

CLOWING AND SEQUENCING OF A cDNA EXPRESSING A
RECOMBINANT HOUSE DUST MITE PROTEIN THAT BINDS
HUMAN IgE AND CORRESPONDS TO AN IMPORTANT
LOW MOLECULAR WEIGHT ALLERGEN

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The cloning of recombinant proteins offers the possibility of producing, identifying, and manipulating the important IgE-binding determinants present on proteins that are responsible for provoking allergic diseases (1). Proteins produced by mites of the genus *Dermatophagoides* are among the most important allergens affecting man in terms of both the frequency of the response by allergic subjects and the proportion of total IgE directed against these allergens (2, 3). Of the 30 or more IgE-binding components demonstrated by our blotting studies (2, 4), at least six may be important allergens on the basis of their frequency and intensity of IgE binding on blots. Only two of these, *Der p* I and *Der p* II, have been extensively investigated. The cDNA coding for *Der p* I has recently been cloned and sequenced (5), although the recombinant protein did not bind IgE.

A cDNA library expressing recombinant mite proteins has been screened using IgE antibodies from mite-allergic subjects. Techniques based on protein blotting have been used to identify the native source of an IgE-binding recombinant protein as the important mite allergen of molecular weight ~14,000.

Materials and Methods

House Dust Mite Extracts. For mRNA preparation, live mites were harvested from culture by centrifugation on NaCl density gradients. For protein blotting, extracts of dried mites containing 10 mg protein/ml were prepared from commercial dried mites, as previously described (2).

Labeled Antisera and Human Sera Used. For screening the cDNA library, goat anti-human IgE antibodies (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) labeled with ¹²⁵I (6) were used. For probing the initial blots of native dust mite extract and inclusion bodies, ¹²⁵I-anti IgE (Kallestad, Austin, TX) was used; for detecting the reprobing of eluted IgE, alkaline phosphatase-labeled anti-IgE (Kallestad) was used. Human sera from mite-allergic subjects was provided by the Allergy Department, Royal North Shore Hospital, from subjects who showed a strong skin-prick test response to mite extract. The pool of mite allergic sera was prepared by combining 20 sera. Before use, the sera from mite allergic subjects was

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preabsorbed with extracts of *Escherichia coli* strain RY 1089 λ gt 11 lysogen to remove IgE directed against those proteins that would otherwise interfere with the screening of the cDNA library.

Production and Screening of cDNA Library to *D. pteronyssinus* Mites. mRNA from live mites was prepared using guanidine isothiocyanate extraction and affinity purified using oligo-dT-cellulose (no. 20003; Collaborative Research, Lexington, MA) (7). A cDNA library was prepared in λ gt 11 (cDNA kit no. RPN 1256; Amersham Corp., Arlington Heights, IL) according to the manufacturer's directions. The primary cDNA library of 1.7×10^5 recombinants was amplified to generate a high titre stock. The library was immunoscreened ($\sim 10^5$ PFU/plate) using serum pooled from mite-allergic subjects following the Amersham protocol. IgE-binding clones were detected using ^{125}I -labeled anti-human IgE antibodies and autoradiography and were purified to single plaques.

Purified recombinant bacteriophage representing the IgE-binding clones were used to infect *E. coli* strain Y1089 to produce fusion polypeptides (8). The IgE-binding polypeptides were identified on protein blots of the lysogen extracts by probing with mite-allergic serum as above. Large scale preparation of the fusion polypeptides was carried out (8).

Identification of the Native Allergen that Shares IgE-binding Determinants with the Cloned Protein. Electrophoretic transfer of unreduced mite extracts after SDS-PAGE on 9–27% gradient gels (SE-400; Hoeffer Scientific Instruments, San Francisco, CA) was performed as previously described (2). The inclusion bodies derived from the phage-infected *E. coli* lysogen containing the recombinant protein were solubilized in 2.5% SDS, 100 mM DTT, 20 mM Tris, pH 7.5, 10 mM EDTA, and 1 mM PMSF by heating at 100°C for 30 min, and were separated on 18% homogeneous gels (Mini-Protean II apparatus; Bio-Rad Laboratories, Richmond, CA). Each blot of mite bodies and inclusion bodies were divided into two portions, and one portion was stored at 4°C. The remaining portion was incubated in 10% pooled mite-atopic serum in PBS, 0.1% sodium azide overnight. The position of IgE-binding components on the blots were located using narrow vertical strips cut from each edge of the blot after incubated with ^{125}I -anti-IgE and autoradiography. The major IgE-binding horizontal band (~ 30 – 40 mm long $\times$ 2–3 mm wide) was excised from blot of inclusion bodies, and six major IgE-binding bands were excised from the blot of mite bodies. An additional strip that did not bind IgE was also excised from the bottom of each blot to function as a control. Each strip was cut into pieces ~ 2 mm² and IgE was eluted by six cycles of gentle vortexing for 1 min each with 100 μl of 0.1 M Glycine-HCl, pH 2.5, 1% (wt/vol) BSA, followed by immediate neutralization with a 3 M Tris-HCl solution, pH 8.8, to give a final pH of 7.0. Sodium azide was added to a final concentration of 0.1% (wt/vol). The solution containing the antibodies eluted from the inclusion body blot was incubated overnight with a stored strip from the mite blot. Similarly, solutions containing the IgE eluted from the six bands of the blot of mite body extract were incubated with strips from the blot of the inclusion bodies. The position of binding of the IgE was located with enzyme-labeled anti-IgE. As an additional control for specificity, IgE samples originally eluted from the blot of the inclusion bodies, after incubation with the mite blot, were then incubated with strips from the blot of the inclusion bodies. Similar controls were performed with IgE from the mite blots.

Sequencing of cDNA. DNA was prepared from the selected IgE-binding cDNA bacteriophage clone (8) and the cDNA insert excised by *Eco* R1 digestion (7), purified after separation on low melting point agarose, and ligated into the plasmid pBTA 502 (Biotechnology, Sydney, Australia). After transformation in the *E. coli* strain JM109 and the isolation of transformed colonies, plasmid DNA was prepared (9). DNA sequencing was carried out on an alkali-denatured plasmid template using a 17-mer primer (no. 1211; New England Biolabs, Beverly, MA) and the enzyme Sequenase (United States Biochemical Corp., Cleveland, OH) according to the manufacturer's directions. ^{35}S -labeled products were run on 8% polyacrylamide gels for 2 and 4 h, autoradiographed overnight, and the sequence of the first 250 bases was determined. For further sequencing, a set of nested deletions was created using the "Erase-a-base" Exo III/S1 nuclease system (Promega Biotec, Madison, WI).

Results

Identification of the Mite Component Corresponding to the Recombinant Mite Protein. Blots of the bacterial inclusion bodies containing the recombinant mite fusion protein showed

a major IgE-binding component of mol wt $\sim 150,000$ (Fig. 1 *b*), whereas blots of nonrecombinant bacterial lysate did not bind IgE (not shown). IgE eluted from this component on an excised portion of the recombinant blot (Fig. 1 *b*, position 1), when incubated with a strip from a blot of mite extract, bound to a component of mol wt $\sim 14,000$ (Fig. 1 *e*). The position on the gels of the component of mol wt 14,000 was of a mol wt $\sim 1,000$ less than *Der p* II. The same procedure was carried out with the control non-IgE-binding portion of inclusion body blot (Fig. 1 *b*, position 2) and no binding of IgE to the mite blot was detected (not shown). When, after incubation with the mite blots, these eluates were reincubated with recombinant blots, the eluted IgE only bound to the inclusion body component of mol wt $\sim 150,000$ (not shown). Similarly, when IgE eluted from the major IgE-binding bands of a mite blot (Fig. 1 *d*, positions 1–6) and a seventh control position (not shown) were incubated with strips from the inclusion body blot, strong IgE binding was only detected with IgE eluted from position 6 of the mite strip (Fig. 1 *f*) and not with IgE eluted from other positions (not shown). When the eluted IgE was reincubated with mite strips, with one exception, each fraction only bound to the component it was eluted from (results not shown). The exception was IgE eluted from the component of mol wt 30,000, which bound to the components of mol wt 30,000, and to a lesser extent to the component of mol wt 25,000 (not shown).

Sequence of the cDNA. The cDNA sequence and the deduced amino acid sequence derived from the cDNA insert is shown in Fig. 2. The insert corresponds to a 148-amino acid polypeptide of estimated mol wt 17,460 and contains no cysteine or tryptophan. At the level of 20 matched bases no homology was found between this sequence and other DNA sequences using the Integenetics IFIND programme (ItelliGenetics, Inc., Mountain View, CA) to search the NIH database.

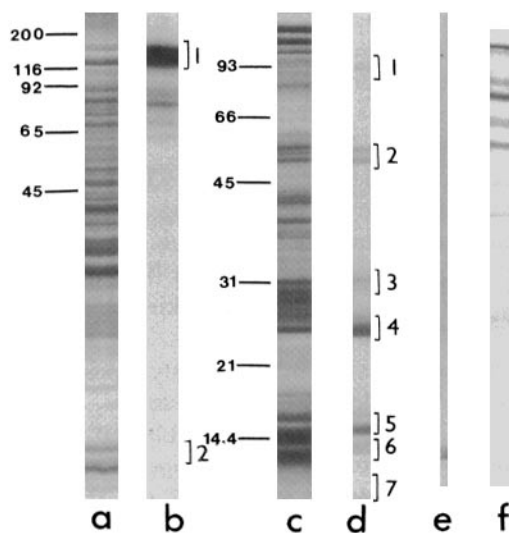


FIGURE 1. Protein staining and immunoprobings of blots of inclusion bodies containing an IgE-binding recombinant mite protein and of a mite body extract. The following are shown: (a) CBB staining of inclusion body proteins separated by SDS-PAGE. The position of molecular mass markers shown as black bars are as follows (200, 116, 92, 65, and 45 kD). (b) Autoradiograph of a protein blot of inclusion bodies showing the position of the IgE-binding component. The position of the bands that were excised for the preparation by elution of clone-specific IgE and of an IgE-free control band are marked as 1 and 2, respectively, on the strip. (c) CBB staining of protein components of a mite extract separated by SDS-PAGE. The position of molecular mass markers 93, 66, 45, 31, 21, and 14.4 kD are shown by black bars. (d) Autoradiograph of a protein blot of the mite extract showing the position of IgE-binding components and the position of the six bands that were excised and used for preparation of specific IgE are

marked 1–6, a seventh control band was excised from the bottom of the strip in a region not binding IgE. (e) A strip of a blot of a mite body extract showing the position of binding of IgE eluted from position 1 of a blot of recombinant inclusion bodies stained using enzyme-labeled anti-IgE. (f) A strip from a blot of recombinant inclusion bodies showing the position of rebinding of specific IgE eluted from position 6 of a mite blot using enzyme-labeled anti-IgE.

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CTT TTT CTC GAA AAT AAA GAT CCC AAA CCG TTA AAA AAA ATA 14
Leu Phe Leu Glu Asn Lys Asp Pro Lys Pro Leu Lys Lys Ile

TCA ATC ATG AAA TTC ATC ATT GCT TTC TTT GTT GCC ACT TTG 28
Ser Ile Met Lys Phe Ile Ile Ala Phe Phe Val Ala Thr Leu

GCA GTT ATG ACT GTT TCA GGT GAA GAT AAA AAA CAT GAT TAT 42
Ala Val Met Thr Val Ser Gly Glu Asp Lys Lys His Asp Tyr

CAA AAT GAA TTT GAT TTC TTA TTG ATG GAA CGT ATT CAT GAA 56
Gln Asn Glu Phe Asp Phe Leu Leu Met Glu Arg Ile His Glu

CAA ATT AAA AAA GGT GAA CTT GCA TTG TTC TAT CTT CAA GAA 70
Gln Ile Lys Lys Gly Glu Leu Ala Leu Phe Tyr Leu Gln Glu

CAG ATT AAT CAT TTT GAA GAA AAA CCA ACA AAA GAA ATG AAA 84
Gln Ile Asn His Phe Glu Glu Lys Pro Thr Lys Glu Met Lys

GAT AAA ATT GTA GCC GAA ATG GAT ACC ATT ATT GCT ATG ATC 98
Asp Lys Ile Val Ala Glu Met Asp Thr Ile Ile Ala Met Ile

GAT GGT GTA CGT GGT GTA CTT GAT CGT CTT ATG CAA CGT AAA 112
Asp Gly Val Arg Gly Val Leu Asp Arg Leu Met Gln Arg Lys

GAT TTA GAT ATT TTT GAA CAA TAT AAT CTT GAA ATG GCT AAA 126
Asp Leu Asp Ile Phe Glu Gln Tyr Asn Leu Glu Met Ala Lys

AAA TCT GGT GAT ATT TTG GAA CGT GAT TTG AAA AAA GAA GAA 140
Lys Ser Gly Asp Ile Leu Glu Arg Asp Leu Lys Lys Glu Glu

GCA CGT GTT AAA AAG ATT GAA GGT TAA
Ala Arg Val Lys Lys Ile Glu Val ***

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FIGURE 2. Nucleotide and predicted amino acid sequence of the cDNA whose expression product shares the IgE-binding determinants with a component of mite extracts of mol wt ~14,000. The numbers at the right refer to amino acid positions. These sequence data have been submitted to the EMBL/GenBank Data Libraries.

Comparison of IgE Binding to Blots of the Recombinant Mite Protein and the Corresponding Component of Mite Extracts. Of the 38 sera tested, 16 did not bind to either the region of the recombinant blot corresponding to components of 150,000 or the region of the mite blots corresponding to mol wt 14,000, two bound weakly to the mite blot only and five bound weakly to the recombinant blot only. All of the remaining 15 sera bound with similar intensities to both blots.

Discussion

The results demonstrate the cloning of an IgE-binding recombinant mite polypeptide recognized by about half the sera from a mite-allergic group of subjects tested. A similar frequency of recognition for the corresponding component on mite blots has been demonstrated in other protein blotting studies from this laboratory using a greater number of sera (4). This component is one of six, of the total of >30, suggested to be important allergens of *D. pteronyssinus* (2, 4).

The blotting strategy used here to identify proteins extracted from a native source carrying the same IgE-binding determinants as the cloned inclusion polypeptides should be generally applicable to the cloning of other allergens. The specificity of the antibody recognition was maximized by the following approaches. The sera used were preabsorbed with a lysate of *E. coli* infected with the phage lacking the cDNA insert to eliminate the possibility of IgE binding to *E. coli* proteins. The IgE was eluted from blots of both the native material and the recombinant polypeptides and then bound to blots of the recombinant polypeptides and native material, respectively. The eluted IgE was shown to faithfully bind to the original blotted source.

The precise molecular weight of the native mite component corresponding to the recombinant protein was not determined. On unreduced SDS-PAGE gels the component had a mol wt of ~14,000, ~1,000 less than the allergen *Der p* II. *Der p* II has a mol wt of 15,000–16,000 by SDS-PAGE, and a mol wt of 14,131 based on amino acid analysis. Differences between molecular weight values based on electrophoretic migration on SDS-PAGE and sequence data are not uncommon (10) and such discrepancies are more pronounced for low molecular weight proteins (11).

From the predicted amino acid sequence, the molecular weight of the cloned mite allergen is ~3,000 greater than the molecular weight determined by nonreducing SDS-PAGE for the corresponding blotted mite component. It may be that the native (mature) component in mite extracts lacks some of the terminal amino acids present in the recombinant protein. A possibility may be the leader sequence, usually of 19–21 amino acids associated with proteins made on membrane-bound ribosomes and normally cleaved off post-translationally during membrane transport (12). The deduced amino acid sequence from a cDNA clone of *Der p* I was demonstrated to contain an additional 23 amino acids when compared with the amino acid sequence of native *Der p* I purified from mite extracts (5).

The strong correlation between the intensity and specificity of binding of IgE from a panel of sera to the electroblotted blotted recombinant protein, and a component of mol wt ~14,000 on blots of the native mite extract, suggests a potential use of such recombinant material for specific diagnostic applications. An aim of the approach described here is to assemble a panel of mite recombinant proteins that include all of the important IgE-binding determinants. Such a selected panel may be more amenable to standardization than the highly variable crude extracts currently available from native sources. Cloned allergens may also be more amenable to modifications, making them safer and more suitable for immunotherapy. Considering the slow progress isolating individual allergens to date, recombinant DNA methods may offer great potential for the future production and sequencing of allergens and for the identification of allergenic determinants.

Summary

A cDNA clone coding for a mite allergen of mol wt ~14,000 has been isolated and its DNA sequence determined. The native component from mite extracts encoded by this DNA was identified by immunoprobings blots of mite body extract with human IgE eluted from the electroblotted cloned fusion polypeptides derived from the expressed cDNA clone. The clone encodes a polypeptide with a deduced mol wt of 17,460. The deduced amino acid sequence was not homologous to any known protein sequences and it contains no cysteine or tryptophan. On blots, 21 of 38 sera from mite-allergic subjects recognized the cloned material, and this recognition strongly correlated with IgE-binding to the native component on protein blots of mite extract.

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