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Short sequence-paper

Cloning and expression of Der f 6, a serine protease allergen from the house dust mite, *Dermatophagoides farinae*[1]

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Abstract

House dust mite allergen is thought to be a major cause of asthma. Characterization of these allergen molecules is therefore an important step for the development of effective diagnostic and therapeutic agents against mite-associated allergic disorders. Here we report molecular cloning and expression of the group 6 (chymotrypsin-like) allergen from the house dust mite, *Dermatophagoides farinae*. Sequencing analysis indicates that cloned cDNA, designated Der f 6, encodes a 279 amino acid polypeptide which conserves a primary structure characteristic for chymotrypsin-like serine proteases found in mammals. Recombinant Der f 6 expressed in *Escherichia coli* bound IgE in a pool made of 20 sera, and induced histamine release from patients' peripheral blood cells.

Keywords: Der f 6; Allergen; House dust mite; Chymotrypsin; Serine protease

Allergens from the house dust mites, *Dermatophagoides farinae* and *Dermatophagoides pteronyssinus*, are major causes of allergic disorders such as bronchial asthma, atopic dermatitis, and allergic rhinitis [1,2]. Identification and characterization of important mite allergens have been done in the last decade via immunochemical, biochemical and molecular biological techniques, and ten major mite allergens are now classified. The group 1 (cysteine protease) and 2 mite allergens are important [3–9], and the groups 5, 7, and 8 (glutathione *S*-transferase) are also thought to be major allergens [10–12]. Our group have cloned several cDNAs encoding additional important mite allergens, including the group 10 (tropomyosin), Mag 29 (heat shock protein 70-like molecule), Mag 1 (39 kDa allergen), and Mag 3 [13–16].

Numerous allergens have proteolytic activities that may contribute to their allergenicity. In the case of allergic bronchial asthma, proteolytic mite allergens are involved in the enhancement of airway inflammation and hyperresponsiveness by accelerating vascular permeability or non-IgE-mediated mast cell de-

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[1] The sequence data reported in this paper have been deposited in the GenBank Sequence Database under accession No. AF125187.

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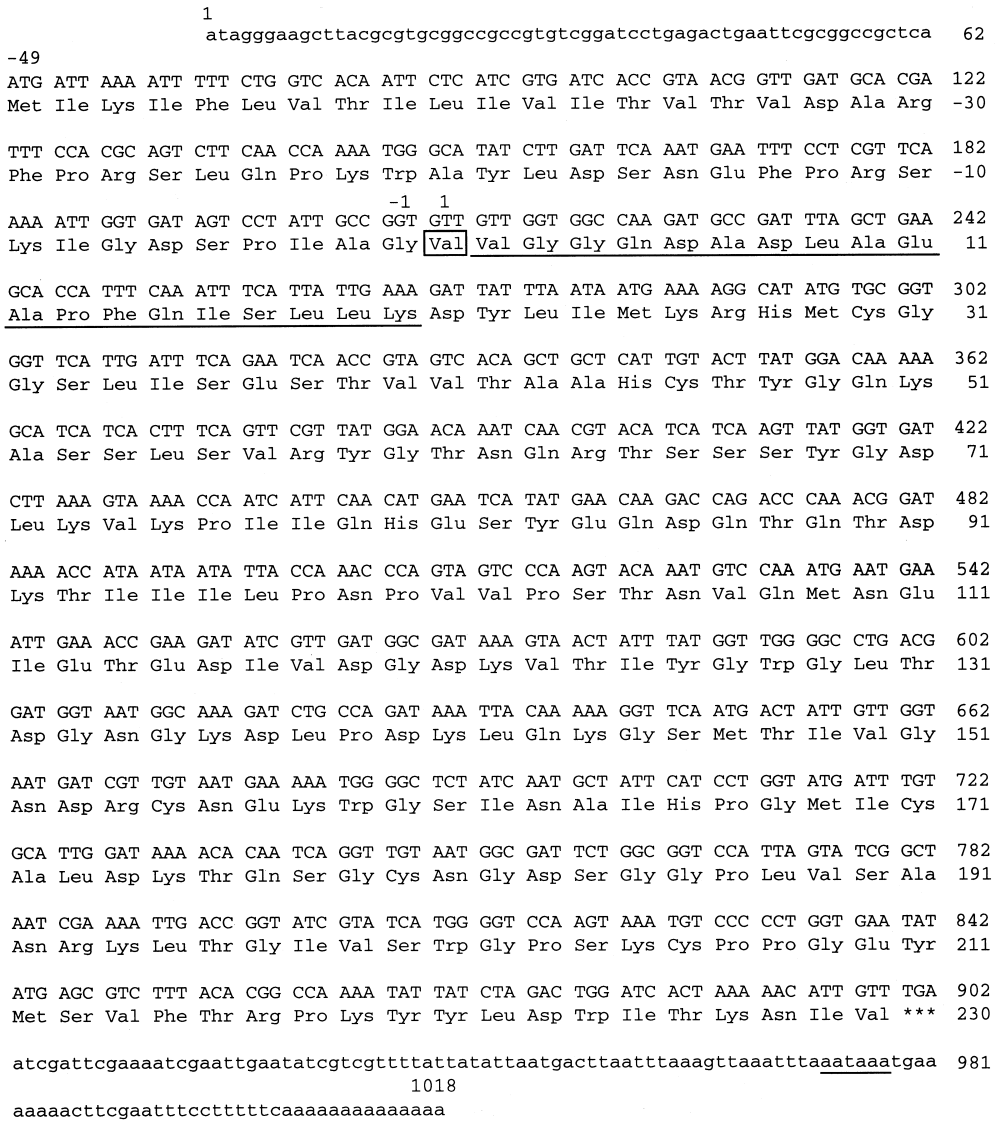

Fig. 1. Nucleotide and deduced amino acid sequences of Der f 6. The amino acid sequence is numbered sequentially from the first amino acid of the putative zymogen. The sequence corresponding to the reported N-terminal Der f 6 polypeptide (Nos. 2–20 [23]) is underlined, and the putative mature N-terminus (Val residue) is shown open boxed. The polyadenylation signal (AATAAA, Nos. 972–977) is also underlined.

granulation [17,18]. In addition to the group 1 allergen, serine proteases in the house dust mite are also recognized as highly immunogenic proteolytic allergens, and are comprised of group 3 (trypsin), group 6 (chymotrypsin), and group 9 (collagenase) allergens [19–24]. Recent evidence has indicated novel roles of these serine protease mite allergens in the development of allergic disorders besides their IgE-binding ability. Maruo et al. [25] have reported that Der 3 members activate the complement system to produce anaphylatoxins. Moreover, King et al. [26] have revealed that Der p 9 triggers the secretion of proinflammatory cytokines from human bronchial epithelium. This evidence suggests a possible involvement of serine protease allergens in the pathogenesis of allergic asthma. However, the detailed immunochemical properties of serine protease mite allergens are poorly understood. In this study, we carried out cDNA cloning and *Escherichia coli* expression of Der f 6, a new member of serine protease allergen from the house dust mite, *D. farinae*.

To clone the group 6 mite allergen cDNA, we first

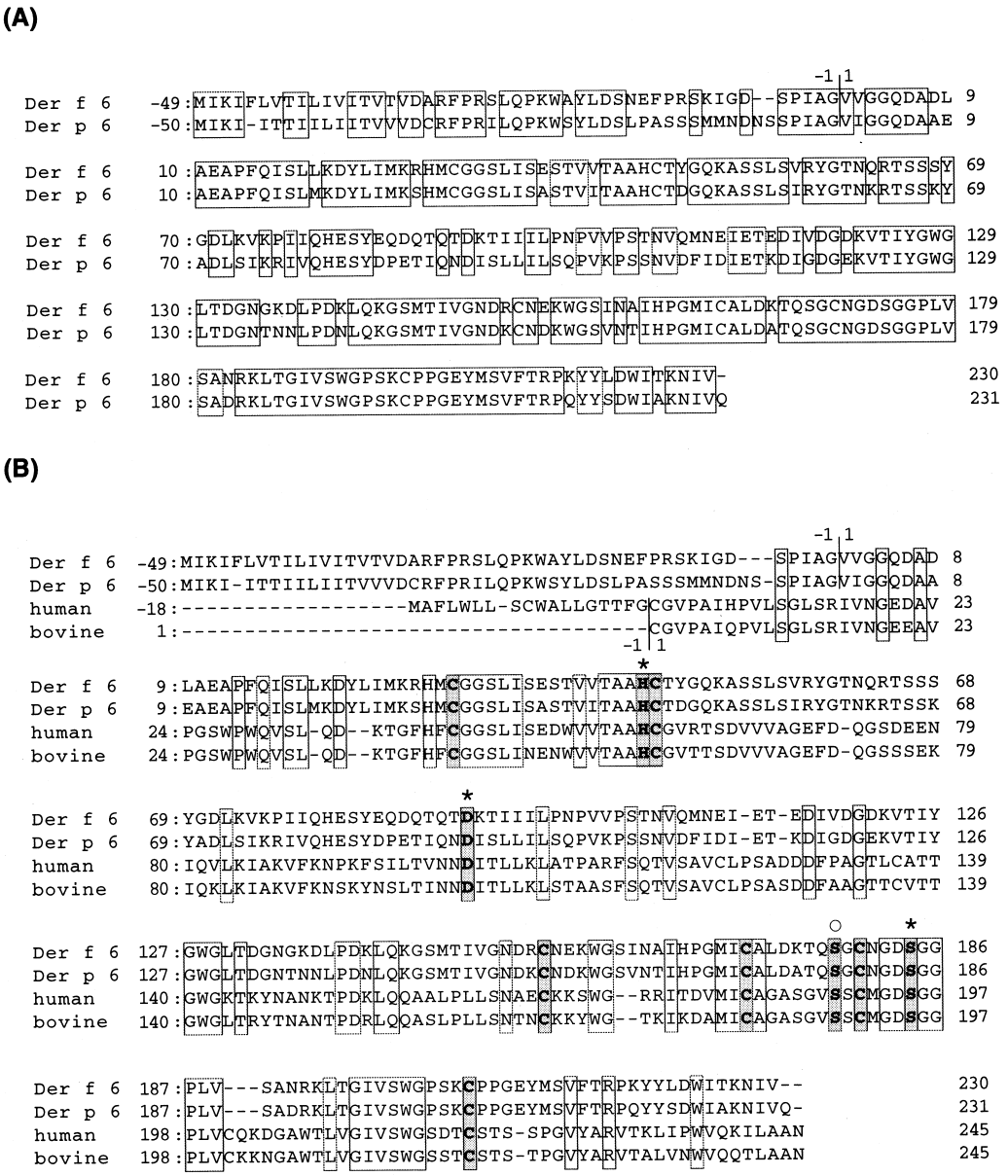

Fig. 2. (A) Amino acid sequence comparison of Der f 6 with another group 6 dust mite allergen Der p 6 [24]. Identical amino acid residues are shown open boxed. (B) Multiple sequence alignment of Der f 6, Der p 6, and chymotrypsinogens from human [29] and bovine [30]. Identical amino acids within four polypeptides are shown open boxed. Conserved cysteine residues (Nos. 30, 46, 155, 171, 180, and 206) are in bold letters and shown gray boxed. The amino acids that comprise the catalytic triad (His-45, Asp-91, and Ser-184; indicated by '*') and the substrate recognition site (Ser-178; indicated by '○') are also in bold and gray boxed.

screened the *D. farinae* cDNA library (constructed in λExcell vector, Amersham Pharmacia Biotech, Uppsala, Sweden) with rabbit antiserum raised against purified natural Der f 6 protein, and isolated a positive cDNA clone (mag132). Insert cDNA was then subcloned into pUC-19 vector (Takara, Kyoto, Japan), and both strands of the cDNA were sequenced as described [16]. Initial sequencing analysis indicated that the mag132 clone did not contain the ATG start codon (the mag132 cDNA consisted of 909 nucleotides; Nos. 110–1018 in Fig. 1). To clone full-length Der f 6 cDNA, the rapid amplification protocol for cDNA end (RACE)-PCR was performed. As a forward primer, the λExcell vector se-

quence (CGCCAAGCTATTTAGGTGAC, nucleotide sequence Nos. 2064–2083) was used. A reverse primer corresponding to the 5′-Der f 6 coding sequence was designed within nucleotide sequence Nos. 147–168 (Fig. 1) as its complementary orientation (CATTTGAATCAAGATATGCCCA). The resulting PCR fragment was subcloned into pGEM-T vector (Promega, Madison, WI, USA), and then sequenced. Cloned 1018 bp cDNA, designated Der f 6, contains an 840 bp open reading frame encoding a 279 amino acid polypeptide with a calculated molecular mass of 30 534 Da (Fig. 1). Yasueda et al. [23] have elucidated the partial N-terminal amino acid sequence of natural Der f 6 as (A)(V) GGQDADLAEAPFQISLLK. In the deduced amino acid sequence, we find the same sequence as for the reported N-terminal Der f 6 protein (VGGQDADLAEAPFQISLLK, amino acids 2–20, shown underlined in Fig. 1), confirming that the cDNA actually encodes Der f 6 protein. Since the initial amino acid residue of the reported sequence (i.e. Ala residue) has been tentatively assigned [23], it is likely that the putative mature N-terminus of Der f 6 polypeptide is a Val residue (shown boxed in Fig. 1). The calculated molecular mass of the mature Der f 6 polypeptide (25 034 Da, amino acids 1–230) roughly coincided with that of its reported natural counterpart (25 kDa).

Amino acid sequence comparison indicated that the putative Der f 6 polypeptide showed significant homology (75.1% identity) with another cloned chymotrypsin-like allergen Der p 6 (Fig. 2A) [24]. This further confirms that our cDNA encodes a new member of the group 6 family mite allergen. Hydropathy plot analysis [27] indicated that the N-terminal 17 amino acid sequence of Der f 6, as well as 16 amino acids of Der p 6, was highly hydrophobic (data not shown), implicating that this portion may serve as a signal peptide. The sequence proximal to the proenzyme-cleavage site (Nos. −5 to 7, see Fig.

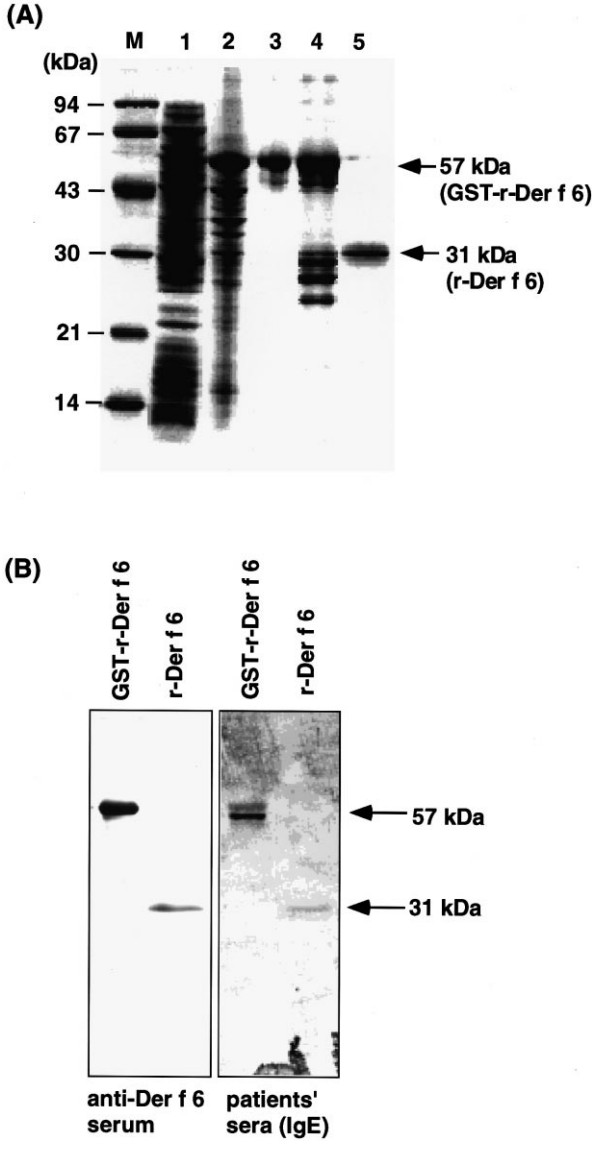

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Fig. 3. (A) Expression and purification of r-Der f 6. Protein samples were run on a 10% polyacrylamide gel, and visualized by Coomassie brilliant blue staining. Lanes: M, molecular weight standard; 1, total cell lysate of the *E. coli* transformant (100 μl culture); 2, precipitate of the cell lysate after sonication (5 μg); 3, purified GST-r-Der f 6 fusion protein (2 μg); 4, thrombin digest of the fusion protein (5 μg); 5, purified r-Der f 6 via preparative SDS-PAGE (1 μg). (B) Immunoblotting analysis of r-Der f 6. GST-r-Der f 6 (2 μg) or r-Der f 6 (1 μg) were electronically transferred onto a PVDF filter (Immobilon P, Millipore, Bedford, MA, USA). After incubation with a blocking buffer (3% skim milk, 0.05% Tween 20 in PBS), the filter was probed with primary rabbit anti-Der f 6 serum (2000-fold dilution in the blocking buffer), followed by staining with 1 μg/ml secondary peroxidase-conjugated goat anti-rabbit IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (left panel). To test IgE binding, the filter was incubated with diluted pooled sera from mite-allergic patients ($n = 20$), and then incubated with 1 μg/ml peroxidase-conjugated goat anti-human IgE antibody (Zymed, San Francisco, CA, USA) (right panel). Protein bands were visualized using the Immunostaining Kit (Konica, Tokyo, Japan).

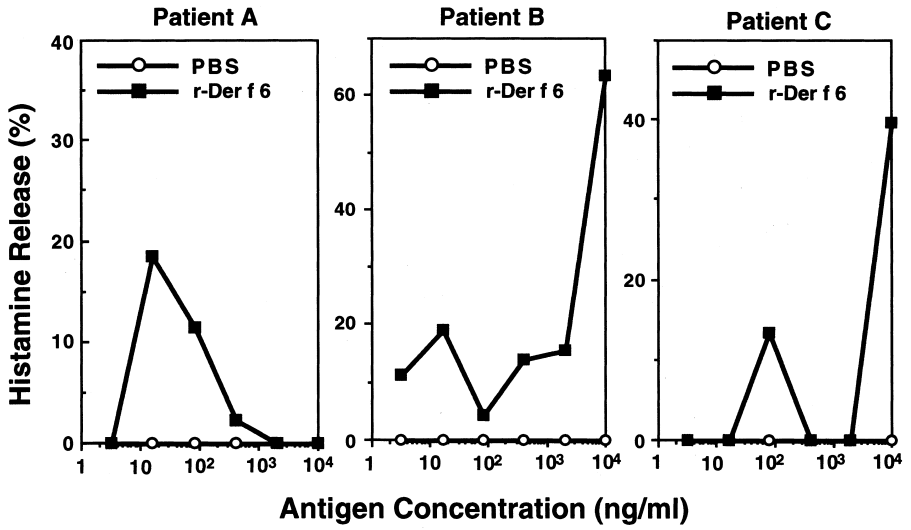

Fig. 4. Purified r-Der f 6 possesses histamine release activity. Peripheral blood cells from mite-allergic patients were suspended in Hanks' buffer, and stimulated with a series of diluted antigens at 37°C for 30 min. Released histamine in the supernatant was analyzed on a HPLC system (Hitachi, Tokyo, Japan) as described [33]. Total or control histamine release was determined using samples from perchloric acid lysis of cell aliquots and cells incubated in Hanks' buffer, respectively. We preliminary confirmed specificity of the reaction using crude mite antigen and non-specific sea squirt protein as positive and negative controls (data not shown). Only the three histamine release-positive cases (among six patients tested) are indicated.

2A) was well conserved (91.7% identity) within Der f 6 and Der p 6. BLAST amino acid similarity searching [28] indicated that the Der f 6 sequence also exhibited homology (30–40%) with serine proteases (trypsin, chymotrypsin, elastase, tryptase, etc.) from various species. Especially, significant alignments were derived when compared with the group 3 trypsin-like mite allergen Der f 3 (36.1% [17]), Der p 3 (37.2% [18]), or chymotrypsin from the African malaria mosquito (37.2%, GenBank accession No. Z18887). Multiple alignment of the deduced Der f 6 polypeptide sequence with those of reported chymotrypsins (Der p 6 [24], human and bovine chymotrypsinogens [29,30]) indicated that six cysteine residues (amino acids 30, 46, 155, 171, 180, and 206), which have been demonstrated to form three disulfide bonds in bovine chymotrypsinogen [31], are conserved in Der f 6 (shown boxed and shaded in Fig. 2B). Moreover, we found that amino acid residues participating to form the chymotrypsin-characteristic substrate recognition site (Ser-178; indicated by an open circle) and the typical catalytic triad (His-45, Asp-91, and Ser-184; indicated by asterisks) of the serine protease family are also conserved in Der f 6 (Fig. 2B). These data indicate that the putative Der f 6 polypeptide possesses a primary structure characteristic for the chymotrypsin family serine protease.

We next constructed an expression system of recombinant Der f 6 (r-Der f 6) in *E. coli*. Der f 6 cDNA fragment (nucleotides 110–1018, corresponding to amino acids −33 to 230) carrying *Eco*RI-*Not*I adaptors (Amersham Pharmacia Biotech) was cleaved from λExcell vector by *Eco*RI digestion, and then cloned into the glutathione *S*-transferase (GST)-tagged pGEX-4T-3 vector (Amersham Pharmacia Biotech). When using this construct, five extra amino acids from the *Eco*RI-*Not*I adaptor sequence (NSRPL) should be added at the Val residue (amino acids −33 in Fig. 1) of r-Der f 6. The transformant was cultured in 2×YT medium (2 l) supplemented with 0.1% IPTG to express r-Der f 6 as a GST-fusion protein. SDS-PAGE analysis indicated that a predicted 57 kDa protein was newly detected in the total cell lysate (Fig. 3A, lane 1) and the insoluble precipitate of cell lysate after sonication (lane 2). Purification using preparative SDS-PAGE [16], we obtained 5 mg of the 57 kDa GST-fusion protein (lane 3). After dialysis against PBS for removing SDS, the fusion protein was subsequently digested with thrombin (10 units/mg protein) to remove the GST tag. Thrombin digestion resulted in four protein frag-

ments (31 kDa, 30 kDa, 26 kDa, and 24 kDa, indicated in lane 4). The 26 kDa fragment was the GST tag, since only this molecule was recognized by rabbit anti-GST serum (data not shown). The other three fragments (i.e. 31 kDa, 30 kDa, and 24 kDa molecules) were shown to be derived from r-Der f 6, as demonstrated by anti-Der f 6 immunoblot analysis (data not shown). The 31 kDa fragment was likely to be r-Der f 6. The 30 kDa fragment might have resulted via internal thrombin digestion of r-Der f 6, since two thrombin cleavage sites [32] are seen in the Der f 6 sequence (RFPR/SL (Nos. −30 to −25) and LQPK/WA (Nos. −25 to −20)) that potentially produce a 30 kDa fragment. The reason why the 24 kDa fragment arises during the thrombin reaction is currently unknown. We purified the 31 kDa molecule using a preparative SDS-PAGE, followed by dialysis against PBS, for further immunochemical characterization (lane 5). The yield of the purified 31 kDa fragment was 91.5 μg protein. Immunoblotting analysis indicated that both the purified 57 kDa GST-fusion protein and the 31 kDa protein were recognized by rabbit anti-Der f 6 serum (left panel of Fig. 3B), confirming successful expression and purification of r-Der f 6.

To test whether our *E. coli*-produced r-Der f 6 has IgE-binding ability, purified 31 kDa r-Der f 6 was immunoblotted with diluted pooled serum IgE from mite-allergic patients ($n = 20$). As shown in Fig. 3B (right panel), 31 kDa r-Der f 6, as well as 57 kDa GST-Der f 6, were shown to be recognized by the patients' IgE, indicating that the r-Der f 6 still retain IgE-binding property even in denaturing condition. This immunoblot assay also suggests that the patients' IgE antibodies should recognize linear epitopes on the Der f 6 polypeptide. Scanning and identification of those linear epitopes is under investigation. Our preliminary ELISA analyses using sera from 38 mite-allergic patients (a patient group which is 80% positive for IgE binding against major mite allergen Der f 1) have indicated that IgE-binding frequency of recombinant Der f 6 was 39% (data not shown), which is comparable to that of reported natural Der f 6 (41% [23]). This suggests that our r-Der f 6 might retain IgE-binding ability comparable to that of natural Der f 6, although further examination is required for critical estimation of the IgE-binding frequency by comparing its reactivity with that of its natural counterpart.

Yasueda et al. have reported that natural Der f 6 exhibited skin test-positive phenotypes [23], implicating potent histamine release ability of Der f 6. To test this possibility, we next examined whether r-Der f 6 is able to induce histamine release from patients' peripheral blood cells. In six patients tested, r-Der f 6-triggered histamine release was observed in three cases (shown in Fig. 4), showing the first evidence of histamine release activity of r-Der f 6. Significantly, patients A and B required a relatively low antigen concentration (2–10 ng/ml) for triggering histamine release, suggesting potent allergenicity of r-Der f 6.

Here we elucidated the primary structure of the group 6 house dust mite allergen Der f 6, and also indicated that r-Der f 6 expressed in *E. coli* still retains allergenicity. Our group has recently noticed that Der 3/Der 6 family members are major components of an immunotherapeutic vaccine prepared from mite feces which is demonstrated to exhibit an excellent clinical score against mite-allergic patients (data not shown). This implicates that serine protease allergens might be candidates for a recombinant vaccine for allergic asthma. Der f 6 cloned in the present paper will facilitate to test this possibility, as well as to elucidate the involvement of the serine protease allergen in the development of house dust mite-associated allergic disorders.

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