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Der f 16: a novel gelsolin-related molecule identified as an allergen from the house dust mite, *Dermatophagoides farinae*[1]

Seiji Kawamoto[a,*], Takayuki Suzuki[a], Tsunehiro Aki[a], Takashi Katsutani[b], Shinji Tsuboi[c], Seiko Shigeta[a], Kazuhisa Ono[a]

[a] *Department of Molecular Biotechnology, Graduate School of Advanced Sciences of Matter, Hiroshima University, 1-3-1 Kagamiyama, Higashi-Hiroshima 739-8530, Japan*
[b] *Katsutani Clinic, Hatsukaichi, Japan*
[c] *Tsuboi Clinic, Otake, Japan*

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Abstract Allergen from the house dust mite (*Dermatophagoides* sp.) is a major trigger factor of allergic disorders, and its characterization is crucial for the development of specific diagnosis or immunotherapy. Here we report the identification of a novel dust mite (*Dermatophagoides farinae*) antigen whose primary structure belongs to the gelsolin family, a group of actin cytoskeleton-regulatory proteins. Isolated mite cDNA, termed Der f 16, encodes 480 amino acids comprising a four-repeated gelsolin-like segmental structure, which is not seen in conventional gelsolin family members. Enzyme immunoassay indicated that recombinant Der f 16 protein, prepared using an *Escherichia coli* expression system, bound IgE from mite-allergic patients at 47% (8/17) frequency. This is the first evidence that the gelsolin family represents a new class of allergen recognizable by atopic patient IgE.

Key words: Allergen; Asthma; Gelsolin; House dust mite; IgE

1. Introduction

Allergens from the house dust mites, *Dermatophagoides farinae* and *Dermatophagoides pteronyssinus*, are major environmental trigger factors for allergic disorders, such as asthma, atopic dermatitis, or rhinitis [1,2]. Toward the development of diagnostics and/or a therapeutic vaccine, important mite allergens have been explored, and now classified as major mite antigens. Among them, the group 1 (cysteine protease) and 2 allergens are recognized primarily important because of their high IgE-binding frequency [3–6]. The groups 3 (trypsin), 6 (chymotrypsin), and 9 (collagenase) family members are serine proteases which provoke hyperreactivity as well as tissue inflammatory reaction [7–12]. The groups 4 (amylase), 5, 7, 8 (glutathione *S*-transferase (GST)), and 10 (tropomyosin) members have been identified as additional major allergens [13–18]. Recent studies on high-molecular size allergens have elucidated new major antigens including the groups 11 (paramyosin, 98 kDa), 14 (also referred to M-177, apolipophorin-like 177 kDa molecule), and 15 (98/109 kDa chitinase-like protein, identified using canine IgE) [19–23]. In addition, the groups 12 and 13 (fatty acid-binding protein) allergens have been isolated from the tropical mite (*Blomia tropicalis*), although their *Dermatophagoides* counterparts have yet to be investigated [24,25]. These major mite allergens thus far identified could be re-categorized as follows: enzymes (groups 1, 3, 4, 6, 8, 9, and 15), cytoskeletal molecules (groups 10 and 11), lipid transport molecules (groups 13 and 14), and others (groups 2, 5, 7, and 12). The cytoskeletal allergens, which are ubiquitously expressed and often highly homologous amongst species, might be important in terms of IgE cross-reactivity. In fact, invertebrate tropomyosin has been recognized as an important cross-sensitizing pan-allergen found not only in house dust mites, but also in shrimp, lobster, or cockroaches [26].

In this study, we provide evidence that a new type of gelsolin-like protein from the house dust mite *D. farinae* potentially serves as an allergen recognized by asthmatic patient IgE. Gelsolin is a highly conserved actin cytoskeleton-regulatory molecule involved in the control of motility, apoptosis, or phosphoinositide signaling pathways [27]. According to the WHO/IUIS allergen nomenclature subcommittee [28], here we propose that this novel gelsolin-like molecule is designated as the group 16 mite antigen, Der f 16.

2. Materials and methods

2.1. Materials, rabbit antiserum, and asthmatic patients' sera

Live mites (*D. farinae*) were supplied from Fumakilla (Hiroshima, Japan), and *D. farinae* body extract (Dfb) was prepared as described [29]. To obtain antiserum against Dfb, a Japanese white rabbit (Hiroshima Experimental Animal, Hiroshima, Japan) was immunized intramuscularly with 1 mg Dfb emulsified in Freund's complete adjuvant and boosted with the same antigen every week. Antiserum was collected 10 weeks after the first immunization. Human sera were collected from mite-sensitive asthmatic patients whose radioallergosorbent test (RAST) scores exhibited over 2 ($n=17$). Control non-allergic sera ($n=3$) were derived from dust mite skin test-negative healthy volunteers ($n=3$) in Hiroshima University.

2.2. cDNA cloning of Der f 16

Construction and immunoscreening of *D. farinae* cDNA library

*Corresponding author. Fax: (81)-824-24 7754.
E-mail address: skawa@hiroshima-u.ac.jp (S. Kawamoto).

[1] The sequence data reported in this paper have been deposited in the GenBank sequence database under the accession number AF465625.

Abbreviations: Dfb, *Dermatophagoides farinae* body extract; GST, glutathione *S*-transferase; IPTG, isopropyl-1-thio-β-D-galactoside; PIP_2, polyphosphoinositide 4,5-biphosphate; RAST, radioallergosorbent test

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were carried out as described [28]. Briefly, poly(A)$^+$ RNA was extracted from live mite bodies using the Fast Track mRNA isolation kit (Invitrogen, San Diego, CA, USA) and double-stranded cDNA was synthesized via a cDNA synthesis kit (Amersham Pharmacia Biotech, Uppsala, Sweden). A cDNA library was constructed on λgt11 (Stratagene, La Jolla, CA, USA). 1.38×10^7 independent clones were immunoscreened with rabbit anti-Dfb serum. Positive immunoreactive clones (273 clones) were then subjected to DNA cross-hybridization analysis [30], and were further classified into nine cDNA groups (A–I). The longest cDNA clone (*mag15*) from one group (group E) was subcloned into a Bluescript II SK(+) phagemid vector (Stratagene), and both strands of the cDNA were sequenced using ABI PRISM 310 Genetic Analyzer (PE Biosystems, Norwalk, CT, USA).

2.3. Expression and purification of recombinant Der f 16 (r-Der f 16)

Der f 16 cDNA fragment (nucleotide no. 129–1571, corresponding to amino acids no. 1–480, see Fig. 1) was prepared via polymerase chain reaction (PCR; conditions were 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s for 30 cycles). PCR primers were synthesized as follows: forward, GCGGATCCATGGCTGCACATGATAAAAAT; reverse, CCGGATCCTCAGTTCCATTTAGGAAAATA. *Bam*HI site (shown underlined) and extra two nucleotides ('GC' in forward, 'CC' in reverse) are added at the 5′ end of each primer for subcloning. The 1459 bp PCR product was cloned into pGEM-T easy vector (Promega, Madison, WI, USA), and *Bam*HI-cleaved Der f 16 cDNA fragment was then cloned into GST-tagged pGEX-2T vector (Amersham Pharmacia Biotech). *Escherichia coli* BL-21 strain was transformed with the expression plasmid, and transformant was cultured in 2×YT medium (1 l) supplemented with 0.1% isopropyl-1-thio-β-D-galactoside (IPTG) to express recombinant GST-fused Der f 16. The recombinant protein was purified by affinity chromatography using a glutathione Sepharose 4B column (Amersham Pharmacia Biotech) according to the manufacturer's instruction. Briefly, harvested bacterial cells were lysed under sonication in an extraction buffer (2% Triton X-100, 150 mM NaCl, 5 mM EDTA, 3 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mg/ml pepstatin A in Tris–HCl pH 7.4). After centrifugation, the supernatant was mixed with glutathione Sepharose 4B beads, and incubated overnight at 4°C with gentle rotation. After washing the column with the extraction buffer, immobilized recombinant protein was cleaved by digestion with thrombin (Amersham Pharmacia Biotech). To monitor expression and purification of r-Der f 16, protein samples were resolved by SDS–PAGE according to the discontinuous buffer system by Laemmli [31] on a 12.5% polyacrylamide slab gel. Fractionated proteins were then visualized by 0.25% Coomassie brilliant blue staining.

2.4. IgE-binding assay

IgE-binding ability of r-Der f 16 protein was assessed by enzyme-linked immunosorbent assay (ELISA). For antigen coating, 50 μl of recombinant antigen solution (1 μg/ml, in 100 mM carbonate buffer, pH 9.3) was added into a 96-well microtiter plate (Falcon 3912, Becton Dickinson Labware, Lincoln Park, NJ, USA), and incubated at 37°C for 2 h. After blocking with 2% skim milk, 1% bovine serum albumin in phosphate-buffered saline, 50 μl of diluted asthmatic patient's serum (×50 dilution with blocking buffer) was supplemented, and incubated overnight at 4°C. Then 50 μl of secondary biotin-conjugated anti-human IgE (×1000 dilution, Zymed, San Francisco, CA, USA) was added and incubated at 25°C for 1 h, followed by addition of 50 μl of alkaline phosphatase-conjugated streptavidin (×1000 dilution, purchased from Zymed). For enzyme reaction, AttoPhos substrate solution (Roche Molecular Biochemicals, Mannheim, Germany) was added and incubated at room temperature for 30 min. Then the fluorescence intensity of each sample well was analyzed using a Cyto-Fluor II microplate reader (PE Biosystems).

3. Results and discussion

3.1. Cloning of a new house dust mite antigen cDNA homologous to the gelsolin family

Immunoscreening of the *D. farinae* cDNA library (1.38×10^7 clones) with rabbit anti-Dfb serum yielded 273 positive clones, and subsequent cross-hybridization analysis classified these cDNAs into nine groups (groups A–I). Among them, we selected one clone (*mag15*, the longest cDNA clone in group E) for further analysis, since preliminary experiments indicated that corresponding recombinant protein (expressed as β-galactosidase fusion protein in *E. coli*) showed IgE-binding ability on immunoblotting (data not shown).

Sequencing analysis revealed that the cDNA (1657 bp in length), named Der f 16, contained a 1443 bp open reading frame encoding a 480 amino acid polypeptide, with a calculated molecular weight of 55 131 Da (Fig. 1). The BLAST amino acid similarity searching [32] revealed that the predicted Der f 16 polypeptide sequence showed striking similarity to gelsolin; a Ca^{2+}- and polyphosphoinositide 4,5-biphosphate (PIP_2)-regulated actin filament severing and capping protein [27,33]. Maximum sequence identity (33%; 470 amino acids alignment) was noted when compared with *Caenorhabditis elegans* hypothetical protein K06A4.3 (GenBank accession number T23355). Significant identity was observed in comparison to gelsolins from American lobster (38% identical

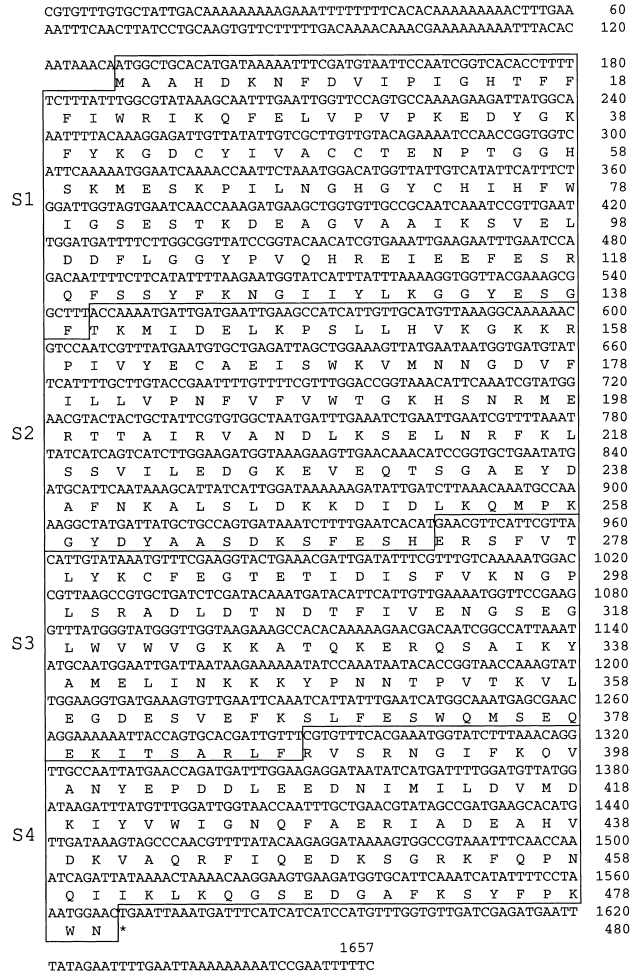

Fig. 1. Nucleotide and deduced amino acid sequence of Der f 16. The gelsolin family characteristic repetitive segments (S1–S4) are shown boxed. Boundaries between each segment (S1/S2, S2/S3) are determined based on a method described by Way and Weeds [45]. A boundary between S3 and S4 is set on the middle of S3 motif C and S4 motif B (detailed motif sequences are shown in Fig. 3A). An asterisk shows a termination codon (TGA). The sequence information is available on the GenBank sequence database under accession number AF465625.

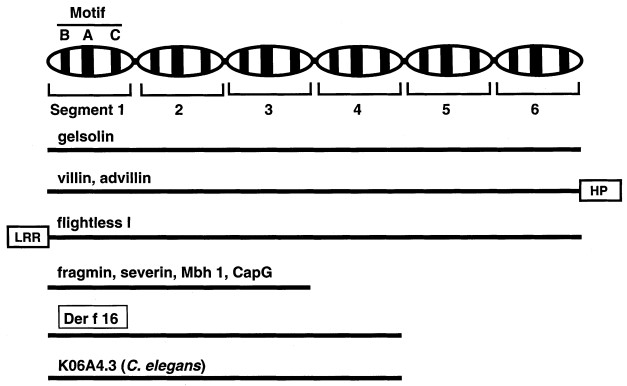

Fig. 2. Domain organization of gelsolin family proteins comprising six (gelsolin, villin, advillin, flightless I), three (fragmin, severin, Mbh 1, CapG), and four segmental repeats (Der f 16, K06A4.3). Each segment contains conserved motifs, B, A, and C [45]. HP, headpiece; LRR, leucine-rich repeat.

around 372 residues, S53373 [34]), *Drosophila melanogaster* (38% within 370 amino acids, A53909 [35]), or from human (34% within 366 amino acids, P06396 [36]). Der f 16 polypeptide also shows significant homology to other gelsolin family members including villin (34% identical within 361 residues, P02640 [37]), advillin (35% within 362 residues, O88398 [38]), fragmin (35% within 356 amino acids, AAC47528 [39]), or severin (32% within 354 amino acids, P10733 [40]).

The gelsolin family consists of characteristic repeated segments [33]. The conventional gelsolin family (gelsolin, villin, advillin, flightless I [41] etc.) has six repeats, whereas another major subgroup including fragmin, severin, Mbh 1 [42], and CapG [43] is composed of three gelsolin repeats (Fig. 2). Stocker et al. [44] have identified a distinct type of gelsolin-related protein (GRP125) from *Dictyostelium* which has five repeats lacking segment 1. In contrast to those known gelsolin family members, we found four gelsolin-like segments in the Der f 16 sequence (see Figs. 1 and 2). To our knowledge, this is the first reported case describing a gelsolin family member with four segmental structures, although the *C. elegans* K06A4.3 hypothetical protein, which also has four repeats, has been deposited in the database. Each of the gelsolin family segments contains conserved motifs, B, A, and C (schematically shown in Fig. 2, [45]). Multiple alignment indicated that those motifs in the Der f 16 segments are also well conserved (shown gray-boxed in Fig. 3A), again confirming that Der f 16 structurally belongs to the gelsolin family.

Previous studies identified several important functional domains on the gelsolin molecule to execute its actin filament severing and capping reaction. It has been demonstrated that two actin-binding regions locate on human plasma gelsolin; one is 'actin-binding helix' in the segment 1 [46], which is also involved in Ca^{2+}-binding (shown boxed in Fig. 3B). The other is 'actin side-binding domain' in segment 2 (shown underlined in Fig. 3B, [47]), that is overlapping with the second PIP_2-

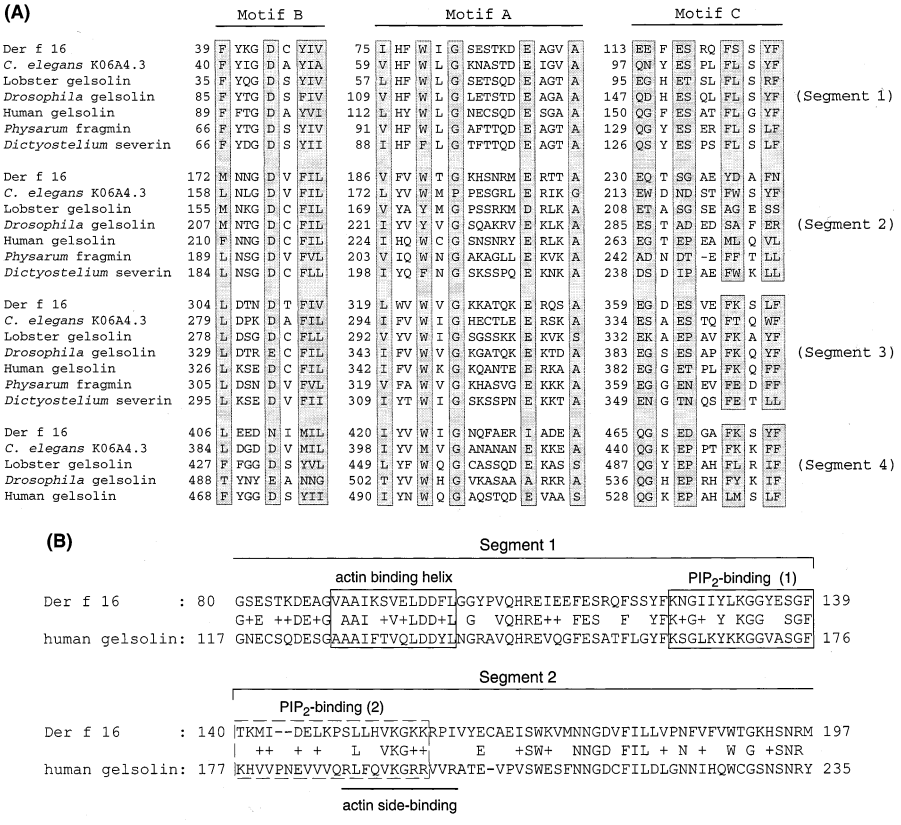

Fig. 3. A: Segmental sequence alignment of conserved motifs in Der f 16 and gelsolin family proteins. Der f 16, *C. elegans* K06A4.3 hypothetical protein (GenBank T23355), gelsolins from lobster [34], *Drosophila* [35], human [36], *Physarum* fragmin P [39], and *Dictyostelium* severin [40] are aligned. The characteristic amino acids in the motifs (B, A, and C [45]) are shown gray-boxed. B: Sequence alignment of Der f 16 and human plasma gelsolin around the boundary of segments 1 and 2. Highly conserved putative actin-binding helix and the first PIP_2-binding domain are shown open-boxed, while the second PIP_2-binding site is boxed by a dashed line. The actin side-binding region is underlined. Identical amino acids are shown by the same letter. Similar amino acid residues are indicated by '+'.

binding domain (described below). Sequence comparison of these domains indicated that the actin-binding helix (amino acids no. 90–102) was highly conserved in Der f 16 (62% identity, 85% similarity) when compared with human plasma gelsolin (Fig. 3B). In contrast, the homology score within the actin side-binding region (no. 149–160) was relatively low (30% identity, 50% similarity). The actin severing activity of gelsolin is positively regulated via Ca^{2+}, whereas PIP_2 negatively modulates its action [33]. Although the detailed structural basis for Ca^{2+}-binding to gelsolin is complex and beginning to be understood [33], PIP_2-binding domains have already been identified. Several lines of evidence have revealed the existence of two PIP_2-binding regions on human plasma gelsolin polypeptide; one is located at the end of segment 1, and another is harbored on the head of segment 2 [48,49]. As shown in Fig. 3B, the first PIP_2-binding domain (no. 125–139, shown open-boxed) was well conserved in Der f 16 polypeptide (60% identity, 73% similarity), whereas the second site (no. 140–157, boxed by a dashed line) showed less homology (20% identity, 50% similarity). These structural similarity data implicate that Der f 16 may serve as a PIP_2-modulated actin severing/capping molecule, although actual gelsolin activity of Der f 16 protein remains to be demonstrated.

3.2. IgE-binding capacity of r-Der f 16

To examine IgE-binding activity, we next tried to express and purify r-Der f 16 using the GST-fusion protein expression system in *E. coli*. Fig. 4 shows SDS–PAGE analysis of recombinant GST–Der f 16 fusion protein expression and confirmation of its purification step. Upon IPTG induction, an 81 kDa protein expression was observed in the BL-21 cells carrying the expression vector (see lane 3). Western blot analysis demonstrated that the 81 kDa protein was recognized by rabbit anti-GST serum (data not shown). Since the recombinant protein was observed in soluble cell lysate (lane 4), we proceeded to its purification via affinity chromatography. After adsorption of the 81 kDa GST-fusion protein on the glutathione Sepharose 4B column, 55 kDa r-Der f 16 was obtained by thrombin digestion (lane 5). This molecular weight (55 kDa) is practically matched with the calculated molecular weight (55 131 Da) deduced from the polypeptide sequence. Amino acid sequencing demonstrated that *N*-terminal 10 amino acids from purified r-Der f 16 coincided completely with those predicted from the sequence data (data not shown).

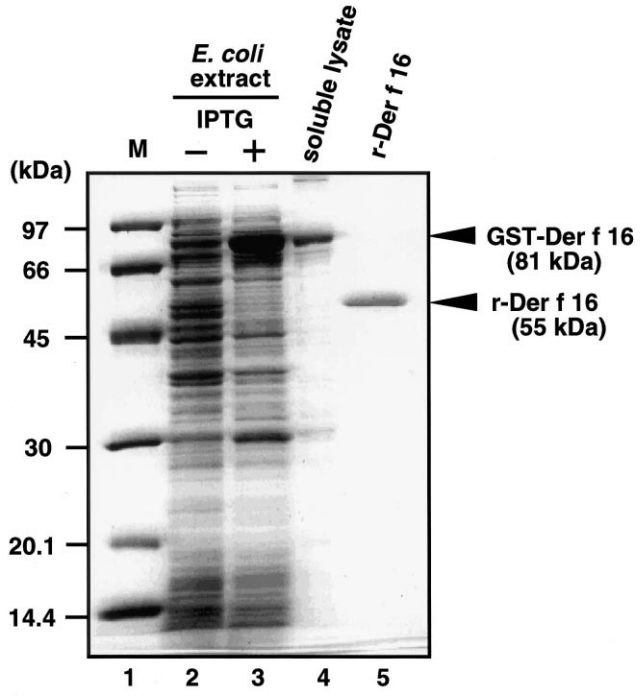

Fig. 4. Expression and purification of r-Der f 16. *E. coli* BL-21 strain was transformed with the pGEX-2T vector carrying full-length Der f 16 cDNA. Recombinant protein expression was then induced by cultivation of the transformant with 2×YT medium supplemented with IPTG. Expression and purification processes were monitored by SDS–PAGE analysis. Lane 1, molecular weight marker; lane 2, total *E. coli* cell extract without IPTG addition; lane 3, total cell lysate upon stimulation with IPTG; lane 4, soluble cell lysate after sonication; lane 5, purified r-Der f 16 via glutathione Sepharose 4B affinity chromatography in conjunction with thrombin digestion. Protein bands corresponding to GST–Der f 16 (81 kDa) and purified r-Der f 16 (55 kDa) are shown by arrowheads.

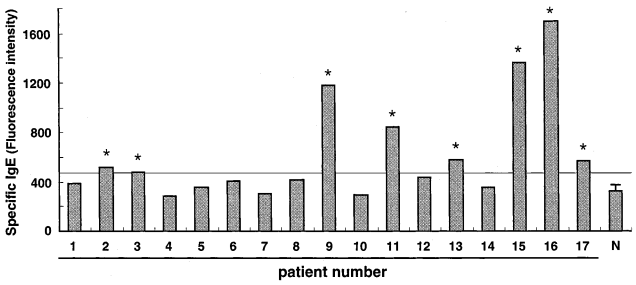

Fig. 5. IgE-binding capacity of r-Der f 16. ELISA was performed against sera collected from *D. farinae*-sensitive allergic patients ($n = 17$, RAST score > 2). As a negative control, mite-insensitive healthy donors ($n = 3$) are also tested. The cut off ELISA value for evaluating positive IgE-binding (mean ELISA value of healthy donors+3 S.D.) is indicated in the graph by a horizontal line. r-Der f 16-positive donors are indicated by asterisks.

To test the IgE-binding profile of purified r-Der f 16, we performed ELISA using sera from 17 mite-sensitive asthmatic patients (RAST score > 2) and three non-allergic healthy individuals. We found that r-Der f 16 was recognized by patients' IgE at the frequency of 47.1% (8/17, positive donors are indicated by asterisks, Fig. 5), whereas no such IgE-binding was obtained when incubated with control sera from dust mite-insensitive healthy volunteers (see the bar indicated 'N' in Fig. 5). A parallel experiment using major mite allergen r-Der f 2 indicated that 76.5% of the patients (13/17) showed positive IgE-binding (data not shown). Another set of ELISA against different 28 asthmatic subjects (whose RAST score > 2) showed that 17.9% of the patients (5/28) were found to exhibit positive IgE-binding to r-Der f 16 (data not shown). All these data suggest that the r-Der f 16 molecule is a novel allergen with low to moderate IgE-binding capacity against house dust mite-sensitive donors. However, a possibility still remains that natural Der f 16 may possess a more potent IgE-binding ability or allergenicity than the *E. coli*-produced recombinant protein. To evaluate the pathophysiological importance of Der f 16 in dust mite-associated allergy, purification and detailed immunochemical characterization of natural Der f 16 are now in progress.

Here we provide the first evidence that the gelsolin family molecule is a new class of house dust mite allergen. Since the gelsolin superfamily is widespread and highly conserved with-

in multicellular organisms, one might expect that this family of proteins serve as a pan-allergen with IgE cross-reactivity. To test whether Der f 16 shows cross-reactivity with atopy-inducible species is therefore an intriguing future issue.

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