

Immunochemical characterization of recombinant and native tropomyosins as a new allergen from the house dust mite, *Dermatophagoides farinae*

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Background: Two predominant mite species, *Dermatophagoides farinae* and *Dermatophagoides pteronyssinus*, are responsible for the immediate hypersensitivity reaction. Although a large number of antigens and allergens was detected in a whole mite culture extract, only the major allergens of Der I and II groups have been extensively studied. Much information on other important allergens remains to be accumulated.

Methods: A new allergen complementary DNA (cDNA) clone was isolated from a *D. farinae* protein cDNA library. The expression product of the cDNA and native tropomyosin from *D. farinae* were purified, characterized, and evaluated.

Results: An amino acid sequence deduced from the cDNA clone revealed significant homology with tropomyosins conserved in a wide range of animals. The amino acid sequences of two fragments obtained by degradation of the purified protein with cyanogen bromide coincided completely with the corresponding portions of the deduced amino acid sequence. The native tropomyosin reacted with specific IgE in the 31 sera tested at a high frequency (80.6%), comparable to that of Der f I (90.3%) and Der f II (74.2%).

Conclusions: The cloned cDNA encodes tropomyosin. The high binding response of specific IgE antibodies to recombinant and native tropomyosins demonstrates that mite tropomyosin is an additional important allergen in house dust mite. (*J ALLERGY CLIN IMMUNOL* 1995;96:74-83.)

Key words: Mite allergen, complementary DNA, sequencing, tropomyosin, *Dermatophagoides farinae*

The IgE-mediated immediate type of allergies are a serious problem for patients with symptoms such as bronchial asthma, perennial rhinitis, and dermatitis. Many patients with allergy, especially in advanced countries, are sensitized with allergens

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Abbreviations used

BSA:	Bovine serum albumin
cDNA:	Complementary DNA
Dfb:	Mite body extract from <i>Dermatophagoides farinae</i>
GST:	Glutathione S-transferase
mag:	Complementary DNA coding for recombinant mite antigen
PAGE:	Polyacrylamide gel electrophoresis
SDS:	Sodium dodecylsulfate

derived from two predominant species of the house dust mite, *Dermatophagoides farinae* and *Dermatophagoides pteronyssinus*.¹⁻³

The total number of allergenic components identified in extracts of mite bodies and mite feces

by immunoelectrophoretic analyses have been reported to be 4 to 26 and 5 to 19, respectively.^{4,5} The frequency and capacity of IgE bindings of the allergens on blots varied with patients sensitive to mite.^{6,7} Only allergens in *Der* I (25 kd), II (15 kd), III (29 kd), IV (56 kd), and VI (25 kd) groups have been isolated as major or important allergens.⁸⁻¹⁴ However, even the major allergens in *Der* I and *Der* II groups that have been intensively studied exhibit multiple components possessing different isoelectric point values on isoelectrofocusing.^{9,10,15-17} Recently, to overcome these problems, the corresponding complementary DNA (cDNA) in *Der* I, II, and III groups were sequenced.¹⁸⁻²² In addition, Tovey et al.²³ reported the sequence of a cDNA, *Der* V, coding for an IgE-binding component of 14 to 15 kd, which apparently is not sequence homologous with the major allergen *Der* II. However, a large number of unknown allergens remain to be characterized.

Recently, Shanti et al.²⁴ identified a shrimp allergen (Sa-II) as tropomyosin and reported the amino acid sequences of two tryptic peptides containing major IgE-binding determinants on the molecule. We report here that the tropomyosin of the house dust mite, *D. farinae*, is a new allergenic component. We found that an amino acid sequence deduced from a newly isolated cDNA clone coding for mite allergen shares high homology with the known amino acid sequences of several forms of tropomyosins that function as actin-binding proteins in skeletal and smooth muscle and in nonmuscle tissues²⁵ and that are highly conserved during the process of evolution. Furthermore, we suggest that the cloned cDNA contains a complete protein-coding sequence corresponding to the amino acid sequence of native tropomyosin isolated from a mite body extract.

METHODS

Material

Mites were supplied by Fumakilla Ltd., Hiroshima, Japan, and were used for preparation of mite body extract (Dfb) as described previously.²⁶ Restriction endonucleases and DNA-modifying enzymes were purchased from Takara Shuzo, Kyoto, Japan. The purified antigens, *Der f* I and *Der f* II, and their antisera were kindly provided by Dr. H. Yasueda of the National Sagamihara Hospital, Kanagawa, Japan.

Human sera and antisera

Thirty-one sera collected from patients sensitive to mite were selected from 121 newly visited patients with bronchial asthma on the basis of higher skin reactions to mite and house dust extracts, and higher reactivity to

Dfb on blots. Control sera from healthy subjects and from patients sensitive to cedar pollen or *Candida* antigen, but insensitive to mite and house dust extracts, were also studied. Rabbit anti-Dfb serum was prepared as described previously.²⁶

Construction and screening of *D. farinae* cDNA library

Poly(A)-mRNA was isolated from live mites with a Fast Track mRNA isolation kit (Invitrogen Co., San Diego, Calif.) after guanidinium isothiocyanate extraction. A cDNA library was prepared in λ gt11 (Stratagene, La Jolla, Calif.) vector²⁷ in accordance with manufacturer's directions. The cDNA library of 1.38×10^7 recombinants was immunoscreened with rabbit anti-Dfb serum as described previously.²⁸

DNA cross-hybridization

After agarose gel electrophoresis of λ recombinant DNAs digested with *Not* I, cDNA fragments were extracted with a GeneClean II kit (Toyobo Co. Ltd., Osaka, Japan). The respective cDNA fragments spotted onto a nylon membrane (Hybond-N, Amersham International plc, Amersham, U.K.) were hybridized with digoxigenin-labeled cDNA probes prepared from the longest cDNA fragment. The DNA hybrid formed was detected with antidigoxigenin antibody according to the manufacturer's direction (DIG DNA labeling and detection kit, Boehringer Mannheim GmbH, Mannheim, Germany). The procedures were repeated with digoxigenin-labeled cDNA probes prepared from the longest cDNA fragment among the unhybridized cDNA fragments. Thus the cross-hybridization enabled us to classify the cDNA inserts coding for immunoreactive proteins into nine discrete groups.²⁸

Expression and purification of recombinant mite allergen

Cultivation of a transformant and purification of glutathione S-transferase (GST)-fused allergenic product were carried out according as described previously.²⁸

In brief, the cDNA insert was subcloned into the *Sma* I site on plasmid pGEX-2T (Amersham International plc). The resultant plasmid was transformed into *Escherichia coli* JM105. The transformant was cultured at 37°C in 2×YT medium. After induction with isopropyl β -D-thiogalactopyranoside, cells were harvested, lysed by sonication, and centrifuged. The recombinant protein fused to GST was specifically recovered on a glutathione Sepharose 4B column (Pharmacia LKB Biotechnology, Uppsala, Sweden) with phosphate-buffered saline solution (pH 7.4) containing 0.1% deoxycholic acid, 2 mmol/L ethylenediaminetetraacetic acid, and 0.1% 2-mercaptoethanol. Simultaneously, nonfused GST was purified. The truncated recombinant protein was purified with the same affinity column after digestion of the recombinant fusion protein with thrombin.

Nucleotide sequencing

The *Not* I-ended cDNAs encoding new mite antigens were subcloned into the same site on a phagemid pBluescript II SK(+) DNA. Fragments excised from the subcloned plasmid by digestions with various restriction endonucleases were ligated into suitable sites on M13-derived vectors, mp18 or mp19. Nucleotide sequencing by the dideoxynucleotide chain termination method²⁹ was performed with an AutoRead Sequencing kit and an A.L.F. DNA sequencer (Pharmacia LKB Biotechnology).

Purification of native tropomyosin

Tropomyosin was isolated by a modification of the method of Smillie.³⁰ Dfb (600 mg) was extracted with 30 ml of 1 mol/L KCl and 0.5 mmol/L dithiothreitol (pH 7.0), and centrifuged. The supernatant was adjusted to pH 4.6 and stirred for 30 minutes. After centrifugation at 5000 g for 15 minutes, the precipitate was dissolved in extraction buffer and centrifuged. The isoelectric precipitation at pH 4.6 and dissolution in extraction buffer at pH 7.0 were repeated twice, and final precipitate was dissolved in 26 ml of 0.5 mmol/L dithiothreitol (pH 7.0). A prechilled solution (29.3 ml) of 100%-saturated ammonium sulfate was added and stirred for 30 minutes. After centrifugation at 10,000 g for 20 minutes, 9.7 ml of the ammonium sulfate solution was added to the supernatant. The suspension was stirred and centrifuged again. The precipitate was dissolved in 1 ml of 0.5 mmol/L dithiothreitol, dialyzed against 2 mmol/L 2-mercaptoethanol, and lyophilized.

Amino acid sequencing

Fifty micrograms of the purified tropomyosin was degraded overnight at room temperature with 7 μ g of cyanogen bromide in 500 μ l of 70% formic acid and evaporated. Resultant peptides were fractionated by reversed-phase chromatography on a TSKgel ODS-120T column (4.6 $\times$ 250 mm, Tosoh Corp., Tokyo, Japan) or by gel filtration chromatography on an Asahipak GS-320 column (7.6 $\times$ 500 mm, Asahi Kasei Corp., Kanagawa, Japan) and successive reversed-phase chromatography on a TSKgel Octadecyl-4PW column (4.6 $\times$ 150 mm, Tosoh Corp.). Amino acid sequence was determined by the automated Edman degradation method³¹ on a protein sequencer (model 473A, Applied Biosystems Japan Inc., Tokyo, Japan).

SDS-PAGE and immunoblotting

SDS-PAGE was performed according to the method of Laemmli.³² The separated proteins were electrotransferred from the gel to an Immobilon-P membrane (Millipore Corp., Bedford, Mass.) with a semidry blotting apparatus and visualized by immunostaining.^{26, 28, 33}

Dot immunoblot test

One microliter of a series of diluted antigen (0.25, 0.05, and 0.01 μ g/ml) was spotted on a nitrocellulose membrane and air-dried. Detection of binding of IgE in individual human serum was the same as in the immunoblotting analysis.

ELISA

ELISA was carried out as described previously.³³

In brief, a microtiter plate (96 wells, Falcon 3912, Becton Dickinson, Oxnard, Calif.) was coated with the native tropomyosin (0.5 μ g/well) in 0.1 mol/L sodium bicarbonate buffer, pH 9.5, containing 0.5 mmol/L dithiothreitol. After the well was blocked with 1% bovine serum albumin (BSA) in phosphate-buffered saline solution containing 0.05% polysorbate 20 and 0.02% thimerosal, a series of serum sample diluted with the same buffer was applied and incubated. The well was thoroughly washed and incubated successively with biotin-conjugated goat anti-human IgE ($\times$ 500, Kirkegaard & Perry Labs, Inc., Gaithersburg, Md.) and peroxidase-conjugated streptavidin ($\times$ 500, Tago Inc., Burlingame, Calif.), followed by enzyme reaction for 10 minutes in 10 mmol/L citrate buffer (pH 4.2), containing 1 mg/ml 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate) and 0.03% hydrogen peroxide.

Allergenic activity

The allergenic activity was evaluated with in vivo skin testing on newly visited patients with bronchial asthma as previously described.³⁴

RESULTS

Sequence analysis of a new mite allergen cDNA

From the nucleotide sequence of the representative cDNA fragments in each group, the amino acid sequence deduced from a cDNA fragment, *mag44*, was found to have remarkable homology with the primary amino acid sequences of tropomyosins from several animals. The complete sequence of *mag44* was shown to be 988 base pairs and included a 45-base-long 5' proximal end sequence, an open reading frame coding for the entire native protein (Mag44) of 284 amino acids (derived molecular weight, 32,954 daltons) terminating with a TAA stop codon, and a 91-base-long 3' noncoding region (Fig. 1). The hexapeptide motif, EAIKKK, important for binding to F-actin, was conserved in the N-terminal region of the deduced amino acid sequence. A periodic sequence constituting the α -helical turn and an extensive leucine zipper motif occurs throughout the sequence as in tropomyosins from other origins. The absence of an Asn-X-Ser(Thr) sequence indicates that there is no N-linked glycosylation site.

Fig. 2 shows the composite alignment of the deduced amino acid sequence with *Drosophila melanogaster* muscle tropomyosin II,³⁵ which has the highest homology score (76.1%), and rabbit³⁶ and human³⁷ α -tropomyosins, with homology scores of 58.8% and 58.1%, respectively. Amino acid sequences of different peptides gen-

TTT TTT TTC GTT GCA GCT AAA CAA CAA CAA CCA TCA ACA AAA ATG GAG GCC ATC AAG	60
Phe Phe Phe Val Ala Ala Lys Gln Gln Gln Gln Pro Ser Thr Lys Met Glu Ala Ile Lys	20
AAA AAA ATG CAG GCA ATG AAG CTC GAG AAA GAT AAT GCT ATC GAT CGA GCT GAA ATT GCC	120
Lys Lys Met Gln Ala Met <u>Lys Leu Glu Lys Asp Asn Ala Ile Asp Arg Ala Glu Ile Ala</u>	40
GAA CAA AAA GCC CGT GAT GCT AAT CTA CGT GCC GAA AAG TCT GAG GAA GAA GTT CGT GCA	180
<u>Glu Gln Lys Ala Arg Asp</u> Ala Asn Leu Arg Ala Glu Lys Ser Glu Glu Glu Val Arg Ala	60
TTA CAG AAA AAA ATC CAA CAA ATT GAA AAT GAA TTG GAT CAG GTC CAA GAA CAA TTA TCG	240
Leu Gln Lys Lys Ile Gln Gln Ile Glu Asn Glu Leu Asp Gln Val Gln Glu Gln Leu Ser	80
GCT GCC AAT ACA AAA TTG GAG GAA AAG GAA AAA GCC CTA CAG ACC GCT GAA GGT GAT GTT	300
Ala Ala Asn Thr Lys Leu Glu Glu Lys Glu Lys Ala Leu Gln Thr Ala Glu Gly Asp Val	100
GCA GCA TTG AAT CGT CGT ATT CAA TTG ATT GAA GAA GAT TTG GAA CGA TCA GAA GAA CGA	360
Ala Ala Leu Asn Arg Arg Ile Gln Leu Ile Glu Glu Asp Leu Glu Arg Ser Glu Glu Arg	120
CTT AAG ATT GCT ACA GCC AAA TTG GAA GAG GCA TCA CAA TCT GCC GAT GAA TCT GAA CGT	420
Leu Lys Ile Ala Thr Ala Lys Leu Glu Glu Ala Ser Gln Ser Ala Asp Glu Ser Glu Arg	140
ATG CGT AAA ATG CTT GAA CAT CGA TCC ATC ACC GAT GAA GAA CGT ATG GAT GGT TTG GAA	480
Met Arg Lys Met Leu Glu His Arg Ser Ile Thr Asp Glu Glu Arg Met <u>Asp Gly Leu Glu</u>	160
AAT CAA CTT AAA GAA GCC CGT ATG ATG GCC GAA GAT GCT GAT AGA AAA TAT GAT GAA GTT	540
<u>Asn Gln Leu Lys Glu Ala Arg Met</u> Met Ala Glu Asp Ala Asp Arg Lys Tyr Asp Glu Val	180
GCC CGT AAA TTG GCA ATG GTT GAA GCC GAT TTG GAA CGT GCT GAA GAA CGT GCC GAA ACC	600
Ala Arg Lys Leu Ala Met Val Glu Ala Asp Leu Glu Arg Ala Glu Glu Arg Ala Glu Thr	200
GGT GAA TCG AAA ATT GTT GAA CTC GAA GAA GAA TTA CGT GTT GTC GGT AAC AAT CTC AAA	660
Gly Glu Ser Lys Ile Val Glu Leu Glu Glu Glu Leu Arg Val Val Gly Asn Asn Leu Lys	220
TCA TTG GAA GTT AGC GAA GAG AAA GCT CAA CAA CGT GAA GAA GCC TAT GAA CAA CAG ATC	720
Ser Leu Glu Val Ser Glu Glu Lys Ala Gln Gln Arg Glu Glu Ala Tyr Glu Gln Gln Ile	240
CGT ATA ATG ACG GCT AAA CTT AAA GAA GCC GAA GCA CGT GCC GAA TTT GCT GAA CGT TCG	780
Arg Ile Met Thr Ala Lys Leu Lys Glu Ala Glu Ala Arg Ala Glu Phe Ala Glu Arg Ser	260
GTA CAA AAA CTC CAG AAA GAA GTC GAT CGT TTG GAA GAC GAA TTG GTC CAC GAA AAG GAA	840
Val Gln Lys Leu Gln Lys Glu Val Asp Arg Leu Glu Asp Glu Leu Val His Glu Arg Glu	280
AAA TAC AAA TCC ATC TCC GAC GAA TTG GAC CAG ACA TTT GCC GAA CTT ACT GGT TAT TAA	900
Lys Tyr Lys Ser Ile Ser Asp Glu Leu Asp Gln Thr Phe Ala Glu Leu Thr Gly Tyr ***	299
TAATAATTCTTTCATATTACGCAGACAGAAATTTTCGATTATTTTAAACAACACCACCACCAGCAACAGCAACAACG	979
AAAAATCAC	988

FIG. 1. Nucleotide sequence of *mag44* and derived amino acid sequence. Deduced amino acid sequence is shown in three-letter code, and terminal codon (TAA) is marked with *asterisks*. Nucleotide and amino acid numbers are represented on *right* and under sequence (*small print*), respectively. Two cyanogen bromide peptides sequenced are indicated beneath deduced amino acid sequence by *underlining*.

erated by proteolysis of shrimp tropomyosin²⁴ reveal a high homology with the deduced amino acid sequence of the cDNA.

Isolation of native tropomyosin

To establish that Mag44 is indeed tropomyosin, the latter was purified from Dfb by isoelectric precipitation and fractionation with ammonium

sulfate. The purified protein was obtained in 0.03% (wt/wt) yield.

The purified protein migrated on a SDS-polyacrylamide gel (Fig. 3, A, lane 5) as a band possessing a mobility corresponding to a molecular weight of 37 kd with a purity of more than 96% as assessed by a densitometer (Toyo Kagaku Sangyo DMU-33C, Tokyo, Japan) (data not shown). In the

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a FFFVAAKQQQPSTKMEAIKKKMQAMKLEKDNAIDRAETAEQKARDANLRAEKSEEEYRALQKKIQQIENELDQV
b . D . . . . . V D . . G . L E . . L V C . . E . . . . T . . . A . . A . Q . . . . T V . . . . T
c . D . . . . . M L . . D . E . . L . . . Q . . A D K K A . E D . S K Q L . D . L V S . . . . L K G T . D . . K Y
d . . . . . M L . . D . E . . L . . . Q . . A E Q K Q . E E . S K Q L . D . L A . M . . L K G T . D . . K Y
e . . . . . A . H E . . . R M . . L . D . . .

a QEQLSAANTKLEEKALQTAEGDVAALNRRILQLIEEDLERSEERLKIATAKLEASQEADESERNRKMLEHRSI
b . . A . T L V T G . . . . N . . . N . S E . . . . . L . . . . . G S . . . . S . . . A . . . . A . I . N . A L
c S . A . K D . Q E . . L A . K A T D . A . . S . . . . V . . E . D . A Q . . A T . . . . . E K A . . . . G M . V I . S . A Q
d S . A . K D . Q E . . L A . K A A D . A E . . S . . . . V . . E . D . A Q . . A T . L Q . . . . E K A . . . . G M . V I . N . A L
e . . S . L K . . I Q . V . . D . . S N . . . E . . . . . L . . . . . A . . . A . . . .

a TDEERNMDGLENQLKEARMMAEDADRKYDEVARKLAMVEADLERAEERAETGESKIVELEELRVVGNLKSLEYS
b A . . . . A . . . . F L . . E . K . . . . . Q . N . . . . .
c K . . K . E I Q . I . . . . K H I . . . . . D . . . . V I I . S . . . . L S . G . C A . . . . K T . T . . . . A Q
d K . . K . E L Q . I . . . . K H I . . E . . . . E . . . . V I I . G . . . T . . . L A . . C S . . . . K N . T . . . . A Q
e . . . . F L . . E . . . . .

a EEKAQQRREEAYEQQIRIMTAKLKEAEARAFAERSVQKLQKEVDRLEDELYHEKEKYKSISDELDQTFABELTCY
b . . . S N . . . E . K N . . K T L N T R . . . . . D . . . R . D . G . D . . T A . V . . I L K E
c A . . Y S . K . D K . . E E . K V L S D . . . . T . . . . T . . E . S I . D . . . . Y A Q . L . . A . . . H A L N D M . S I
d A . . Y S . K . D K . . E E . K . L . D . . . . T . . . . A . . E . T I . D . . . . Y A Q . L . . A . . . H A L N D M . S I
e . . S N . . . E . K N . . K . . . . . . . . . . . . . . . N . . . . Q

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FIG. 2. Composite alignments of deduced amino acid sequence of *mag44* (row a) with *Drosophila melanogaster* muscle tropomyosin II (row b), rabbit skeletal muscle α -tropomyosin (row c), human skeletal muscle α -tropomyosin (row d), and shrimp tropomyosin (partial peptide fragments) (row e). Sequences are shown by one-letter code, and amino acid residues identical to those in *Mag44* molecule are indicated by dots in rows b to e.

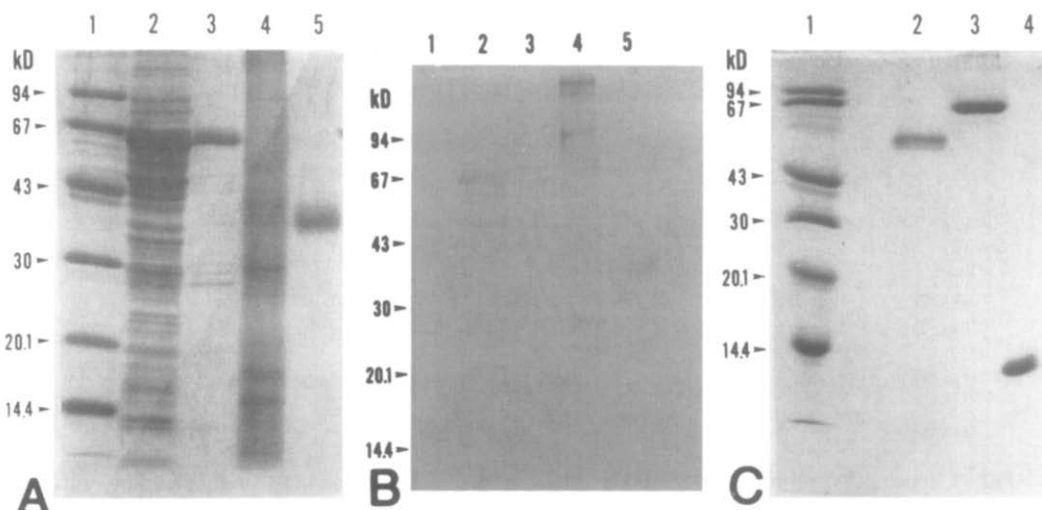


FIG. 3. SDS-PAGE of recombinant fusion protein and purified protein. Proteins were separated under reducing condition on 12.5% SDS-polyacrylamide gel in absence (A and B) or presence (C) of 6 mol/L urea. A, Separated proteins were visualized by staining with Coomassie Brilliant Blue R-250. Molecular weights in kilodaltons are represented at left. Lane 1, Molecular weight markers; lane 2, total cell lysate (10 μ l) of *E. coli* JM105 transformant carrying *mag44*-inserted pGEX-2T; lane 3, purified GST-*mag44* fusion (2 μ g); lane 4, Dfb (10 μ g); lane 5, purified protein (2 μ g). B, Separated proteins were electrotransferred to membrane and immunostained by probing with IgE antibodies in pooled serum of 10 patients sensitive to mite. Symbols are same as in A. C Band of purified protein was detected at position corresponding to apparent molecular weight of 50 kd when protein was stained with Coomassie Brilliant Blue R-250. Lane 1, Molecular weight markers; lane 2, purified protein (2 μ g); lane 3, BSA (2 μ g); lane 4, cytochrome c (2 μ g).

presence of 6 mol/L urea, the mobility of the purified protein on SDS-polyacrylamide gel changed to give an apparent molecular weight of 50 kd, whereas BSA (67 kd) and cytochrome C

(12.5 kd) controls migrated at the positions expected for their molecular weights (Fig. 3, C). This change in electrophoretic behavior is characteristic of tropomyosins and indicates that the

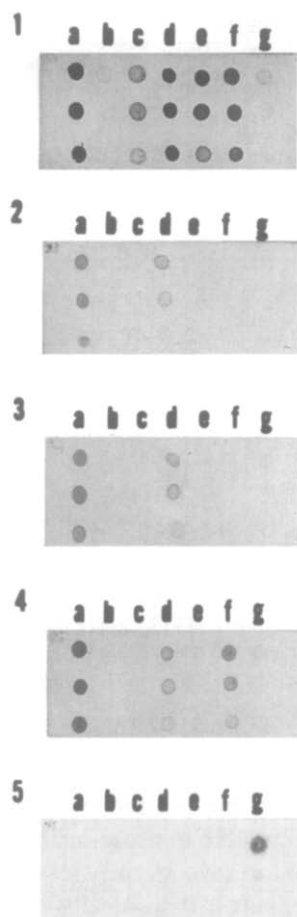


FIG. 4. Dot spot tests. Series of diluted proteins (upper row, 0.25 μ g; middle row, 0.05 μ g; lower row, 0.01 μ g) were spotted on nitrocellulose membranes. Each membrane was incubated with sera from patients sensitive to mite (panels 1 to 4) and pool of sera from subjects with cedar pollen and *Candida* allergy with high IgE titers (1500 and 1600 IU/ml, panel 5). Binding specific IgE was detected as described in text. Column a, Dfb; column b, recombinant GST; column c, purified recombinant GST-mag44; column d, purified native tropomyosin; column e, native *Der f* I; column f, native *Der f* II; column g, cedar pollen extract.

purified protein is indeed native tropomyosin. However, the IgE-binding response of immunoblotting showed that the protein in the Dfb preparation was barely included in the gel (Fig. 3, B, lane 4).

Because the N-terminal amino acid residue of the native tropomyosin is blocked, the protein was degraded with cyanogen bromide. Only two peptide fragments were purified as nonoverlapping and symmetric peaks, respectively, and these were sequenced by the Edman degradation method. The identified sequences perfectly matched amino acids 12 to 31 and 142 to 153 in the sequence deduced from the *mag44* cDNA (see Fig. 1). Thus we conclude that this cDNA fragment codes for mite tropomyosin.

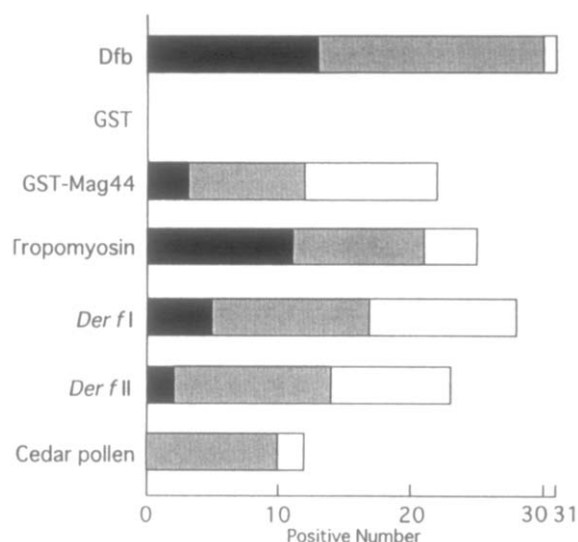


FIG. 5. Frequency and capacity of IgE bindings. Numbers of positive sera were indicated as abscissa when 0.25 μ g (white bar), 0.05 μ g (shaded bar), and 0.01 μ g (black bar) of respective antigens were spotted on membranes and probed with peroxidase-conjugated anti-human IgE after membranes were overlaid with individual serum of patient sensitive to mite.

IgE-binding study of recombinant fusion and native tropomyosins

An expression plasmid carrying *mag44* was constructed on plasmid pGEX-2T and transformed into *E. coli* JM105. A soluble fraction containing the recombinant tropomyosin fused to GST (GST-Mag44) was recovered from the whole cell lysate of the transformant and affinity-purified on a glutathione Sepharose column (Fig. 3, A, lane 3). Fifty micrograms of the GST-Mag44 fusion protein was obtained from 150 ml of the culture.

The IgE-binding response of the GST-Mag44 fusion protein was qualitatively similar but at least 25 times weaker than that of the purified native tropomyosin (data not shown). The native tropomyosin was also immunoreactive to IgE in a pool of 10 sera from GST-Mag44-reactive patients allergic to mites and house dust (Fig. 3, B, lane 5) but was not immunoreactive to IgE in patients nonreactive to GST-Mag44 (data not shown).

The frequency and capacity of IgE bindings of the tropomyosins (the fusion protein and the purified native tropomyosin) were compared by dot spot tests with those of the native major allergens, *Der f* I and *Der f* II. IgE in sera of 31 patients allergic to mites and house dust reacted with the native tropomyosin, *Der f* I, or both. The IgE-binding responses of the patients to the tropomyosin molecule did not correlate with those to the cedar pollen allergens, although IgE specific to cedar pollen extract was detected in 12 sera. The

TABLE I. Allergenicity of native and truncated recombinant tropomyosins

Case No.	Extract		Tropomyosin		<i>Der f</i>	
	House dust	mite	Native	Recombinant	I	II
1	+++	++	+++	+++	+	++
2	+++	++	++	++	++	++
3	+++	++	++	++	++	++
4	+++	++	+++	+	-	+
5	++	++	-	-	-	-
6	-	++	-	-	-	-
7	-	++	-	-	-	-
8	+++	+	++	-	-	+++
9	-	+	-	-	-	-
10	-	+	-	-	-	-
11	++	-	+++	+++	+	+++
12	±	-	-	-	-	-
13	-	-	-	-	-	-

Allergenic activity was evaluated with in vivo skin test on newly visited patients with bronchial asthma by injecting intracutaneously 20 μ l of solution containing native tropomyosin (5 μ g/ml), truncated recombinant tropomyosin (5 μ g/ml), *Der f* I (5 μ g/ml), *Der f* II (5 μ g/ml), house dust extract (1:1000) (Torii & Co., Tokyo, Japan), or mite extract (1:10,000) (Torii & Co.) dissolved in 0.9% NaCl and 0.5% phenol. When erythema, appearing after 15 minutes, was 0 to 9 mm, 10 to 19 mm, 20 to 29 mm, 30 to 39 mm or more than 40 mm in mean diameter, score of -, $\pm$, +, ++, or +++ was assigned, respectively.

intensity and the selectivity of the binding reactions of these antigens to IgE were largely dependent on the differences in persons. Representative results are shown in Fig. 4. A good correlation for IgE-binding selectivity between the recombinant fusion tropomyosin and native tropomyosin implies that they both possess IgE-binding determinants. It is noteworthy that the IgE antibodies of the serum of one patient (Fig. 4, panel 2) were specifically bound to the tropomyosins except for Dfb, and similar results were obtained with two other sera (data not shown).

The results of the IgE bindings are summarized in Fig. 5. The frequency of the IgE binding to 0.25 μ g of the native tropomyosin was 80.6% (25/31), which is comparable to those for the native major allergens, that is, 90.3% (28/31) for *Der f* I and 74.2% (23/31) for *Der f* II. Higher frequencies of IgE binding occurred at lower concentration of the native tropomyosin than for the other antigens. On the other hand, positive IgE-binding reactions to the tropomyosin, *Der f* I, and *Der f* II were observed in five, three, and two of 18 sera, respectively, from persons with allergy who had negative skin reactions to mite and house dust extracts. Of the five sera that bound to the tropomyosin, two also reacted with *Der f* I and cedar pollen (data not shown). Moreover, IgE antibodies in four sera of healthy donors did not bind to the tropomyosin. Table I shows that the allergenic activities of the

native and the truncated recombinant tropomyosins were comparable to those of *Der f* I and *Der f* II as examined in vivo by skin test, but the truncated recombinant tropomyosin was allergenically less active than the native one. These results confirm that tropomyosin reacts significantly with serum IgE from patients allergic to mites and that it represents as an important allergen.

The antibody-binding specificities of native tropomyosin were also assessed by ELISA. IgE antibodies in two sera of patients who possessed positive reaction to the native tropomyosin (in dot spot tests) bound to the tropomyosin coated on wells in proportion to the amount of the antibody. No significant IgE binding was observed in four sera of tropomyosin-insensitive patients, including two with high IgE titers (1500 and 1600 U/ml) from patients allergic to cedar pollen or to *Candida* antigen (Fig. 6, A). No reaction was observed for any sera when BSA and cytochrome c were used as coating antigen in place of the tropomyosin (data not shown). Furthermore, specific bindings of rabbit anti-*Der f* I or anti-*Der f* II IgG antibody to the tropomyosin were not significant in comparison with anti-Dfb serum (Fig. 6, B). Similar antibody specificities were reproduced by immunoblotting with rabbit anti-*Der f* I or anti-*Der f* II IgG antibody after SDS-PAGE (data not shown). These results indicate that the ability of the

tropomyosin to bind IgE is specifically displayed by patients allergic to mites and that the tropomyosin can be discriminated from the two major mite allergens by the antibody specificity.

DISCUSSION

Screening for an allergen gene usually requires the acquisition of antiserum raised against the target protein, the collection of a large amount of human sera allergic to the protein, or the synthesis of DNA probes encoding certain sequences of the target protein. When none of these are available, we find that it is very effective to concentrate in advance the target clones from the allergen cDNA library by screening with antisera raised against crude allergen preparation. The primary screening of the cDNA library with rabbit serum may be suitable for detection of all clones coding for a relatively weak-reactive allergen such as the gene expression product. With this strategy we have succeeded in cloning a new 39 kd allergen,²⁶ a heat shock protein 70,²⁸ and, as described in this report, tropomyosin, all of which may be important agents in mite allergies.

The predicted amino acid sequence of the new mite allergen Mag44 was completely coincident with partial sequence fragments of the purified native tropomyosin. It is well known that tropomyosin is a regulatory protein for the process of contraction in skeletal muscle in association with the troponin complex.²⁵ The highly conserved hexapeptide sequence, comprising DAIKKK, in the N-terminal region of the α -tropomyosins contributes to their binding to F-actin.³⁸ Aspartic acid residue in N-terminus of the motif is replaced by glutamic acid residue for the Mag44 molecule. However, this substitution applies to the sequence of human α -tropomyosin. It is interesting to note that profilin, which binds to actin and regulates a polymerization of the actin molecules, might be one of the important pan-allergens in grass pollen allergy.³⁹ Furthermore, it was recently reported that shrimp tropomyosin binds to the specific IgE antibodies in sera from subjects with shrimp allergy,²⁴ but cross-reactivity of the mite and shrimp tropomyosins is not evident.

It is remarkable that the IgE-binding frequency of the native tropomyosin (80.6%) was a match for that of the major allergens (Fig. 5). The occurrence of patients with allergy (3/31) who had IgE reactions positive to tropomyosin but negative to the major allergens confirms that tropomyosin is a discrete house dust mite allergen (Fig. 4, panel 2). IgE-binding capacity of the recombinant fusion

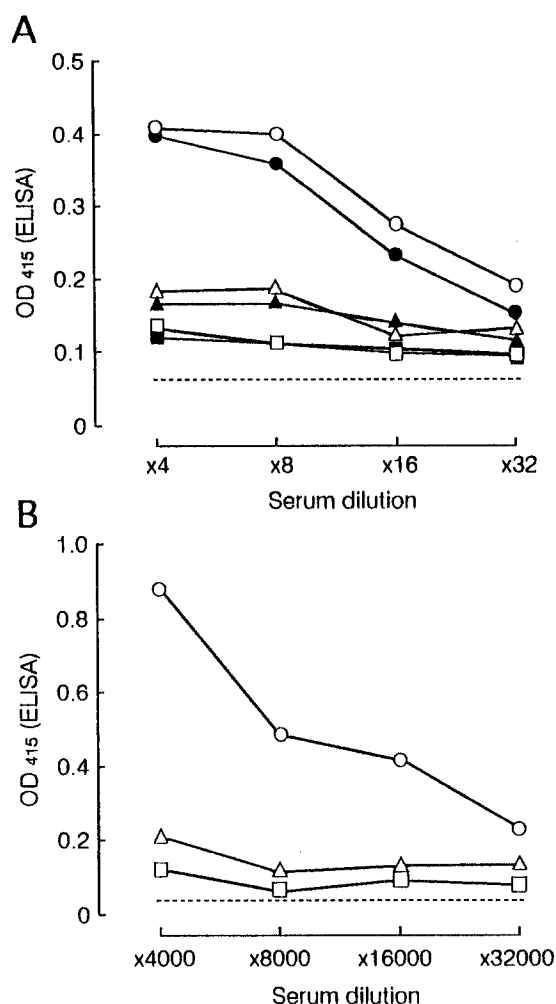


FIG. 6. ELISA plates coated with native tropomyosin. **A**, Detection was carried out by probing with peroxidase-conjugated anti-human IgE after wells were overlaid with series of properly diluted sera of two patients sensitive to mite (○, ●), two patients sensitive to mite but insensitive to tropomyosin (△, ▲), and two patients sensitive to cedar pollen or *Candida* but insensitive to house dust and mites (□, ■). Dotted line shows control. **B**, Detection was carried out by probing with peroxidase-conjugated anti-rabbit immunoglobulins after wells were overlaid with series of properly diluted sera of rabbit anti-Dfb (○), anti-Der f I (△), or anti-Der f II (□). Dotted line shows control.

tropomyosin was significantly weaker than that of the native protein, but it is not unusual for biologic activity of a recombinant protein produced in prokaryotic cells to be weaker than that of the corresponding native protein.¹⁸ In addition, allergenic activity of the truncated recombinant tropomyosin was slightly weaker than that of the native one. Unfolding of the recombinant protein may be a contributory factor but does not fully explain the deficiency in immunochemical activity of the re-

combinant protein. Attempts to produce the recombinant proteins by other host-vector systems are in progress.

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