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

By Christina Mayer,* Ulrich Appenzeller,* Heike Seelbach,‡ Gernot Achatz,§ Hannes O Berkofer,§ Michael Breitenbach,§ Kurt Blaser,* and Reto Crameri*

From the *Swiss Institute of Allergy and Asthma Research (SIAF), CH-7270 Davos, Switzerland; ‡Hodgebirgsklinik, CH-7265 Davos-Wolfgang, Switzerland; and the †Institut für Genetik und Allgemeine Biologie, Universität Salzburg, A-5020 Salzburg, Austria

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

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 pathophysiological 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 cytoplasmic 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...
riboosomal 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 A fumigatus and C ladosporium 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 PBM C's 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 pJuo (19) and displayed on the surface of filamentous phage M13 as described (5, 6). Serum IgE from phagemid pJuo (19) and displayed on the surface of filamentous phage M13 were sequenced as described (23) on an ABI prism 373A. Clones were enriched from an ELISA. Absorbance 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 gel electrophoresis (4–20%), transferred to nitrocellulose, incubated with patient sera diluted 1:10, and processed as described (16, 25).

Proliferative Responses of PBM C's. PBM C's 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 x 10^3 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 wheel 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. 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 allergic systems (26). Starting from a phage surface–displayed cDNA library generated from rRNA A. fumigatus (6), we selectively enriched phage able to bind human serum IgE from individuals sensitized to the fungus. cDNAs isolated from single phage-migrating 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). A 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 cDNA s 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 protein, 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 mass of 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.

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 N-terminus.

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).

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 naïve 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 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.

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 µl of the protein solutions (10^{-2} µg/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.

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