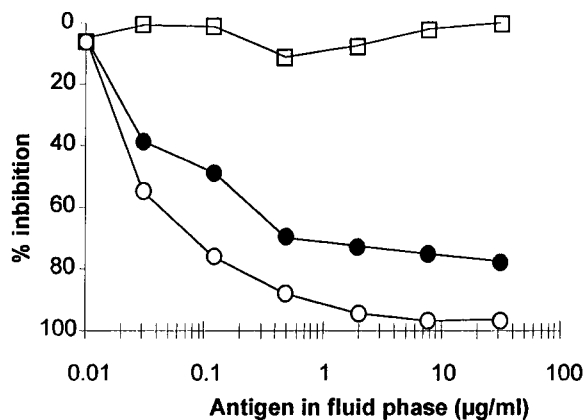


Figure 2. Competitive inhibition of IgE binding to solid phase-coated *A. fumigatus* recombinant P₂ protein. Serum from *A. fumigatus*-sensitized patients was preincubated with increasing amounts of recombinant *A. fumigatus* (○), human P₂ protein (●), or Asp f 1 as negative control (□). Preincubated serum samples were transferred to wells coated with *A. fumigatus* P₂ protein, and bound IgE was analyzed by antigen-specific ELISA (8, 16).

Proliferative Responses of PBMC from Individuals Sensitized to *A. fumigatus* P₂ Protein. T cell help is required for the production of allergen-specific IgE (27). Therefore, we measured the proliferative responses of mononuclear cells from six individuals sensitized to the *A. fumigatus* P₂ protein to fungal extract, recombinant *A. fumigatus*, and human P₂ protein. The mean proliferative responses to optimal concentrations of extract, fungal, and human P₂ protein were 39,500; 20,092; and 11,790 cpm, respectively (mean SI 11.9, 6.5, and 5.7). The mean proliferative responses of 15,608; 1,601; and 1,273 cpm (mean SI 12.6, 0.8, and 1) to fungal extract, *A. fumigatus*, and human P₂ protein, respectively, obtained for four individuals sensitized to *A. fumigatus* lacking IgE antibodies against the fungal P₂ protein indicate that the recombinant antigens did not induce nonspecific effects. Three control individuals did not respond to any of the antigen preparations (mean SI < 1). Comparison of the values by the rank sum test indicates highly significant differences (*P* < 0.01). The proliferative

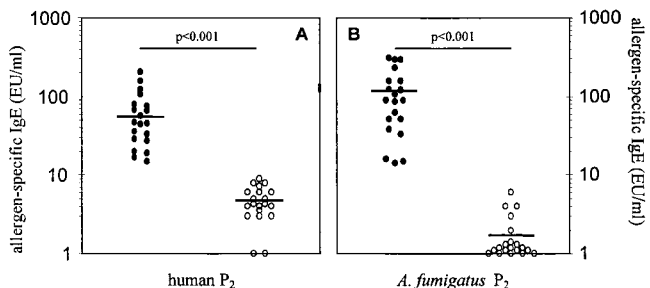


Figure 3. 20 sera from individuals sensitized to (■) and 20 sera of individuals lacking IgE to the *A. fumigatus* P₂ protein (○) were analyzed for their content of serum IgE able to recognize human P₂ protein (A). (B) IgE binding of the same sera to *A. fumigatus* P₂ protein is shown. Units are expressed as arbitrary ELISA units (EU/ml) calculated from the absorbency of an in-house reference serum pool, which was set as 100 EU/ml (8). Bars, mean values.

responses induced by human P₂ protein in individuals sensitized to the *A. fumigatus* P₂ protein indicate a pathogenesis related to autoreactive T cells. Intense local inflammatory responses to *A. fumigatus* occurring in the lungs of patients suffering from ABPA (11, 12) and in the skin of patients suffering from severe atopic dermatitis might result in release of autoantigens as a consequence of tissue damage due to the inflammatory process (28). Exposure to autoantigens containing cross-reactive determinants (molecular mimicry) can recruit the memory T cell repertoire at the site of inflammation where lymphokine expression is upregulated. These lymphokines can induce expression of MHC II on naive T cells and upregulate accessory molecules that function as costimulatory signals for T cell activation, creating a microenvironment in which all requirements for priming a T cell response are present (29). Molecular mimicry at the T cell level could be a possible pathogenic mechanism to explain autoaggression remaining confined to the local area of inflammation (29).

Allergenicity of the Recombinant P₂ Proteins In Vivo. The ability of a protein to bind IgE in ELISA and Western blots provides strong evidence for the allergenicity of the protein. However, the final demonstration that a protein preparation acts as an allergen and therefore possesses biological activity in vivo is its ability to elicit a type I skin reaction in sensitized individuals. We have investigated whether the IgE-mediated cross-reactivity against *A. fumigatus* and human P₂ protein shown in vitro is sufficient to provoke allergic reactions in vivo through skin tests (8, 16). Four individuals with high IgE levels against *A. fumigatus* P₂ protein, four individuals allergic to the fungus lacking IgE responses to the P₂ protein, and two nonallergic control individuals were investigated for their ability to respond to intradermal challenge with recombinant *A. fumigatus* and human P₂ proteins. As expected, none of the individuals without detectable IgE antibodies to *A. fumigatus* P₂ protein reacted against the recombinant protein preparations. A positive skin reaction to *A. fumigatus* P₂ protein was detected only in individuals who had IgE levels >10 EU/ml to the fungal protein. The amounts of recombinant protein needed to elicit a classical type I reaction ranged from 1 to 10 ng, depending on the subject. All individuals reacting to *A. fumigatus* P₂ protein also showed strong skin reaction to challenges with comparable amounts of human P₂ protein (Fig. 4). These results show that human P₂ protein can cross-link IgE on mast cells in vivo (30) and suggest humoral autoimmune response in some patients suffering from mold allergy. IgE reactivity to fungal and human P₂ protein was, however, only detectable in individuals sensitized to *A. fumigatus* suffering from ABPA or severe atopic

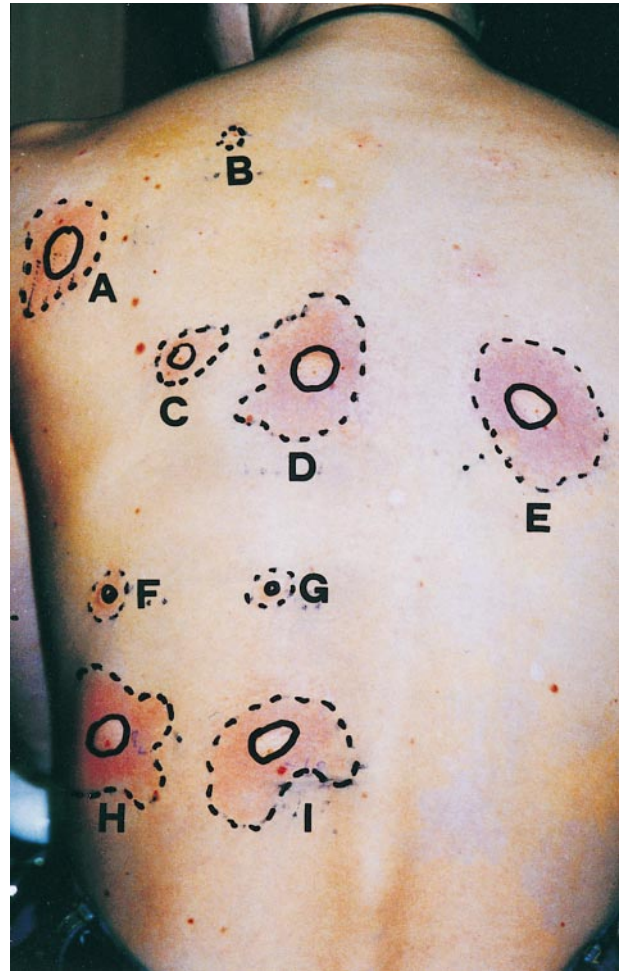


Figure 4. Skin test reactivity to recombinant *A. fumigatus* and human proteins in a patient sensitized to MnSOD and P₂ protein. For intradermal skin tests, 100 μ l of the protein solutions (10^{-1} μ g/ml) was injected with a syringe. 0.01% histamine dihydrochloride (A) and 0.9% saline (B) were used as positive and negative controls, respectively. The reactions show that 10 ng fungal (D) and human (E) P₂ protein or fungal (H) and human (I) MnSOD are able to elicit a wheal that is comparable to the size of the skin reaction induced by the positive histamine control. C shows the reaction to a challenge with 1 ng human P₂ protein. The patient lacks IgE to rAsp f 3 (F) and rAsp f 11 (G), two additional *A. fumigatus* allergens (10). The absence of reactions to skin challenges with these allergens demonstrates the specificity of the test.

dermatitis. This was also the case for the IgE autoreactivity to human MnSOD described earlier (16). Therefore, these two human proteins may serve as a tool to study the role of IgE autoreactivity in tissue damage and release of autoantigens at the site of inflammation.

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