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Molecular Cloning of IgE-Binding Fragments of *Alternaria alternata* Allergens

Abstract

Exposure to the hyphomycete *Alternaria alternata* is recognized as an important risk factor for asthma. IgE immunoblotting has been used to catalogue the number and M_r of allergens in *A. alternata* extracts, with estimates ranging from 10 to 30, although few are present in nearly all extracts studied. Several *A. alternata* allergens have been cloned, including a subunit of the major allergen Alt a 1, ribosomal P2 phosphoprotein, aldehyde dehydrogenase and a yeast YCP4 homolog. We have cloned two sequences encoding IgE-binding fragments of allergens from an *A. alternata* λ gt11 cDNA library using pooled atopic sera from *A. alternata*-sensitive individuals. One is homologous to a region near the C-terminus of *hsp70* from *Cladosporium herbarum*; a near-complete isoallergen variant of *A. alternata* ribosomal P2 protein was also cloned. Their *lacZ* fusion proteins had reactivities of 5 and 14%, respectively, with individual atopic sera, indicating that the corresponding allergens are both minor. This study describes one new *A. alternata* allergen candidate and implicates ribosomal P2 protein as an allergen that is stable between independently isolated clones.

Key Words

Allergen
Fungi
Alternaria alternata
cDNA cloning
IgE immunoblot
hsp 70
Ribosomal P2 phosphoprotein

Introduction

Characterization and epitope mapping of fungal allergens are complicated by extensive variability between strains with respect to allergen content and composition and by inability to produce large amounts of purified allergen [1, 2]. *Alternaria alternata* is recognized as a prominent source of fungal aeroallergens, and exposure to this fungus represents a risk factor for severe asthma [3, 4]. Consequently, several groups have attempted to catalogue the number and size of allergens in extracts of *A. alternata* by IgE immunoblotting [5–8]. Estimates range from 10–30 resolved bands although only a small number of these have been observed in the majority of independent studies.

Molecular characterization of allergens is essential to a better understanding of the pathogenesis of atopic disease

and the development of specific diagnostic and immunotherapeutic reagents. It has been envisaged that allergens of consistent composition and purity can be prepared using recombinant DNA techniques. Gene-cloning strategies have been applied to allergens of *A. alternata* to obtain cloned reference standards for this fungus. Allergens that have been cloned and expressed as IgE-binding proteins include a subunit of the major allergen Alt a 1, ribosomal P2 phosphoprotein, aldehyde dehydrogenase and a yeast YCP4 homolog [9, 10]. Because only Alt a 1 has been recognized as a major *A. alternata* allergen thus far, it is important to search for and characterize additional, and preferably major *Alternaria* allergens for potential use as immunodiagnostic reference standards. In this study, we have characterized clones immunoselected from an *A. alternata*-specific λ gt11 library using human atopic serum IgE.

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Material and Methods

RNA Isolation and Library Construction

An *A. alternata* cDNA library was constructed in bacteriophage λ gt11 by a commercial firm (Clontech), using total RNA isolated in our laboratory. An *A. alternata* isolate (34-016, originally obtained from Dr. E.G. Simmons, Amherst, Mass., USA), available from the American Type Culture Collection) was cultured on liquid synthetic revised tobacco medium at 22°C as described [11]. Mycelia were harvested on days 4, 6 and 10 by washing with ice-cold PBS, drying and storing at -70°C. The mycelial material was suspended at approximately 1 ml/g in 4.7 M guanidine isothiocyanate containing 1% β -mercaptoethanol and homogenized. After centrifugation at 10,000 g for 10 min, the supernatant was extracted twice with phenol/chloroform (1:1, v/v), and precipitated with 0.2 M potassium acetate pH 4.0 and ethanol at -20°C overnight. RNA pelleted at 14,000 g for 20 min at 4°C was resuspended in 20–30% of the original volume of guanidine isothiocyanate solution, layered on a cushion of 5.7 M CsCl₂ and ultracentrifuged overnight at 30,000 rpm with Beckman SW50.1 rotor at 18°C. The RNA pellets were resuspended in RNase-free water, quantitated by determination of A₂₆₀, and equal amounts of the time point samples were pooled.

After purification of poly A⁺ RNA, oligo(dT)-primed double-stranded cDNAs were synthesized using reverse transcriptase, DNA polymerase I and RNase H. *Eco*RI sites within the cDNA were methylated prior to ligation of *Eco*RI adaptors, and *Eco*RI-digested cDNA was ligated into dephosphorylated vector arms. Functional phage particles containing the recombinant genomes were generated using packaging extracts.

Antisera and Immunoscreening

Sera from individuals recognized as *A. alternata*-sensitive by positive skin test were collected and assayed by a radioallergosorbent test (RAST) using *A. alternata* extracts prepared in our laboratory [12]. Sera scoring 19% or greater by RAST were utilized in pools for immunoscreening and individually when assessing reactivity of recombinant fusion proteins.

Screening of the library using pooled atopic sera and alkaline-phosphatase (AP)-conjugated IgG directed against human IgE was carried out using standard protocols [13] and is described in greater detail elsewhere [9]. Initial high-density screening of the library was carried out at 10,000 plaque-forming units (PFU) per 100-mm petri dish. Pools of atopic sera were preabsorbed with bacterial lysate [9], diluted 5× in TBST (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% Tween-20) and applied to duplicate nitrocellulose membrane lifts of the plated phage that had been blocked in 1% BSA in TBST for > 1 h at room temperature. After overnight incubation at room temperature, the membranes were washed with TBST for 3×5 min and incubated with AP-conjugated goat anti-human IgE at 1:1,000 dilution for 2 h, also at room temperature. After washing as before, staining was carried out using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate in AP buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM MgCl₂). Immunoselected phage plaques were purified by several rounds of sparse plating and screening, until all plaques on a membrane stained positively.

Polymerase Chain Reaction and DNA Sequencing

DNA from purified plaque lysates (10 μ l) denatured at 99°C for 5 min were amplified by the polymerase chain reaction (PCR) in re-

actions containing 10 pmol each of λ gt11 forward and reverse primers, 0.2 mM dNTPs, and 5 U of Taq polymerase in 50 μ l of PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl₂). Reactions were heated to 95°C for 5 min and subjected to 30 cycles of 95°C for 36, 51°C for 36 and 72°C for 90 s. Amplified sequences were ligated into plasmid pCR II (Invitrogen) and mini-prep DNAs were sequenced using the ALF laser fluorescence system and fluorescein-conjugated M13-40 and reverse primers (Pharmacia). The BLAST algorithm was used for homology searches of GenBank and EMBL databases [14]. Sequence analyses and alignments were carried out using DNAsis, PROsis (Hitachi Biotech) and Align Plus version 2.0 (Scientific & Educational Software).

Generation of λ gt11 Lysogens and Immunoblotting of Fusion Protein

A culture of Y1089 cells was grown at 37°C in Luria-Bertani broth containing ampicillin and 0.4% maltose until approaching saturation. The cells were spun at 3,000 rpm for 10 min and resuspended in sterile 10 mM MgSO₄. Aliquots of 2×10⁸ viable cells were infected with amplified clone stocks at a multiplicity of infection of 5, and incubated at 32°C for 15 min. To determine the percentage of infected cells, an aliquot was serially diluted 10⁶ in LB, and 50 μ l was plated on each of two plates that were incubated overnight at either 32°C or 42°C. Individual colonies from the plate grown at 32°C were subjected to replica plating and again incubated at either 42 or 32°C overnight. Cultures of representative lysogens that grew at 32°C but were lysed at 42°C were frozen at -80°C and served as a cell stock for the generation of β -galactosidase (*lacZ*) fusion protein.

To produce fusion protein, recombinant and nonrecombinant lysogen cultures were grown at 32°C until reaching log phase, divided into aliquots of equal volume and incubated at 42°C for 20 min. After induction of one aliquot with isopropyl- β -D-galactopyranoside (IPTG) to 10 mM and incubation at 37°C for 45 min, the cells were pelleted and resuspended in 75 μ l of resuspension buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA) per milliliter of culture. After addition of phenylmethylsulfonyl fluoride to 2 mM cultures were frozen at -80°C and thawed rapidly. DNase I was added to 5 μ g/ml, followed by incubation at 37°C for 15 min. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer concentrate (5×) was added, and the samples were heated at 95°C for 5 min. Aliquots of 5–10 μ l from each sample were blotted as previously described [9] and immunostained using 10×-diluted sera, 1:8.5-diluted human atopic serum pool 7P or 1:10-diluted serum pool from 6 nonatopic individuals. Coomassie blue R-250 staining of blots was carried out to ensure that similar amounts of lysate proteins were loaded into the gels.

Data Base Submissions

Sequences of the hu12, hu11 and hu31 allergen clones isolated in this study were submitted to GenBank under the accession numbers U87806, U87807 and U87808, respectively.

Data obtained from this study have also been submitted to the WHO-/UIS Allergen Nomenclature Subcommittee for approval of the new allergen names Alt a 6.0102 (ribosomal P2 protein isoallergen variant) and Alt a 3 (*hsp*70 fragment).

Hu11	(1)	aagaccaacaagattgtcatcaccaacgacaagggccgtctctccaagga
Hu11	(51)	ggagatcgagcgcatgttggtgaggccgagaagtacaaggctgaggacg
Hu11	(101)	aggcagaggctgcccgtatctctgctaagaacgctctcgagtcctacgct
Hu31	(1)	-----GAGGCTGCCCGTATCTCTGCTAAGAACGCTCTCGAGTCCTACGCT
Hu11	(151)	TACTCGCTCCGCAACACTCTCTCCGACTCCAAGGTCGACGAGAAGCTTGA
Hu31	(46)	TACTCGCTCCGCAACACTCTCTCCGACTCCAAGGTCGACGAGAAGCTTGA
Hu11	(201)	TGCTGGCGACAAGCAGAAGCTCACTGCCGAGATTGACAAGACCGTCCAGT
Hu31	(96)	TGCTGGCGACAAGCAGAAGCTCACTGCCGAGATTGACAAGACCGTCCAGT
Hu11	(251)	GGTTGGACGACAACCAGACCGCCACCAAGGACGAGTACGAGTCTCAGCAG
Hu31	(146)	GGTTGGACGACAACCAGACCGCCACCAAGGACGAGTACGAGTCTCAGCAG
Hu11	(301)	AAGGAGCTTGAGGGTGTGCCAACCCCATCATGATGAAGTTCTACGGAGC
Hu31	(196)	AAGGAGCTTGAGGGTGTGCCAACCCCATCATGATGAAGTTCTACGGAGC
Hu11	(351)	TGGTGGTGAGGGTGGCATGCC-----
Hu31	(246)	TGGTGGTGAGGGTGGCATGCCGggcggtatgccgggtggcgcatgcccg
Hu31	(296)	gtggcgccccaggtggtgcccgggtgatgacggccaaccgtcgaggag
Hu31	(346)	gtcgact <u>aa</u> gcggatcgcttcactgcagtctcttcttcttcttcttctgt
Hu31	(396)	gcattcttcaagcatagacttcttttccatgggtggtagttcacggagttt
Hu31	(446)	cacggccaattaattgagccagtttcatggtttcttcttatgcatcggat
Hu31	(496)	gaataacgatcaacgattgggaggaaagttaggtggtgtcagcatgtac
Hu31	(546)	tctaatagtgggatagttctatatctgtgtagaga <u>aattgag</u> agaataact
Hu31	(596)	tcaaaaaaaaaaaaaaaaaaaaaa

Fig. 1. Nucleotide sequence alignment of human atopic IgE-binding recombinant clones hu11 and hu31. Sequence matches are capitalized. The termination codon is double underlined. The closest approximation to a polyadenylation signal is underlined.

Results

Immunoselection and PCR Amplification

Initially, a pool of human sera (7P) was prepared from sera of 7 atopic individuals exhibiting high RAST scores and positive skin test reactions against *A. alternata* antigens. Immunoselection of the cDNA library was carried out after preabsorption of pool 7P with bacterial lysate to reduce nonspecific background. From a screen of 60,000 pfu, two strong, authentic positives were obtained, as they were detected on duplicate membranes. The clones (hu11, hu31) were purified in preparation for PCR amplification.

Additional clones encoding different *A. alternata* allergens were obtained by further screening using a pool of 4 atopic sera chosen from pool 7P and additional individual sera to avoid reselection of cloned sequences similar to

hu11 and hu31. This second pool of human atopic sera (designated pool 6789) yielded five additional clones (hu12, hu22, hu32, hu61, hu71) from a screen of 205,000 pfu.

A rapid PCR protocol was employed to amplify insert DNAs for subcloning into sequencing vectors. Clones hu11 and hu31 contained inserts of ≈ 300 –400 bp and ≈ 700 bp (respectively), whereas the sizes of amplified product from clones selected using pool 6789 ranged from 700 to 1,600 bp (data not shown). All PCR-amplified inserts were subsequently subcloned into plasmid-sequencing vector pCR II.

Sequence Analysis of hu11 and hu31

Nucleotide sequence comparisons of the hu11 and hu31 inserts revealed that they are 372 and 617 bp in length, respectively (fig. 1). Their sequences also overlap, encoding C-terminal fragments of the same protein. Clone hu11 lacks

Reference molecule:	A. alternata	36 - 124	(89 aa)	Homology
Sequence 2:	N. crassa	531 - 620	(90 aa)	79%
Sequence 3:	C. herbarum	166 - 256	(91 aa)	72%
Sequence 4:	G. max	537 - 626	(90 aa)	48%
Sequence 5:	D. carota	540 - 628	(89 aa)	45%
Sequence 6:	C. elegans	532 - 620	(89 aa)	46%
Sequence 7:	R. norvegicus	532 - 620	(89 aa)	36%
Sequence 8:	H. sapiens	530 - 618	(89 aa)	40%

A. alternata	(1)	-----KTNKIVITNDKGRLSKEEIERMLAEAEKYKAEDEA
A. alternata	(36)	EAARISAKNALESYAYSLRNTLSDSKVDEKLDAGDKQKLTAIEDKTQVWL
N. crassa	(531)	..K.VA...G.....A..E..KS....I.A..
C. herbarum	(166)	..G..Q...G.....K..V..P..E...S.E..ET..GA.....A.I
G. max	(537)	VKKKVE...S..N...NM...IK.E.IGG..SPDE...IEKAVEDAI...
D. carota	(540)	VKKKVE.....N...NM...IK.D.IPG.....E.IETAVNEAIE...
C. elegans	(532)	QKD..G...G.....FN.KQ..IE.E.LKD.ISPE..K.IEDKC.EILK...
R. norvegicus	(532)	QRE.VA.....FNMKSAVE.EGLKG.ISEA..K.VLDKCQEVIS...
H. sapiens	(530)	QRE.V.....FNMKSAVE.EGLKG.ISEA..K.VLDKCQEVIS...
A. alternata	(86)	DDNQATATKDEYESQQKELEGVANPIMMKFY-GAGGE--GGMP
N. crassa	(581)	.E..Q..RE...ER.....AI.....-...AP-....
C. herbarum	(216)	.E.....E...AE..Q..S...V...I...-E.GAP....
G. max	(587)	EG..M.EV..F.DK.....IC...IA.M.Q..A.P--D.V
D. carota	(590)	EK..L.EV..L.DKL.....LC...IARL.-QGR.D--VPIG
C. elegans	(582)	.S....E.E.F.....D...L.K.DLS.L.-QSA.G--APPG
R. norvegicus	(582)	.S.TL.E.E.FVHKRE...R.C...ISGL.-QGA.A--P.AG
H. sapiens	(580)	.A.TL.E...F.HKR....Q.C...ISGL.-QGA.G--P.PG
A. alternata	(125)	GGMPGGGMPGGAPGGAAGDDGPTVEEVD

Fig. 2. Sequence comparisons between an *A. alternata* *hsp70* C-terminal peptide sequence common to clones hu11 and hu31 and the corresponding sequences from *N. crassa* (GenBank Accession No. U10443), *C. herbarum* (X81860), *G. max* (X62799), *D. carota* (X60088), *C. elegans* (M18540), *R. norvegicus* (X74271) and *Homo sapiens* (M11717) [17–21]. Matched amino acids are indicated by (.)

3' untranslated sequence and poly(A) that is present in clone hu31, but contains similar amounts of translatable sequence, accounting for the similar sizes that had been observed for recombinant *lacZ* fusion proteins produced by lysogens of the two clones (data not shown). GenBank searches using the BLAST algorithm [14] indicated that these sequences exhibit 65–70% nucleotide sequence identity to 70-kD heat shock protein (*hsp70*) from numerous other species.

Figure 2 illustrates peptide sequence homology between the region common to clones hu11 and hu31 and corresponding regions of *hsp70*s from other representative species. The two strongest homologies among the *hsp70* peptide species shown are to fungal representatives, specifically *Neurospora crassa* (79%) and *Cladosporium herbarum* (72%) [15, 16]. However, it may be noted that two representative plant *hsp70* species, those of *Glycine max* (soybean) and *Daucus carota*, (carrot) also show some-

what more homology to this region of *A. alternata hsp70* (≈ 45 –50%) than do *Caenorhabditis elegans*, *Rattus norvegicus* or human *hsp70*s (≈ 35 –45%) [17–21]. When conservative substitutions are taken into account, amino acid similarities to this region of *A. alternata hsp70* are 90% for *N. crassa*, 89% for *C. herbarum*, 72% for *C. elegans*, 69% for *G. max* and *D. carota*, 60% for rat and 63% for human. Because this region is common to both clones, and the same individual serum within pool 7P is responsible for their immunoselection, it is possible that a common IgE-binding epitope resides within this region.

Sequence Analysis of Clones hu12, hu22, hu32, hu61, hu71

Sequencing from the insert termini inward of clones selected using serum pool 6789 revealed that all five contain a sequence that is nearly identical to an *A. alternata* minor allergen previously isolated by Achatz et al. [10] and desig-

HU12	(1)	AAQNENPRHHSPTTRSLHPSTPKKHLAAYLLLGLGGNTSPSAADVKAVLE
ALTA6	(1)	-----M.....
CLADP2	(1)	-----M.YM.....A..S...E.I.T..S
HU12	(51)	SVGIEADSDRLDKLISELEKGDINELIASGPEKLASVPSGGAGGAAASGG
ALTA6	(29)	S.....
CLADP2	(29)	D..EE..SS.LK.....S..SQ.....S.A.PSA..
HU12	(101)	AAAAGGSAEAEAAPEAAKEEKEEEDMDGFGLFD
ALTA6	(79)	
CLADP2	(79)	--AT.....D.....

Fig. 3. Peptide sequence comparisons between clone hu12 and ribosomal P2 protein allergens from *A. alternata* and *C. herbarum* [10, 22]. Matched amino acids are indicated by (.) and gaps by (-).

nated Alt a 6 (data not shown). The two longest clones (hu32 and hu61) are partial Alt a 6 clones, lacking the first 21 and 120 bases of the Alt a 6 ORF (respectively), but which contain an additional cDNA sequence ligated to the 3' end of the ribosomal P2 sequences that exhibit strong homology to rRNA, the result of rRNA contamination of the mRNA used for library construction. Similarly, two clones (hu22 and hu71) were also revealed to be partial Alt a 6 clones, lacking the first 27 and 42 bases of the Alt a 6 ORF, respectively. However, clone hu71 does contain 14 additional bases at its 5' end that is distinct from the Alt a 6 sequence. The fifth clone (hu12) consists of a complete Alt a 6 ORF and 3' untranslated sequence, except for the AUG initiation codon, which is replaced by a region of 68 bases at the 5' end of the clone. Sequence data base searches of the 68-bp stretch failed to demonstrate strong homology with known vector, ribosomal or fungal sequences. We speculate that, like clone hu71, this additional 5' sequence was ligated onto a near-complete Alt a 6 ORF cDNA during library construction, although conclusive proof of this hypothesis is lacking.

Sequences collected from all five clones were aligned with that of Alt a 6 as obtained by Achatz et al. [10] to determine the degree of similarity between Alt a 6 allergen sequences recovered from the two *Alternaria* isolates (data not shown). Although only clones hu12 and hu22 were sequenced in their entirety, sequences obtained from the other three clones covered enough of the Alt a 6 ORF such that all regions other than the first 27 bases were represented by two or three clones other than hu12. The alignment strongly indicated that the two different fungal isolates encode virtually identical P2 proteins except for one amino acid, as a single-base deviation from the Alt a 6 ORF was evident in all five clones, resulting in a proline for serine substitution

at amino acid 81 of clone hu12 (fig. 3). This deviation, and one present in the 3' untranslated sequences of clones hu12, hu22 and hu71 represent the only two deviations from Alt a 6 observed in all sequenced clones, indicating that they could not have resulted from Taq polymerase misincorporation during PCR or sequencing ambiguity.

Nucleotide sequence homology between hu12 and ribosomal P2 protein of *C. herbarum* [10, 22] is 77% over the entire open reading frame and 54% in the 3' untranslated sequence, whereas the peptides are 74% identical and 91% similar (fig. 3).

Reactivity of Recombinant Fusion Proteins with Individual Atopic Sera

Lysogens were generated by infecting host bacteria with amplified clone stocks, growing cultures at the permissive temperature of 32°C and replica plating. Lysate proteins from IPTG-induced and uninduced recombinant and nonrecombinant lysogens were immunoblotted using 10×-diluted human sera from atopic, *A. alternata*-sensitive donors. Initially, 19 individual sera were tested, including sera from those donors that comprised the serum pools used in the initial screening of the *A. alternata* cDNA library. Using preabsorbed serum, fusion proteins were stained with minimal background from λgt11 *lacZ* or other bacterial protein (fig. 4). Leaky expression of fusion protein accounts for the fainter bands observed in lanes containing uninduced hu12 and hu31 lysate proteins. An additional 24 sera were tested later, using preparative blots and a Bio-Rad Multi-Screen manifold (data not shown). In total, 2 sera (A and C, fig. 4) reacted strongly with the *lacZ*-hu31 fusion protein at 1:10 dilution. These 2 sera were similarly reactive with *lacZ*-hu11 fusion protein (data not shown). These blots were stained more intensely than the positive control, since only

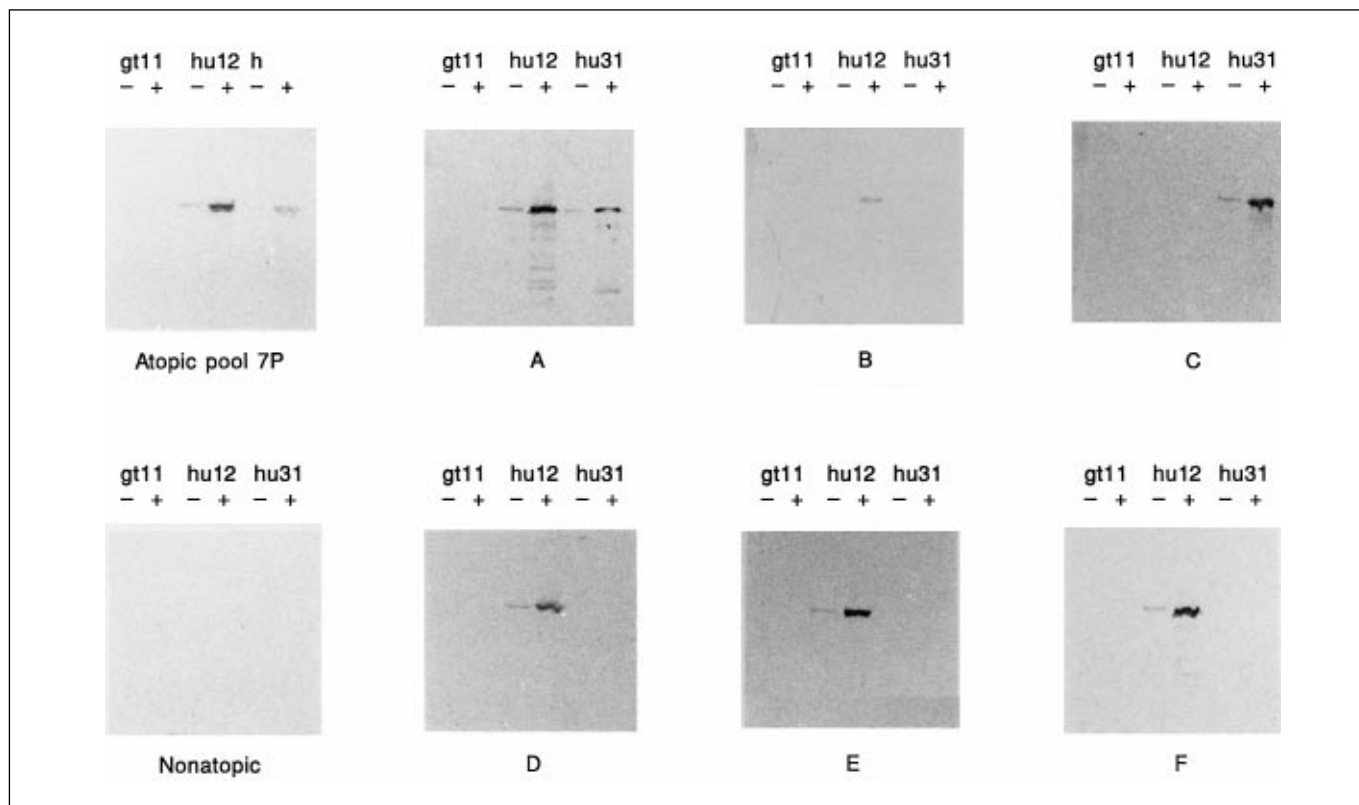


Fig. 4. Reactivity of individual human atopic sera against recombinant hu31 and hu12 *lacZ* fusion proteins. Proteins from IPTG-induced (+) or uninduced (–) nonrecombinant λ gt11 (gt11), hu12 or hu31 lysogen lysates were blotted and immunostained with individual sera (A–F), atopic pool 7P or nonatopic pool.

1 of the 7 sera which comprised the 8.5 $\times$ -diluted pool (serum A) was reactive with the *hsp70*-derived epitope and, therefore, at 1:60 dilution within the pool. With respect to clone hu12 (ribosomal P2 protein), a total of 6 of the 43 sera tested were immunoreactive with the *lacZ*-hu12 fusion protein at 1:10 dilution, of which five are shown in figure 4. Of these, only serum F was present in the 6789 serum pool, and serum A was reactive with both fusion proteins. The data indicate that clones hu31 and hu12 encode fragments of minor allergens that are reactive with 5 and 14% of *A. alternata*-sensitive individuals.

Discussion

In this study, we attempted to obtain and clone novel *A. alternata* allergen sequences by IgE immunoscreening of a λ gt11 cDNA library. Although cloned *A. alternata*-specific sequences encoding Alt a 1, ribosomal P2 phosphoprotein, aldehyde dehydrogenase and a homolog of yeast YCP4 have

been isolated and characterized [9, 10], all except Alt a 1 encode minor allergens that are recognized by fewer than 50% of *A. alternata*-specific serum IgEs. In addition, these clones only account for 25–50% of the total number of potential allergens estimated in extracts of *A. alternata* [8]. Because of the extreme variability between allergens from different isolates of a given fungal species, it is important to identify and clone allergen sequences that remain stable among different isolates, so that they may be expressed in biological systems better suited to purification and potential use as immunodiagnostic reagents.

Initially, we isolated two clones that encode overlapping fragments of the C-terminal portion of *A. alternata*-specific *hsp70*. The 89-amino-acid overlap encompasses a region extending from 29 to 117 amino acids upstream of the C-terminus. Because the same 2 atopic sera reacted with both hu11 and hu31 *lacZ* fusion proteins, and an IgE-binding epitope has also been localized to a homologous region in *hsp70* from *C. herbarum* [Zhang, unpubl. data], it may be hypothesized that the overlapping region encodes a com-

mon *hsp70* IgE-binding epitope responsible for the immunoselection of clones hu11 and hu31. However, it should be noted that the overlapping region is sufficiently large to harbor several independent IgE epitopes. Moreover, these observations do not preclude the possible presence of IgE-binding epitopes within different nonoverlapping regions of the hu11 and hu31 fragments. Strong homology between *A. alternata*, and both *C. herbarum* (72%) and *N. crassa* (79%) *hsp70* DNA sequences indicates that similar epitopes may be detected in homologous regions of *hsp70* from other fungal species. This portion of *hsp70* is also the most divergent region of the molecule between humans and fungi, with only 35–40% peptide sequence identity, although sequence similarity is >60% in all cases. However, other regions of *A. alternata hsp70* have not necessarily been ruled out as sites for IgE epitopes, especially since the remainder of the *A. alternata hsp70* sequence has not yet been sequenced. Further epitope mapping of these clones, and of the entire *hsp70* of *A. alternata* once sequenced, is essential to pinpoint the exact sites of epitopes within the cloned fragments and to determine whether other regions of *hsp70* are potentially allergenic.

Unlike other characterized allergens of *A. alternata*, *hsp70* is not a constitutively expressed 'housekeeping' protein, but a heat-shock- or stress-induced chaperone that may be expressed during fungal sporulation as well as upon heat shock [23]. Spore-specific extracts may contain higher levels of *hsp70* relative to mycelial extracts grown under nutritionally rich conditions, provided coinduction of *hsp70* and sporulation were to occur in other fungal species. One other example of a stress-induced allergen in fungi is *hsp90* from *Aspergillus fumigatus* [24]. Clearly, the appearance of *hsp70* in extracts of *A. alternata* would be expected to be dependent on the culture growth conditions employed. The stress-inducible character of this allergen emphasizes the need for standardized extracts that contain a maximal number of allergens, obtained under nutritionally rich as well as starvation conditions. Comparison of *hsp70* expression in spore and mycelial extracts of *A. alternata* would be desirable.

The second clone isolated encodes a near-complete acidic ribosomal P2 phosphoprotein that has been recognized, cloned and designated Alt a 6 [10]. Nucleotide sequence homology between corresponding regions of clone hu12, the most complete of the five partial clones isolated, and Alt a 6 is >95%, and the only reproducibly distinct deviation between the two allergen sequences is a conservative proline for serine substitution in hu12 at amino acid 81. On this basis, hu12 may be considered as an isoallergen variant of Alt a 6, although the single substitution may have

little effect on its antigenicity or allergenicity. The clone also featured replacement of the first two bases of the Alt a 6 initiation codon by 68 additional nucleotides at the 5' end of the insert, likely ligated onto the cDNA during library construction. However, this additional sequence is not believed to be intrinsic to the Alt a 6, nor did the 23 extra amino acids encoded by this sequence inhibit IgE-binding to the *lacZ* fusion protein of the clone during library screening. Hu12 and Alt a 6 also share extensive homology with *C. herbarum*-derived ribosomal P2 phosphoprotein allergen [10, 22], although the IgE-binding regions of these allergens have not yet been determined. However, the C-terminal 22 amino acids of ribosomal P2 proteins show homology with autoantigens involved in systemic lupus erythematosus [25], and in this study, clone hu61 was immunoselected from the λ gt11 library despite the absence of Alt a 6 sequence encoding the N-terminal 40 amino acids of the protein. These observations point to the C-terminal regions of Alt a 6 as most likely to feature IgE-binding epitopes. Surprisingly, the 3' untranslated regions of hu12 and Alt a 6 are also highly conserved, with only one consistent nucleotide substitution over 204 bp. This implies that the 3' untranslated region of ribosomal P2 protein may serve an important and conserved function. The near identity between variants obtained from independent sources also implies that the sequence of this protein is highly stable among different *A. alternata* isolates and of potential importance as an immunodiagnostic reagent.

Both isolated allergens are considered minor as they react with 5% (*hsp70*) and 14% (P2) of sera from individuals allergic to *A. alternata*. These values are consistent with the 2–8% reactivities previously observed for all other *A. alternata* allergens cloned by Achatz et al. [10]. However, assessment of the reactivity of *Alternaria*-sensitive individuals to *hsp70* may yet be underestimated, since the clones only encode a small fraction of the complete *hsp70* sequence and the reactivity of sensitized individuals was assessed using the fragments as a small part of a *lacZ* fusion protein. Cloning and expression of *A. alternata hsp70* as a full-length nonfusion protein, and its use to determine the reactivity of sensitive individuals to *hsp70*, are required to determine conclusively whether other regions of the allergen contain additional IgE-binding epitopes.

Finally, it should be noted that most of the cloned *A. alternata* allergens isolated at present have proven to be minor allergens and relatively small cytoplasmic proteins within fungal mycelia. Previous characterizations of *A. alternata* allergens indicate that a substantial number of large and important glycoprotein allergens are present in extracts from this species. Difficulties in preparing undegraded mRNA from *A. alternata* mycelia as well as bacterial in-

ability to glycosylate expressed sequences from λ bacteriophage libraries prevent more of these allergens from being immunoselected. Purification and preparation of monospecific IgG against these allergens, an approach used with Alt a 1 [9], may prove to be the only means of isolating other major allergens of *A. alternata*.

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