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Cloning and Characterization of cDNA Coding for a New Allergen from the House Dust Mite, *Dermatophagoides farinae*

Abstract

A cDNA clone encoding a new allergen from the *Dermatophagoides farinae* cDNA λ gt11 library was isolated and sequenced. There was no amino acid sequence homology with other known allergens. The gene product, β -galactosidase fusion protein, of the truncated cDNA on blot reacted with IgE in 13 of 43 sera from patients allergic to mites. The affinity-purified fusion protein had a potent ability to release histamine from washed blood cells of the mite-allergic patients. Human specific IgE eluted from the fusion protein band on blots recognized a 39-kD component on blots of a mite body extract.

Introduction

Allergens from two predominant and closely related mite species, *Dermatophagoides farinae* and *Dermatophagoides pteronyssinus*, are major triggers of allergic diseases such as asthma, rhinitis, and dermatitis [1]. Many efforts from various groups have identified three groups as major mite allergens [2]. Among them, emphasis has been on the *Der* I and *Der* II groups that react with IgE in 60–90% of allergic sera [3–12]. However, of the 30 or more IgE-binding and heterogeneous components detected by crossed radioimmuno-electrophoresis or immunoblotting analysis using allergic sera, at least six were reported to be possibly important allergens on the basis of their frequency and intensity of IgE binding on blots [13–18]. Progress in characterization of these mite allergens has been hampered by the small or variable quantities and the complexity of mite allergens present in the extracts of whole mite cultures.

Recently, to help overcome these limitations and to facilitate further studies, the molecular cloning of mite allergens was introduced. However, this approach is still focussed on the identification of total nucleotide sequences of the *Der* I and *Der* II groups [19–23]. Tovey et al. [24, 25] reported the sequencing of a cDNA clone coding for a mite allergen with a molecular weight of about 14 kD as one of six important allergens. However, other important allergens remain to be characterized [15, 26].

In this paper, we describe the cloning and sequencing of a cDNA that encodes a new allergen corresponding to the house dust mite protein. The recombinant mite allergen produced in *Escherichia coli* reacted with considerable frequency to IgE in the sera of mite-allergic patients. Based on protein blotting, the native antigen sharing IgE-binding determinants with the cloned protein was identified as a 39-kD protein.

Materials and Methods

Live mites, *D. farinae*, and the spent growth media, in which mites were bred at 25°C and 75% humidity for 30 days, were supplied by Fumakilla Ltd. (Hiroshima, Japan). Mite body extract (Dfb, Lot 1) and mite feces extract (Dff, Lot 1) were prepared from the spent growth medium according to essentially the same method of Sasa et al. [27]. The major allergens, *Der f*I and *Der f*II, and their rabbit antisera were kindly provided by Dr. H. Yasueda of the National Sagami Hospital (Kanagawa, Japan). Cedar pollen extract was purchased from Torii Pharmaceutical Co. (Tokyo, Japan). Restriction endonucleases and DNA-modifying enzymes were obtained from Takara Shuzo Co. Ltd. (Kyoto, Japan), Toyobo Co. Ltd. (Osaka, Japan), or Boehringer Mannheim GmbH (Mannheim, FRG). The radioactive tracers used in this study were purchased from ICN Biochemicals Inc. (Costa Mesa, Calif., USA). All other reagents used were of analytical grade.

Phage and Plasmids

Phage vector λ gt11 (Stratagene, La Jolla, Calif., USA), plasmid vectors pUC18 (Takara Shuzo), and pUEX2 (Amersham International plc, Amersham, UK) were used for preparation of the cDNA library, subcloning, and expression, respectively. Plasmid vectors M13mp18 and M13mp19 (Takara Shuzo) were used for DNA sequencing.

Bacterial Strains and Culture Media

E. coli Y1090 (Stratagene) and pop2136 (Amersham International plc) were used as host strains for λ gt11 and pUEX2, respectively. *E. coli* JM109 (Takara Shuzo) was used for pUC18, M13mp18, and M13mp19. These strains were routinely grown at 28 or 37°C in LB medium. Super broth medium composed of 2.4% yeast extract, 1.2% bacto polypeptone, 0.5% glycerol, and 100 mM potassium phosphate buffer (pH 7.5) supplemented with 100 μ g/ml ampicillin and 2xTY medium were used for expression and DNA sequencing, respectively.

Human Sera

Allergic sera were collected from 43 asthmatic adults who were selected at random from newly visited cases with positive skin reactions to mite and house dust. Three sera with high IgE titers (600, 1,200, and 1,500 IU/ml) and a serum were also selected as specificity controls from patients and a healthy donor, respectively, with negative skin reactions to both allergens.

Before use, the sera were preadsorbed with an equal volume of non-recombinant *E. coli* lysates containing about 10 μ g/ml of β -galactosidase (β -Gal) to remove the β -Gal-specific antibody and other antibodies that would interfere with the screening of the mite cDNA library.

Preparation and Screening of *D. farinae* cDNA λ gt11 Library

Polyadenylated mRNA from live mites was isolated sequentially using guanidinium isothiocyanate extraction, ultracentrifugation in cesium trifluoroacetic acid, and affinity chromatography on oligo (dT)-cellulose column (Boehringer Mannheim) [28] according to protocols of a cDNA synthesis kit (Pharmacia LKB Biotechnology, Uppsala, Sweden). Approximate 3.2 μ g of polyadenylated mRNA was isolated from about 1 g of live mites. A cDNA library was prepared in λ gt11 [29] using a cDNA synthesis kit (Pharmacia LKB Biotechnology) [30] and an in vitro packaging kit (Stratagene) to construct 3.2×10^6 recombinant clones, and one twentieth of which

was screened with rabbit anti-Dfb serum. All purified recombinant bacteriophages representing the 53 rabbit anti-Dfb sera-reacting clones were used to infect *E. coli* Y1090 to produce fusion protein. The sole clone was selected on the basis of immunoreactivity of fusion protein on protein blots of lysogen extracts against IgE in human pooled mite-allergic sera according to the manufacturer's directions (SuperScreen Immunoscreening System, Amersham International plc) and used for further study.

Nucleotide Sequencing

The *Kpn*I-*Sac*I fragment (3.24 kbp) of λ gt11 containing a cDNA insert (1.14 kbp), named *mag* (mite antigen gene), was subcloned into plasmid pUC18. The resultant plasmid, pAK1, was used to transform *E. coli* JM109. To subclone *mag* and two derivative fragments (*mag*1, 0.94 kbp and *mag*1', 0.2 kbp) from pAK1 into pUC18, plasmid pAK1 DNA was partially digested with *Eco*RI and then used to ligate with *Eco*RI-digested pUC18 DNA. The recombinant plasmids were designated as pAK101, pAK102, and pAK103 in the order of base pairs in length. The sequences of *mag*, *mag*1, and *mag*1' cDNAs were determined by the dideoxynucleotide chain termination method [31] using a Sequenase Ver.2.0 kit (Toyobo) or a Tth DNA sequencing kit (Toyobo) with [α - ^{32} P]dCTP or [γ - ^{32} P]dCTP (1,000 Ci/mmol), respectively, after subcloning into the M13-derived vectors, mp18 and mp19 [32].

Expression of *mag*1 in *E. coli* and Purification of the Recombinant Fusion Protein (β -Gal-Mag1)

The expression vector, pAEX102, was constructed by insertion of *mag*1 cDNA into the downstream of the *lacZ* gene on a plasmid pUEX2 [33] and used to transform *E. coli* pop2136. The *E. coli* pop2136 strain carrying the constructed plasmid was grown at 28°C in 400 ml of super broth medium. After a temperature shift from 28 to 42°C by adding an equal volume of the same medium preheated at 56°C, β -Gal-Mag1 was induced for 90 min.

Harvested cells producing β -Gal-Mag1 were lysed in PBS containing 70 μ g/ml lysozyme, 10 mM EDTA and 0.1 mM PMSF on ice for 30 min. Furthermore, the lysate was sonicated 10 times for 1 min and centrifuged. The inclusion bodies containing β -Gal-Mag1 were solubilized with 2% SDS and applied to an AG11A8 column (50–100 mesh, Bio-Rad Laboratories, Richmond, Calif., USA) [34]. One-tenth volumes of 100 mM PBS were added to pooled effluent and applied to an anti- β -galactosidase (Promega, Madison, Wisc., USA)-immobilized GM (1.5 ml, Kurita Kogyo, Tokyo, Japan) column. After extensive washing, the target protein was eluted with 0.2 M glycine-HCl buffer (pH 1.6) into the tubes containing one-fifth volumes of 1 M Tris-HCl buffer (pH 8.5). The purified fusion protein was dialyzed against water and lyophilized.

Simultaneously, β -Gal was inductively produced by the *E. coli* pop2136 strain carrying the intact plasmid and purified by the same procedures.

Assay of Histamine Released from Washed Blood Cells of Mite-Allergic Patients

The amount of histamine released during incubation of washed blood cells from mite-allergic patients with antigen was determined by a liquid chromatograph apparatus (Shimadzu LC-6A, Kyoto, Japan) equipped with a fluorescence HPLC monitor (Shimadzu RF-535; manuscript in preparation).

Southern Blot Analysis

Analysis of genomic DNA by Southern hybridization was performed according to the methods of Sambrook et al. [35]. A *Bam*HI-*Eco*RI fragment (0.84 kbp) of *mag1* was labeled using a DIG DNA labeling and detection kit (Boehringer Mannheim) to prepare a probe. Prehybridization, hybridization, and posthybridization washes were carried out according to the manufacturer's recommendations (Boehringer Mannheim).

Identification of the Native Antigen that Shares Antigenic Determinants with the Cloned Protein

The protein blot of whole cellular components containing β -Gal-Mag1 was blocked with 10 mM Tris-buffered saline (pH 8.0) containing 3% skim milk, 0.02% Thimerosal, and 0.05% Tween 20 and coated with individual mite-allergic sera ($\times 10$) preabsorbing with *E. coli* lysates. One portion of the blot was immunostained. From the remaining portion of the blot, the band (2–3 cm²) corresponding to about 50 μ g of β -Gal-Mag1 was excised and washed four times with the blocking buffer. Antibody (equivalent to 500 μ l of a mite-allergic serum) specific for Mag1 was eluted three times with 1 ml of 200 mM glycine-HCl (pH 2.5) and immediately dialyzed against Tris-buffered saline.

After blocking the protein blot of Dfb with the blocking buffer, the blot was overlaid with the Mag1-specific antibody solution. The membrane was thoroughly washed and incubated successively with biotin-conjugated goat anti-human IgE (The Binding Site, Birmingham, UK; $\times 500$) and peroxidase-labeled streptavidin (Tago Inc., Burlingame, Calif., USA; $\times 500$), followed by staining using 3,3'-diaminobenzidine tetrahydrochloride and NiCl₂.

Results

Determination of Nucleotide Sequence of the cDNA

The nucleotide sequence analyses of the cDNA insert (*mag*) and two derivative fragments (*mag1* and *mag1'*) were performed on both strands. The cDNA sequence and the deduced amino acid sequence derived from the cDNA insert is shown in figure 1. A termination codon TGA was located at position 1,024–1,026, followed by a possible polyadenylation signal (AATAAA) at position 1,125–1,130 within a 3'-untranslated region of 121 nucleotides. Neither possible N-glycosylation site nor cysteine residue was evident in the predicted amino acid sequence.

No homology between this sequence or the derived amino acid sequence and other DNA sequences or proteins, respectively, was found by searching the sequences with those present in EMBL/GenBank/DDBJ. The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank Nucleotide Sequence Databases with the following accession number D13961.

Southern blot of *Eco*RI-digested and *Bam*HI and *Eco*RI double-digested genomic DNA from *D. farinae* by probing with the digoxigenin-labeled 0.84-kb *Bam*HI-

*Eco*RI fragment of *mag1* suggested that no intron is included in the *Bam*HI-*Eco*RI region in *mag* DNA (data not shown).

Reaction of β -Gal-Mag1 with IgE in Sera from Mite-Allergic Patients

A significant amount of β -Gal-Mag1 or β -Gal, located at the position of 150 or 166 kD on SDS-PAGE, was accumulated as insoluble inclusion bodies during 1.5-hour cultures of the *E. coli* pop2136 strain carrying pAEX102 or pUEX2 after the heat induction, respectively. The recombinant fusion protein on blots was incubated with 43 sera from mite-allergic patients along with 3 sera with high IgE titers from patients allergic to other allergens and 1 serum from healthy donors (fig. 2). Among them, 13 cases showed positive reactions to β -Gal-Mag1 on blots, but the rest were negative on blots. Similarly, blots of β -Gal did not bind IgE at all. The binding of β -Gal-Mag1 to IgE was inhibited with Dfb (fig. 2D, lanes b) or β -Gal-Mag1 (data not shown), but not with cedar pollen allergen (fig. 2D, lanes c). Consequently, the recombinant fusion protein was found to bind to specific IgE in mite-allergic sera with comparatively high frequencies.

In vitro Allergenicity of the Purified β -Gal-Mag1

From 400 ml of each culture, purified β -Gal-Mag1 and β -Gal were obtained in 2.4- and 3.6-mg yields, respectively, giving practically a single band with a slight amount of non-immunoreactive proteins on SDS-PAGE in each case (fig. 3A).

In the histamine release test (fig. 3B), about 30% of maximum histamine release was observed by stimulation of blood cells of mite-allergic patients with purified β -Gal-Mag1 at a concentration of less than one hundredth in Dfb. No significant release was observed by stimulus with purified β -Gal. Blood cells of patients allergic to cedar pollen released histamine when stimulated with cedar pollen extract, but did not with β -Gal-Mag1 (fig. 3C). Moreover, no histamine release was observed for healthy donors, who showed a negative histamine release response to Dfb at all concentrations of antigens used (data not shown). These results suggested that β -Gal-Mag1 would be potent in stimulating histamine release from patients' basophils.

Identification of the Native Mite Component Corresponding to the Recombinant Protein

Specific IgE in 8 allergic sera, which were selected over the range of apparent intensities of these stains as shown in figure 2B, was eluted from the blots of the β -Gal-Mag1.

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TTT GTC ATG AAA CGA GAA CCA TTG CGA TTC AGA GAC ATC ACT GTC GAA GGA AAC GAA AAT 60
F V M K R E P L R F R D I T V E G N E N 20

GCC TAT ATC AAA AAT GGC AAA CTT CAT TTG TCG CTT ATG GAT CCG TCA ACA TTG AGT TTA 120
A Y I K N G K L H L S L M D P S T L S L 40

GTC ACG AAA GCC GAT GGA AAA ATC GAC ATG ACA GTA GAC TTG ATA TCG CCA GTC ACA AAA 180
V T K A D G K I D M T V D L I S P V T K 60

CGT GCA TCG TTG AAA ATT GAT TCA AAG AAA TAC AAC CTT TTC CAT GAA GGT GAA TTG AGT 240
R A S L K I D S K K Y N L F H E G E L S 80

GCA TCG ATC GTA AAC CCA CGA TTG TCA TGG CAT CAA TAC ACG AAA CGC GAT TCT CGT GAA 300
A S I V N P R L S W H Q Y T K R D S R E 100

TAC AAG AGT GAT GTA GAA CTA TCG TTG CGA TCG TCG GAC ATT GCT CTC AAG ATT ACG ATG 360
Y K S D V E L S L R S S D I A L K I T M 120

CCT GAT TAT AAT TCG AAA ATT CAT TAT TCA CGA CAA GGT GAT CAA ATC AAC ATG GAC ATC 420
P D Y N S K I H Y S R Q G D Q I N M D I 140

GAT GGT ACA TTG ATC GAA GGT CAT GCA CAA GGA ACC ATC AGA GAA GGT AAA ATC CAC ATT 480
D G T L I E G H A Q G T I R E G K I H I 160

AAA GGT AGA CAA ACT GAT TTC GAG ATC GAA TCC AAC TAC CGA TAC GAA GAT GGC AAA CTA 540
K G R Q T D F E I E S N Y R Y E D G K L 180

ATC ATC GAA CCG GTC AAG AGT GAA AAT GGC AAA TTG GAA GGC GTT CTT TCC CGT AAG GTG 600
I I E P V K S E N G K L E G V L S R K V 200

CCA TCA CAT CTG ACA CTA GAA ACA CCA CGA GTC AAG ATG AAT ATG AAA TAT GAT CGA TAT 660
P S H L T L E T P R V K M N M K Y D R Y 220

GCA CCA GTC AAA GTG TTC AAA TTG GAT TAT GAT GGC ATC CAC TTC GAG AAA CAT ACC GAT 720
A P V K V F K L D Y D G I H F E K H T D 240

ATT GAA TAC GAA CCT GGC GTT CGA TAC AAG ATC ATC GGC AAT GGA AAA CTC AAG GAT GAT 780
I E Y E P G V R Y K I I G N G K L K D D 260

GGC CGC CAC TAT TCT ATC GAT GTG CAA GGT ATT CCA CGC AAA GCA TTC AAT CTG GAC GCT 840
G R H Y S I D V Q G I P R K A F N L D A 280

GAC TTG ATG GAT TTC AAA CTG AAA GTG AGC AAG CCA GAA GAT AGC AAT AAA GCT CAA TTC 900
D L M D F K L K V S K P E D S N K A Q F 300

AGC TAC ACA TTC AAC GAA TAT ACC GAG ACC GAA GAA TAT GAA TTC GAT CCA CAT CGT GCC 960
S Y T F N E Y T E T E E Y E F D P H R A 320

TAT TAT GTT AAT TGG TTG AGT TCC ATT CGC AAA TAC ATC CAG AAT TTC ATC GTC GAA GAC 1020
Y Y V N W L S S I R K Y I Q N F I V E D 340

AAC TGA acaccattaatgcacattgattgagaatgcaatgagcaaatgtcttcattgattcattcgattattccatt 1097
N * 341

ctctaatttttatatctattgacttgtataaaatctaaggaaaac 1144

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Fig. 1. Nucleotide and deduced amino acid sequence of *mag*. The nucleotide sequence is numbered from the nucleotide (T) adjacent to the 3' end of the *Eco*RI adaptor. The upper and lower numbers refer to nucleotide and amino acid positions, respectively. The terminal TGA codon is located above* in the line expressing the predicted amino acid sequence. The polyadenylation signal is underlined.

The pooled IgE reacted specifically with a molecular weight of 39-kD on blots of the mite body extract (fig. 4A). The antibody was specifically absorbed by purified β -Gal-Mag1 (fig. 4B). These results suggested that the 39-kD protein shares the IgE-binding determinants with the cloned protein.

Discussion

Several strategies have been used to clone the major allergen genes from a mite protein cDNA library. Whichever strategy one chooses, human mite-allergic sera [20, 24, 25], antisera raised against purified target allergens [19, 22], and/or information on their amino acid sequences [19, 21, 23] are required for cloning. However, antisera

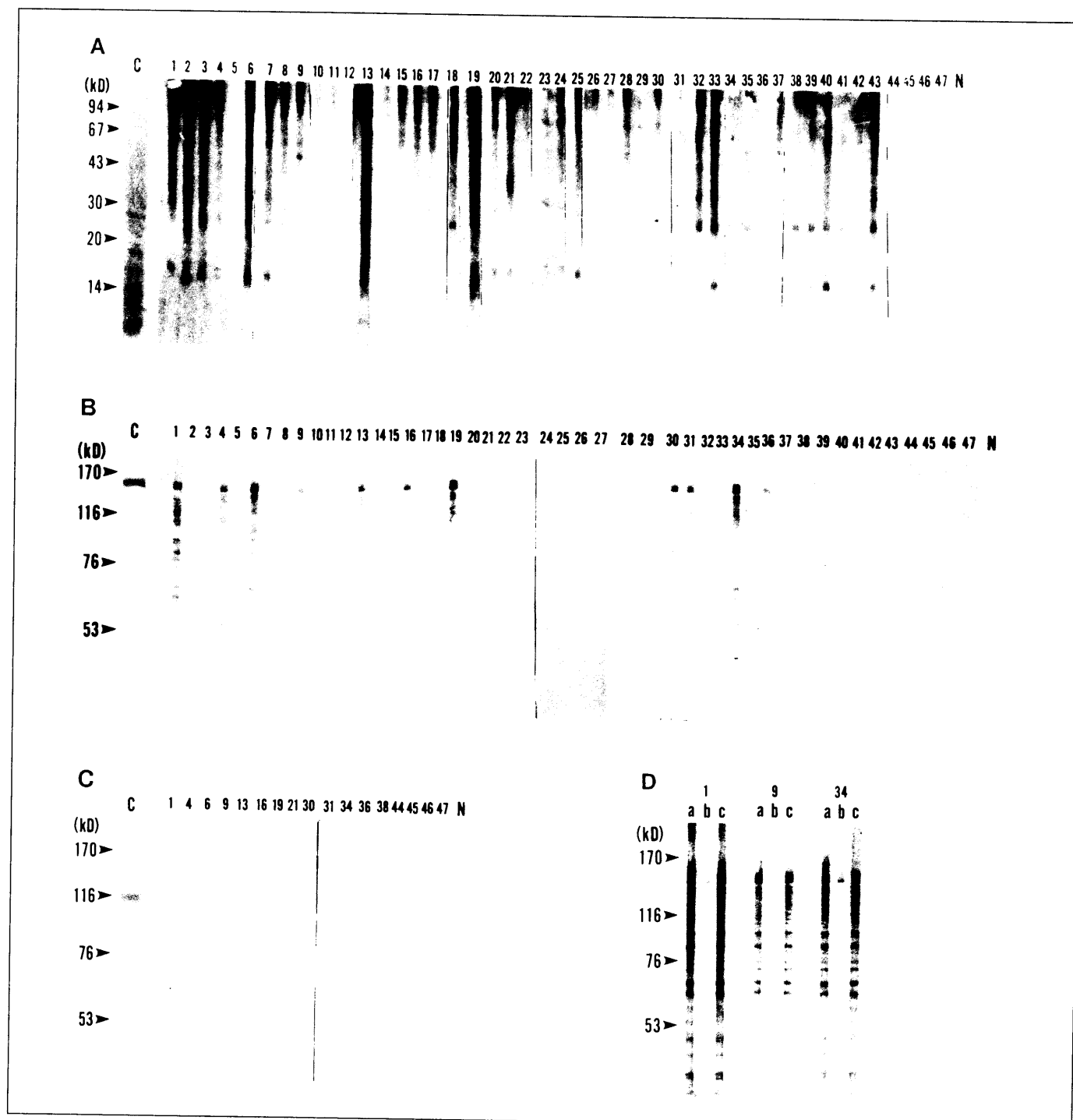


Fig. 2. Reaction of Dfb, β -Gal-Mag1, and β -Gal with specific IgE in individual allergic sera. Dfb (**A**) and whole cell lysates prepared from 50 μ l cultures of *E. coli* pop2136 carrying pAEX102 (**B, D**) or pUEX2 (**C**) by boiling with 2% SDS were separated on a 12.5% polyacrylamide gel. After SDS-PAGE [36], proteins were visualized with Coomassie brilliant blue R-250 (lanes C). Electrophoretic transfer was performed on Immobilon membranes (Millipore Corporation, Bedford, Mass., USA) [37]. The blots (**A–D**) were overlaid with mite-allergic sera (lanes 1–43), sera with high IgE titers from patients allergic to other allergens (lanes 44–46), serum from a healthy donor (lane 47), and skim milk (lane N) and then visualized by immunostaining. **D** Before overlaying the membrane with primary mite-allergic sera, the sera were preabsorbed with 3% skim milk (lanes a), 100 μ g of Dfb (lanes b), and 100 μ g of cedar pollen extract (lanes c) containing the same skim milk. The positions of molecular mass makers are shown by arrows.

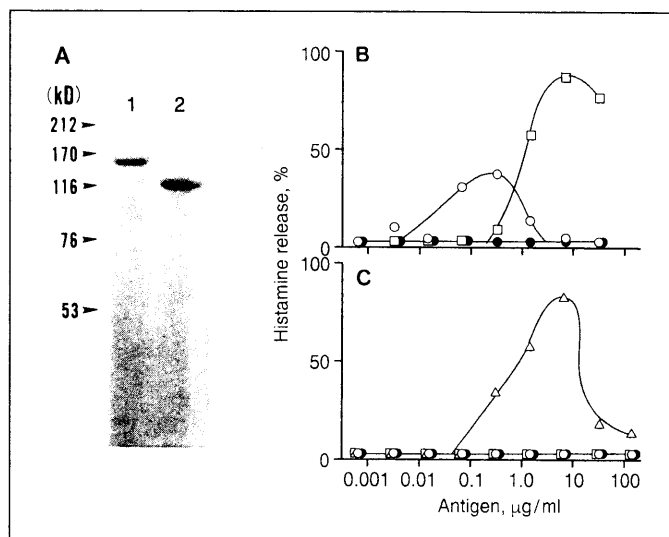


Fig. 3. SDS-PAGE and histamine release tests of purified β -Gal-Mag1 and β -Gal. **A** Twenty micrograms of purified β -Gal-Mag1 (lane 1) and β -Gal (Lane 2) was electrophoresed and stained under the same conditions used in figure 2. The arrows are the same as those used in the legend to figure 2. **B, C** Washed blood cells from 2 donors with mite allergy (**B**) and cedar pollen allergy (**C**) were stimulated with an appropriate diluted series of an antigen solution. Histamine released in the supernatant during the incubation was measured by HPLC. β -Gal-Mag1 ($\circ$) and β -Gal ($\bullet$) were used as stimuli. Simultaneously, Dfb ($\square$) and an extract of cedar pollen ($\triangle$) were used as positive and negative controls, respectively.

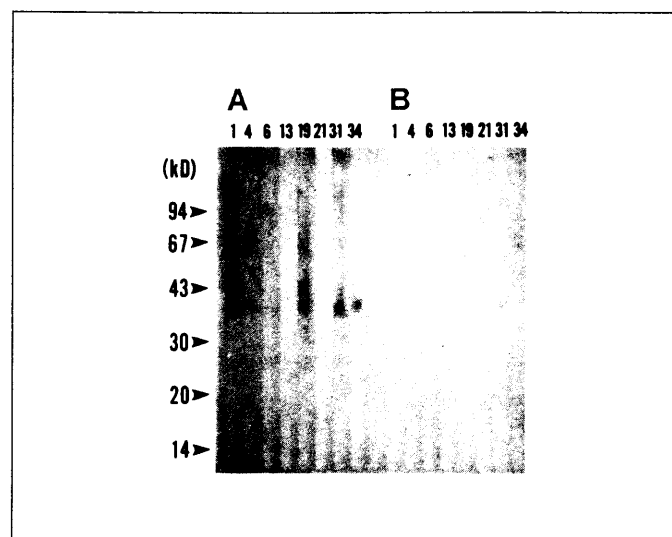


Fig. 4. Immunoblotting of blots of the native mite component that shares IgE-binding determinants with Mag1. One hundred micrograms of Dfb was separated on a 12.5% polyacrylamide gel. After electroblotting, a blot of Dfb was overlaid with intact Mag1-specific human IgE (**A**), or Mag1-specific human IgE pretreated with 50 μ g of purified β -Gal-Mag1 (**B**). The membrane was visualized by immunostaining.

and relevant information are not available for other unknown allergens, because of the difficulty in their purification from a spent mite culture. Furthermore, there are two shortcomings when primary screening for other allergen genes is carried out using mite-allergic sera. One default is derived from the poor reproducibility due to remarkable differences in IgE specificities to various mite allergens in the sera. The other is low reactivity owing to incorrect folding of the recombinant allergen [19]. Thus, we used serum raised against Dfb for the primary screening.

Major allergens have been selected so far in terms of both the frequency of the response by allergic subjects and the proportion of total IgE directed against these allergens. In fact, *Der f*I and II corresponding to 25–30 and 14–16 kD, respectively, on SDS-PAGE bound to specific IgE with a considerably high frequency (22/43 and 24/43, fig. 2A). However, purified *Der f*I and II, spotted on an Immobilon membrane, reacted with relatively low frequency (2/15 and 2/15, respectively, data not shown) with specific IgE of newly visited mite-allergic patients, who were selected at random under milder criterion so that

histamine release was elicited from their washed blood cells by Dfb stimulation. On the other hand, the reaction of purified β -Gal-Mag1 to specific IgE was detected with a significantly high frequency (5/15, data not shown) on blots, although a fairly low reactivity (13/43) to specific IgE was observed for a 39-kD component on blots (fig. 2A). Moreover, the results obtained on the histamine release test suggested that β -Gal-Mag1 had a potent allergenicity that accounted for about 30% release of the total histamine in their washed blood cells (fig. 3). Accordingly, results on blots and the histamine release test suggest that the antigen would be of clinical importance, although the total number of allergic cases is not sufficient for clinical assessment.

When comparing with the antigen concentration giving 50% of the maximum release rate in each case, results obtained on the histamine release test indicated that the native antigen, sharing IgE-binding determinants with Mag1, in the Dfb preparation would be present at a concentration of less than 1/400th by weight at the most. Simultaneously, similar results were obtained by the inhibition test using β -Gal-Mag1 as competitor in an ELISA

(Dfb/rabbit anti-Dfb serum) (data not shown). However, if the native antigen actually occurred even at such a low concentration in the Dfb preparation, the antigen would make an about 30% contribution to the histamine release elicited by allergens in the Dfb preparation. The real contribution of the native antigen to the allergen concentrations in the Dfb preparation or other preparations is now under investigation.

The nucleotide sequence analysis and the homology search of cDNA revealed that this cDNA encodes a new allergen. At present, we do not yet know whether the first ATG codon at nucleotide positions 7–9, 97–99, or 148–150 is the translation initiation site that corresponds to a calculated molecular weight of 39,421, 35,896, or 34,123, respectively, as a mature protein. On the other hand, several

bands in the range of more than 39-kD were occasionally detected on blots (fig. 4A) by using IgE in several mite-allergic sera and frequently detected when human IgG from mite-allergic patients was used as a probe. Whether the reaction was due to cross-reactivity or non specific absorption is now under investigation. Furthermore, the N-terminal analysis of the native antigen remains unsolved and will be discussed in the near future.

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