

Der f 34, a Novel Major House Dust Mite Allergen Belonging to a Highly Conserved
Rid/YjgF/YER057c/UK114 Family of Imine Deaminases

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ABSTRACT

The high prevalence of house dust mite

(HDM) allergy is a growing health problem worldwide, and the characterization of clinically important HDM allergens is a prerequisite for the development of diagnostic and therapeutic strategies. Here, we report a novel HDM allergen that belongs structurally to the highly conserved Rid/YjgF/YER057c/UK114 family (Rid family) with imine deaminase activity. Isolated HDM cDNA, named *der f 34*, encodes 128 amino acids homologous to Rid-like proteins. This new protein belongs to the Rid family and has seven conserved residues involved in enamine/imine deaminase activity. Indeed, we demonstrated that purified *Der f 34* had imine deaminase activity that preferentially acted on leucine and methionine. Native *Der f 34* showed a high IgE-binding frequency as revealed by two-dimensional immunoblotting (62.5%) or ELISA (68%), which was comparable to those of a major HDM allergen *Der f 2* (77.5% and 79%, respectively). We also found that *Der f 34* showed cross-reactivity with another prominent indoor allergen source, *Aspergillus fumigatus*. This is the first report showing that the Rid family imine deaminase represents an additional important pan-allergen that is conserved across organisms.

Asthma is a serious global health problem that significantly reduces quality of life. In a cross-sectional World Health Organization survey of 178,215 individuals from 70 countries conducted in 2002-2003, it was estimated that 4.3% of adults (range, 0.2-21.0%) had been

diagnosed with asthma by a medical doctor (1). Inhaled airborne allergens cause allergic inflammation *via* the cross-linking of allergen-specific IgEs bound on high affinity IgE receptor (FcεRI) located on the surface of airway or lung mast cells, causing the secretion of inflammatory chemical mediators (*e.g.* histamine and leukotrienes) from activated mast cells. House dust mites (HDMs) and fungi are major sources of airborne allergens and trigger asthmatic attacks in allergic patients concomitant with air pollutants (2).

Allergen-specific immunotherapy (AIT) is the only curative treatment for allergic asthma that changes the natural course of an allergy (3). AIT induces allergen-specific tolerance by administering increasing doses of causative allergens subcutaneously or sublingually (4). The precise mechanisms underlying AIT remain to be fully elucidated, but the involvement of regulatory T cells and B cells or the induction of T cell anergy is suggested to be a key mechanism for an efficient AIT. AIT induces antigen-specific immuno-modulatory cells or the anergic response of T cells specific to major allergens included in an allergen extract as an AIT vaccine. Therefore, the standardization of AIT vaccines using important major allergens and the characterization of major component allergens in an allergen source are important issues for the development of an effective AIT (5, 6).

An HDM extract commonly used for AIT of HDM-allergic rhinitis and asthma is standardized

by its concentrations of major allergens, Der f 1 and 2 or Der p 1 and 2. Only Der f 1/2 and Der p 1/2 are used for the standardization of a vaccine for AIT of HDM. On the other hand, many allergens have been identified and characterized from HDM, including *Dermatophagoides farinae* (American HDM) and *D. pteronyssinus* (European HDM) as Der f/Der p allergens or mite allergen gene (Mag) allergens, such as Der f 6 and Mag3 (7-14). Among the allergens, 28 from *D. farinae* have been formally registered in a database for the systematic allergen nomenclature approved by the World Health Organization and the International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-committee. These allergens have been identified and characterized from mite body, mite feces, or a cDNA library of mite bodies. Der f 1 (cysteine protease), Der f 2 (MD2-like lipid binding domain protein), and Der f 23 (peritrophin-like protein) are serodominant allergens in *D. farinae* (7, 15-17). Serine proteases from *D. farinae*, including Der f 3 (trypsin), Der f 6 (chymotrypsin), and Der f 9 (collagenase) act on mucosal epithelial cells to induce chemokine production that triggers the migration of inflammatory cells (13, 18-23). Tropomyosin as Der f 10 and gelsolin as Der f 16 are also important HDM allergens as pan-allergens (10, 14).

We previously reported that subcutaneous AIT using an allergen fraction prepared from *D. farinae* feces extract (HM2 fraction) significantly reduced symptom-medication scores for bronchial

symptoms without any severe adverse events, such as asthmatic attacks or anaphylaxis (24, 25). Interestingly, although HM2 fraction has the highest allergenicity and tolerability, it does not contain major HDM allergens Der f 1 and Der f 2. This suggests that new HDM allergens, other than Der f 1 and Der f 2, are responsible for the immunotherapeutic effects of HM2 fraction. These findings prompted us to characterize the component allergens in HM2 fraction, which should contain important uncharacterized allergens applicable to AIT.

In this study, we identified a novel allergen, Der f 34, belonging to the Rid/YjgF/YER057c/UK114 family (Rid family) with imine deaminase activity from HM2 fraction. The Rid family protein is broadly conserved from prokaryotes to mammals with their high homology of amino acid sequences (26). We further demonstrated that native Der f 34 showed a high IgE-binding frequency comparable to that of a major HDM allergen Der f 2 by two-dimensional allergenome analysis and ELISA. Furthermore, our ELISA inhibition analysis showed that Der f 34 shared its immunoglobulin-binding epitopes with those of *Aspergillus fumigatus*. Taken together, these results indicate that Der f 34 could be a novel and important cross-reactive allergen for HDM allergy.

RESULTS

Molecular cloning and characterization of

der f 34 in *Dermatophagoides farinae*—To identify uncharacterized allergens in HM2 fraction, that has the highest allergenicity and tolerability in HDM extract, we immunoscreened cDNA library of *D. farinae* with anti-HM2 fraction pAb. Consequently, we cloned a cDNA named *der f 34* from a cDNA library for *Dermatophagoides farinae* by immunoscreening with anti-HM2 fraction pAb. The cDNA for *der f 34* consisted of 522 nucleotides containing a 387-bp open reading frame encoding a 128-amino acid polypeptide with a calculated isoelectric point of 6.25 and a molecular weight of 13,998 Da (Fig. 1A). The deduced amino acid sequence contained a characteristic pattern as an “uncharacterized protein family UPF0076 signature consensus pattern”, whose characteristic sequence was referred to as [PA]-[ASTPV]-R-[SACVF]-x-[LIVMFY]-x(2)-[GSAKR]-x-[LMVA]-x(5,8)-[LIVM]-E-[MI] (indicated by underlining in Fig. 1A) deposited as Prosite entry PS01094 on the ExPASy server (Swiss Institute of Bioinformatics). The sequence also contained a domain conserved in the YjgF/YER075c/UK114 family, which was recently renamed the Rid family (26), and deposited in the NCBI conserved domain database (indicated in bold in Fig. 1A). These features of the deduced sequence show that Der f 34 is a new member of Rid family protein.

Rid family proteins share catalytically critical arginine residues for their enamine/imine deaminase enzymatic activity and serve to

hydrolyze the reactive enamine/imines generated by pyridoxal 5'-phosphate (PLP)-dependent enzymes or FAD-dependent amine oxidases (33). The deduced amino acid sequence of Der f 34 contains seven amino acid residues (19Y, 33G, 59N, 91N, 105R, 114P, and 120E; indicated in red color in Fig. 1A) predicted to be involved in an active site structure or in a catalytic domain for enamine/imine metabolisms (33).

Der f 34 showed the highest identity with the Rid-like protein from marine gamma Proteobacterium HTCC2143 (NCBI GenBank Accession No. EAW32813), with 57.0% identity and 81.2% similarity (35). The sequence of the Rid-like protein also contains the signature pattern for the UPF0076 family, a member of the Rid family (26). Der f 34 was also homologous to endoribonuclease from *Halorhodospira halochloris* (NCBI No. AHK77905), with 50.8% identity and 88.3% similarity; endoribonuclease (No. AEG00088) from *Methylobacterium methanica*, with 50.4% and 80.5%; and translation initiator inhibitor from *Limnobacter* sp. MED105 (No. EDM82235), with 46.9% and 81.2% (Fig. 1B) (36, 37). Der f 34 is also homologous to enamine/imine deaminase from *Candidatus Accumolibacter* (No. JDVG02000346) with 50.4% and 79.1%, suggesting that Der f 34 may be an enamine/imine deaminase of *D. farinae*. Importantly, Der f 34 is also homologous to the UK114 protein from *Homo sapiens* (No. CAG33125), with 42.3% identity and 75.2% similarity, and to the Rid-like protein from

Aspergillus ruber (No. EYE94158), with 39.1% and 73.4% (Fig. 1B). These data suggest that proteins homologous to Der f 34 are broadly conserved in various species including mammals, and that Der f 34 may act as a cross-reactive allergen in a variety of allergen sources.

Purification of native Der f 34—We made recombinant Der f 34 (rDer f 34) to prepare anti-rDer f 34 pAb available for immunoaffinity chromatography to purify native Der f 34 (nDer f 34). The rDer f 34 appeared at 16 kDa on SDS-PAGE, coinciding with a calculated molecular weight of 14 kDa (lane rDer f 34 in Fig. 2A). We immunized rabbits with the rDer f 34 to produce rabbit antisera to rDer f 34 and then purified rDer f 34-specific pAb by affinity chromatography using a rDer f 34-immobilized affinity column. Subsequently, we isolated nDer f 34 from HM2 fraction of *D. farinae* feces extract by immunoaffinity chromatography using the anti-rDer f 34 pAb. Prior to purification, nDer f 34 was concentrated by anion-exchange chromatography from HM2 fraction (lane Conc. Fr. in Fig. 2A). Then, nDer f 34 was purified by immunoaffinity chromatography with anti-rDer f 34 pAb from the Der f 34-enriched fraction (lane nDer f 34 in Fig. 2A). The resultant nDer f 34 appeared at 16 kDa in the same position with rDer f 34 (lanes nDer f 34 and rDer f 34 in Fig. 2A). We confirmed that the purified protein was indeed nDer f 34 by immunostaining with anti-rDer f 34 pAb as shown in Fig. 2B and also by N-terminal amino acid sequencing of the protein as

‘SPKRIIST’ (indicated with daggers in Fig. 1A). We found that a faint nonspecific signal appeared at 30 kDa in HM2 fraction by immunostaining with anti-rDer f 34 pAb, but the protein disappeared after anion-exchange chromatography (lane HM2 Fr. and Conc. Fr. in Fig. 2B). nDer f 34 appeared at 16 kDa with pI 6.5 (calculated pI is 6.25) on a two-dimensional SDS-PAGE gel, and sequence of this spot was confirmed to be Der f 34 by *de novo* protein sequencing by MALDI-TOF MS (spot with an arrow in Fig. 2C).

Imine deaminase activity of Der f 34—The functions of Rid family proteins have been reported to include those of enamine/imine deaminase, molecular chaperon, endoribonuclease, chorismatase, 2-aminomuconate deaminase, and so forth (26, 38-42). Although Der f 34 contains conserved amino acid residues for enamine/imine deaminase activity, Der f 34 also showed higher homology with endoribonuclease from *H. halochloris* (Fig. 1B). Therefore, we analyzed the imine deaminase activity of Der f 34 using Leu, Met, or Phe as a substrate. We utilized rTF-Der f 34 instead of rGST-Der f 34 for the analysis of imine deaminase activity using rTF as a negative control, because GST may interfere with the imine deaminase activity. We also analyzed the imine deaminase activity for mutant of Der f 34, whose conserved seven amino acid residues for enamine/imine deaminase activity were substituted for alanine. RidA protein from *Salmonella enterica* (NCBI GenBank accession number AF095578) and a nonfunctional variant

(RidA^{R105A}) were used as a positive and negative control for the imine deaminase assay (32, 33). The imine deaminase activity was determined as an inhibitory activity to produce semicarbazone, which was produced by a reaction with imine and semicarbazide (31). The rTF-Der f 34 showed imine deaminase activity and preferentially digested Leu and Met in cooperation with L-amino acid oxidase. rTF-Der f 34 poorly digested Phe, similar to the case of other imine deaminases of the Rid family (Fig. 3) (26). The imine deaminase activity of rTF-Der f 34 was comparable to that of the known enamine/imine deaminase, RidA protein from *Salmonella enterica*. Mutant rTF-Der f 34 did not show the enzymatic activity comparable to the inactive variant RidA^{R105A} protein or rTF as a negative control (Fig. 3) These results indicate that Der f 34 possesses an imine deaminase activity and the seven conserved residues (red characters in Fig. 1A) are responsible for the imine deaminase enzymatic activity for Der f 34.

Potent IgE-binding capacity of Der f 34 and its cross-reactivity with fungal allergens—We analyzed the importance of Der f 34 as an HDM allergen by allergenome analysis, which is an omics analysis for allergens capable to bind patients' IgE by two-dimensional immunoblotting (representative data is shown as Fig. 2D), and ELISA using plasma from patients allergic to HDM. Our allergenome analysis revealed that a corresponding spot of nDer f 34 (indicated with an arrow in Fig. 2C and D) showed positive binding

with IgE in 62.5% (25/40) of patients, which is comparable to that of Der f 2 (77.5%, 31/40) (data not shown). We further confirmed the IgE-binding capacity of nDer f 34 by ELISA using plasma from 19 patients allergic to HDM. We defined IgE-binding of nDer f 34 as positive if a patient's nDer f 34-specific IgE titer exceeded a threshold (indicated by the broken line in Fig. 4A) (mean+3×SD of 4 healthy volunteers, 2.8 U/ml). Sixty-eight percent (13/19) of the patients reacted positively with nDer f 34, while 79% (15/19) reacted positively with a major allergen, Der f 2 (Fig. 4A and data not shown). These data suggest that Der f 34 is a major allergen for HDM-allergic patients comparable to Der f 2.

Rid proteins are highly conserved from prokaryotes to mammals and their amino acid sequences are highly homologous. Therefore, we analyzed whether Der f 34 shares its immunoglobulin-binding epitopes with those of other Rid family proteins. Importantly, Der f 34 showed significant homology with a Rid protein from a fungus (*Aspergillus ruber*), as indicated in Fig. 1B. Fungi are also important sources of indoor allergens and trigger respiratory disorders such as allergic asthma and aspergillosis (43). Therefore, we evaluated the cross-reactivity of Der f 34 and fungal allergens by ELISA inhibition assay using allergenic fungal crude extracts and Der f 34-specific rabbit IgG (Fig. 4B). The crude fungal extract from *Aspergillus fumigatus* completely prevented the binding between rTF-Der f 34 and rabbit IgG specific to the

rTF-Der f 34 in a dose-dependent manner, but the extract from *Alternaria alternata* did not inhibit the binding (Fig. 4B). These data strongly suggest that Der f 34 shares its immunoglobulin-binding epitopes with *Aspergillus fumigatus* allergens.

DISCUSSION

To achieve a more effective AIT for HDM allergy, it is important to characterize component allergens from the fraction of HDM that had the most potent allergenicity and tolerability by subcutaneous AIT. We characterized a novel allergen named Der f 34 as an imine deaminase belonging to the Rid family. This is the first report to show the IgE-binding capacity of a Rid family protein in allergic patients. Der f 34 showed that imine deaminase activity involved in amino acid metabolisms cooperated with amino acid oxygenase or hydrogenase. Imine deaminase catalyzes the hydrolysis of imine to produce ammonia and ketone (33). Rid family proteins are highly conserved in various species, including prokaryotes and eukaryotes, and their sequences for the UPF0076 signature pattern and residues for enamine/imine deaminase activity are highly conserved (Fig. 1A and B) (26). Due to sequence similarities among members of the Rid family, Der f 34 is a candidate for a novel pan-allergen in various allergen sources. We elucidated that Der f 34 shares its immunoglobulin-binding epitopes with those from *Aspergillus* species (Fig. 4B). This strongly suggests that Der f 34-specific IgE can bind with allergens that share their

immunoglobulin-binding epitopes from other allergenic sources, because binding between epitopes and antigen-binding regions of immunoglobulins (Fab region) is crucial for antigen recognition of immunoglobulin, and this binding is independent of the immunoglobulin isotype (Fc region). Although we found its cross-reactivity only in *Aspergillus* species, it seems to cross-react with other allergens in the Rid family or with enamine/imine deaminases. Elucidation of the cross-reactivity of Der f 34 with other homologous allergens in various allergen sources may also be important for the precise diagnosis of HDM allergy.

High doses (100 and 300 µg/ml) of crude extract from *Aspergillus fumigatus* showed statistically significant inhibition of binding between Der f 34 and Der f 34-specific IgG, although excess amount of a non-specific negative control protein (rTF) fails to block the binding. This clearly indicates the observed competition with rTF-Der f 34 as well as with *Aspergillus fumigatus* extracts is not a non-specific binding. The present data also indicated that the non-specific binding kinetics of rTF is distinct from those seen in rTF-Der f 34, *D. farinae* feces extract, and *Aspergillus fumigatus* extract (Fig. 4B). Our estimation from the inhibition assay suggests the content of rDer f 34-cross-reactive allergens in *Aspergillus fumigatus* is only 0.001% in the crude extract. One possible reason for the low allergen content is that those cross-reactive allergens in *Aspergillus*

fumigatus might be lost during the extraction procedures, because the Rid proteins are small molecules (less than 20 kDa), albeit the inhibition is statistically significant compared to that of *Alternaria alternata* and rTF as a negative control. To further gain insight into the cross-reactivity, experiments using purified native *Aspergillus fumigatus* Rid proteins and Rid proteins-deficient *Aspergillus fumigatus* extract (prepared by immunodepletion with anti-Der f 34 pAb or made from *rid* genes disruptants) are needed.

We characterized Der f 34 as a candidate for a major allergen available to develop a more efficient AIT vaccine. UK114 protein in the Rid family was reported to induce Th2-skewing responses in murine splenocyte and to induce high IL4 with low IFN-gamma production by costimulation with concanavalin A (44). We analyzed the potential for such an immunological skewing property of rDer f 34 produced by the COS-7 (monkey kidney fibroblast) expression system, which is a lipopolysaccharide-negative protein expression system, by culturing with murine splenocytes. However, we could not conclude that Der f 34 possesses the capacity to skew the Th2 response in mice (data not shown). It will be interesting to elucidate whether Der f 34 has the potency to skew the Th1/Th2 response in humans. Interestingly, UK114 protein was also identified in humans as a tumor-associated antigen (NCBI GenBank accession number P80601; 47.4% identical to and 75.2% similar with Der f 34), and human monoclonal antibody against the

UK114 antigen showed complement-dependent cytotoxicity to tumor cell lines but not to normal cells, except for a small fraction of hepatocytes of fetal origin (45, 46). The UK114 protein also inhibits the growth of mammary carcinomas induced in Sprague-Dawley rats, and the authors of that report speculated that the UK114 antigen may be involved in antigen presentation and tumor immunosurveillance (47). We tested cross-reactivity between rTF-Der f 34 and human recombinant GST-tagged UK114, but failed to find a significant cross-reactivity assessed by ELISA inhibition assay and immunoblotting using anti-Der f 34 pAb (data not shown). One possible reason for this poor cross-reactivity is that UK114 was GST-tagged recombinant protein produced by wheat germ cell-free protein expression system, whose conformation could be different from its natural counterparts. We could not clarify the cross-reactivity between native Der f 34 and native UK114 nor whether anti-Der f 34 antibody was involved in the progression or clinical condition of tumors or other human diseases. Further analysis is needed to clarify the cross-reactivity between Der f 34 and human UK114 protein. However, if native UK114 is cross-reactive antigen with Der f 34, it may possible that Der f 34-specific antibody might be involved in the mechanisms underlying the inverse association between an allergy and specific types of cancer (48). These reports implicated that Der f 34 is capable of immunomodulation and may be involved in the

therapeutic mechanisms of AIT.

Native Der f 34 had a high IgE-binding capacity, comparable to that of Der f 2 (62.5% and 68% for Der f 34, 77.5% and 79% for Der f 2 by two-dimensional immunoblotting and ELISA, respectively; Fig. 4A and data not shown), and Der f 34 cross-reacted with fungal allergens (*A. fumigatus*, Fig. 4B). These data strongly suggested that Der f 34 is an important cross-reactive allergen for HDM and other allergen sources, and that the Rid family will be a new category for pan-allergens, such as profilins and nonspecific lipid transfer protein families (49). Allergen sources that digested amino acid in their metabolic pathways may contain enamine/imine deaminase in the Rid family homologous to Der f 34. Therefore, we consider that Der f 34 is also a clinically important major allergen for HDM-allergic rhinitis and asthma.

In this paper, we identified and characterized a novel mite imine deaminase, named *der f 34*, from HDM as a pan-allergen for the first time. Our results suggested that Rid family proteins are important as novel pan-allergens for the cross-reactivity among various allergen sources. It will be important to elucidate in the near future Der f 34's contribution to AIT using HDM extract.

EXPERIMENTAL PROCEDURES

Human plasma—HDM-allergic plasma samples were obtained from 19 patients selected on the basis of their clinical history and a positive

CAP-RAST score for HDM (RAST score 2 or higher). Non-allergic plasma from four healthy volunteers with negative RAST scores were used as negative controls. The study protocol conformed to the standards set by the declaration of Helsinki. All experiments were approved by the Institutional Review Board of Takanobashi Central Hospital and were described in detail to all participants before they provided their informed consent and were admitted into the study. The patients' plasma samples for allergenome analysis (N=40) and ELISA (N=19) were collected separately for each experiment as a different data set.

Extraction of house dust mite allergens—House dust mites (*Dermatophagoides farinae*) were cultured on purina rodent chow (Orient Yeast, Tokyo, Japan) for 30 days and the whole mite culture (1 kg) was suspended in a saturated sodium chloride solution (3 L). The solution was centrifuged at 6,400g for 30 min and supernatant was collected, dialyzed against water, and then lyophilized. The lyophilizate was used as mite feces extract. The mite feces extract was loaded onto UF-20 ultrafiltration apparatus (Tosoh, Shin-Nanyo, Japan) to eliminate small molecular weight allergens. The resultant high molecular fraction was further fractioned by gel filtration chromatography using ultrogel AcA54 column (Sigma Aldrich, St. Louis, MO, USA) monitoring with an absorbance at 280 nm. A faction eluted between BSA (67 kDa) and OVA (45 kDa) was named as a high molecular fraction 2 (HM2

fraction) (27, 28).

cDNA synthesis and recombinant Der f 34 (rDer f 34) in Escherichia coli—The cDNA library was constructed with mRNA from *Dermatophagoides farinae* as described previously (8). Anti-HM2 fraction polyclonal antibody (pAb) was prepared using a male rabbit immunized with 100 µg HM2 fraction mixed with Freund's complete adjuvant (total volume: 1 ml) every 2 weeks until when 2,000-fold diluted serum can detect 100 µg HM2 fraction immobilized on PVDF membrane by colorimetric dot blot analysis. Subsequently, peripheral blood was drained, and serum containing pAb to HM2 fraction was collected after centrifugation with 10,000g for 20 min at 4°C. The serum was aliquot and stored at -20°C until use. The cDNA library of 1.38×10^7 recombinants expressed in *E. coli* NM522 was immunoscreened with the 500-fold diluted rabbit anti-HM2 pAb. Positive bindings were detected by HRP-conjugated anti rabbit IgG with 3, 3'-diaminobenzidine tetrahydrochloride. Seven clones positively bound with anti-HM2 fraction pAb and a positive clone was named *der f 34* (initially named *mag133*, unpublished data). The sequence for *der f 34* was determined by sequencing one positive clone. The sequencing, the construction of plasmid using pGEX-4T-1, transfection into *Escherichia coli* BL21, protein expression of GST-tagged Der f 34 (GST-Der f 34) induced by 0.1 mg/ml IPTG, and purification of recombinant GST-Der f 34 (rGST-Der f 34) using glutathione Sepharose 4B column (GE

Healthcare Japan, Tokyo, Japan) were performed by methods previously described, with slight modifications (8). The rGST-Der f 34 was digested by thrombin on a glutathione column for 16 hr at room temperature, after which only recombinant Der f 34 (rDer f 34) was eluted without GST-tag by 50 mM Tris-HCl containing 150 mM NaCl and 2.5 mM CaCl₂ (pH 8.0). The rGST-Der f 34 was used to prepare anti-rDer f 34 pAb. In enzymatic assay for imine deaminase activity and ELISA inhibition assay for cross-reactivity with fungal allergens, cDNA for *der f 34*, mutant *der f 34*, or *der f 2* (NCBI GenBank accession number CAI05849) was cloned into pCold TF DNA vector (Takara, Tokyo, Japan) and expressed as a recombinant trigger factor (rTF) fusion protein (rTF-Der f 34) with His-tag in Rosetta-gamiTM (DE3) competent cells (Merck Millipore, Billerica, MA, USA). rTF-fusion proteins was purified using His-trap HP (GE Healthcare Japan). cDNA for mutant *der f 34* was chemically synthesized from the sequence of *der f 34* by substitution of seven amino acid residues responsible for enamine/imine deaminase activity (19Y, 33G, 59N, 91N, 105R, 114P, and 120E) by alanine.

Purification of native Der f 34 (nDer f 34) from crude mite extract—Two male rabbits were immunized with rDer f 34 with Freund's complete adjuvant every 2 weeks for eight times. Peripheral blood was drained, and serum containing pAb to rDer f 34 was collected after centrifugation with 10,000g for 20 min at 4°C. Anti-rDer f 34 pAb

was further purified using a rDer f 34-immobilized CNBr-activated Sepharose 4B column (GE Healthcare Japan). HM2 fraction (10 mg protein) fractionated from mite feces extract was loaded onto a Resource Q anion-exchange column (GE Healthcare Japan) in 20 mM Tris-HCl, and the gradient was eluted with 0-1.0 mM NaCl. The fraction containing 16 kDa molecules which bound with anti-Der f 34 pAb by western blotting analysis, and then the nDer f 34-enriched fraction was loaded onto an anti-Der f 34-immobilized protein G-Sepharose 4FF column (GE Healthcare Japan), and nDer f 34 was eluted with 0.1 M glycine-HCl (pH 2.8). Animal experiments were carried out using protocols approved by the Animal Studies Committee of Hiroshima University.

SDS-PAGE and immunoblotting—The purified rDer f 34 and nDer f 34, HM2 fraction, and the enriched nDer f 34 fraction after anion-exchange chromatography were loaded onto 12.5% SDS-PAGE gel under reduced condition, and the proteins were visualized with silver staining following the manufacturer's instructions (2D silver staining kit, Daiichi Kagaku Yakuhin, Tokyo, Japan). Two-dimensional SDS-PAGE and allergenome analysis of *D. farinae* extract were performed using methods similar to those described previously (29). The two-dimensional immunoblotting analysis in the allergenomics study was carried out using plasma samples from 40 patients allergic to HDM (unpublished data). The HM2 fraction, the enriched nDer f 34 fraction,

rDer f 34, and nDer f 34 were immunostained with 20,000-fold-diluted rabbit anti-rDer f 34 pAb, followed by 20,000-fold-diluted peroxidase-conjugated goat anti-rabbit IgG (Cell Signaling Technology, Danvers, MA, USA) and visualized with ECL Plus Western blotting detection reagents (GE Healthcare Japan). The area of PVDF membrane corresponding to the protein weight marker was cut after protein transfer, and then stained with Coomassie Brilliant Blue.

Enzyme-linked immunosorbent assay (ELISA) and ELISA inhibition assay—ELISA and ELISA inhibition were performed according to a method previously reported (30). Briefly, 25 ng/well nDer f 34 was coated onto a 96-well microtiter plate, which was then blocked with PBS containing 2% skim milk and 1% BSA. Serial dilution of purified human IgE (Athens research Technology, Athens, GA, USA) was coated on the plate as a calculator to estimate the positive IgE-binding. Positive binding equal to the immobilized 10 ng IgE was defined as 1 arbitrary unit (AU). Then 50-fold-diluted plasma samples from 19 allergic patients or 4 healthy volunteers were added to the plate, followed by incubation with 1,000-fold-diluted biotin-conjugated anti-human IgE (epsilon chain-specific; Biosource International, Camarillo, CA, USA). After incubation with 1,000-fold-diluted alkaline phosphate-conjugated streptavidin (Jackson ImmunoResearch Laboratories, West Grove, PA, USA), Attophos substrate solution (Promega,

Fitchburg, WI, USA) was added to the plate, and fluorescence intensity was measured using a Wallac 1420 ARVOsx Multilabel Counter (Perkin-Elmer, Downers Grove, IL, USA). To analyze the cross-reactivity of Der f 34 with fungal allergens, rTF-Der f 34 was coated onto a microtiter plate and reacted with a 25,000-fold-diluted rabbit anti-HM2 fraction pAb preincubated with serial concentrations (300 µg/ml, and 10-fold serial dilutions from 100 µg/ml) of fungal crude extract from *Aspergillus fumigatus* or *Alternaria alternata*, crude extract from *D. farinae* feces, rTF-Der f 34, or rTF as a negative control. Both extract from *Aspergillus fumigatus* and *Alternaria alternata* were purchased from ITEA (Tokyo, Japan). The percent of inhibition was calculated using the positive binding without inhibitor as 0% and saturated binding (average of 300, 100, 10, and 1 µg/ml) of rTF-Der f 34 as 100%.

Enzymatic activity for imine deaminase of Der f 34—Imine deaminase activity was analyzed as reported elsewhere with slight modifications (26). Briefly, 300 nM rTF-Der f 34, mutant

rTF-Der f 34, wild type RidA protein from *Salmonella enterica* (NCBI GenBank accession number AF095578), inactive variant RidA^{R105A} protein or rTF as a negative control was mixed with 10 mM semicarbazide-HCl, 1 µg (2-5 units) bovine liver catalase, 10 µg (30 milli-units) of L-amino acid oxidase from *Crotalus adamanteus* (Sigma-Aldrich, St Louis, MO, USA), and substrate amino acid; 5 mM L-leucine (Leu), 6.25 mM L-methionine (Met), or 10 mM L-phenylalanine (Phe) in 50 mM potassium pyrophosphate (pH 8.7) (26, 31). OD248 nm was monitored every 1 min for 15 min at 22°C for the assay of enzymatic activity. The wild type RidA protein from *Salmonella enterica* and inactive RidA^{R105A} variant protein were prepared as previously described (32, 33). The amount of keto acid formation determined as OD248 was calculated as $0.6 \times 10^3 \text{ M}^{-1} \text{cm}^{-1}$ (34).

Statistical analysis—Non-repeated ANOVA was applied to compare data among the groups and, when the differences was significant ($p < 0.01$), post hoc analysis using a Student-Newman-Keuls (SNK) test was performed.

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Conflict of interest: The authors declare that they have no conflicts of interest regarding the contents of this article.

Author contributions: KGR, TF, TaA, KH, JWK, KB, CK, and TI performed experiments including production of recombinant fusion proteins; YA, TH, TK, and ST contributed to obtaining plasma from HDM patients and healthy volunteers, as well as the clinical data for them; KGR, TF, and SK analyzed experimental data; TF, TuA, KO, and SK conceived, arranged, and supervised the study; KH, DMD, IHR and DBA analyzed experimental data and reviewed the manuscript prior to submission; TF and SK wrote the paper and revised the manuscript; KGR, TF, and SK have agreed to be accountable for all aspects of the work and in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved; SK has given final approval of the version of the manuscript to be published.

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FOOTNOTES

The abbreviations used are: HDM, House dust mite; nDer f 34, native Der f 34; rDer f 34, recombinant Der f 34 without tag; rGST-Derf 34, rDer f 34 with GST-tag; rTF-Der f 34, rDer f 34 as a TF fusion protein; rTF, Trigger factor.

FIGURE LEGENDS

FIGURE 1. A. nucleotide sequence of *der f 34* cDNA and its deduced amino acid sequence. A conserved domain for the YjgF/YER057c/UK114 family (residues 19-125) and a signature motif conserved in the UPF0076 family (residues 103-121), [PA]-[ASTPV]-R-[SACVF]-x-[LIVMFY]-x(2)-[GSAKR]-x-[LMVA]-x(5,8)-[LIVM]-E-[MI] are shown in boldface and by a single underline, respectively. Seven residues predicted to be involved in the active site structure of catalytic activity are indicated in red color: 19Y, 33G, 59N, 91N, 105R, 114P, and 120E (also shown with red asterisks in Fig. 1B). The amino acid sequence coincided with that from N-terminal amino acids sequencing of purified nDer f 34 is indicated with daggers. B. multiple sequence alignment of Der f 34 polypeptide. Sequences of Der f 34 and Rid family members from bacteria, fungi, and mammals were aligned using ClustalW, including Rid-like protein (marine gamma proteobacterium HTCC2143, NCBI GenBank accession No. EAW32813), endoribonuclease (*Halorhodospira halochloris*, AHK77905), endoribonuclease L-PSP (*Methylobacter methanica*, AEG00088), translation initiation inhibitor (*Limnobacter* spices, EDM82235), enamine/imine deaminase (*Candidatus Accumulibacter* BA91, JDVG02000346), UK114 (*Homo sapiens*, CAG33125), and Rid-like protein (*Aspergillus ruber* CBS135680, EYE94158). Identical amino acid residues are marked with coloring. Red asterisks indicate seven residues predicted as active sites of enamine/imine deaminase. Percent identity and similarity between Der f 34 and each sequence is indicated following to the each sequence.

FIGURE 2. Purification of native Der f 34. A, purification of native Der f 34 from *D. farinae* extract. Native Der f 34 was purified by anion-exchange chromatography followed by immunoaffinity chromatography using an anti-rDer f 34 pAb-immobilized column. HM2 fraction, the nDer f 34-enriched fraction after anion-exchange chromatography, and the resultant nDer f 34 and rDer f 34 were loaded onto SDS-PAGE gel under reduced conditions, and are shown as HM2 Fr., Conc. Fr., nDer f 34, and rDer f 34, respectively. The fractionated proteins were visualized by silver staining. The marker represents a molecular weight marker. B, immunoblotting with anti-rDer f 34 pAb. The proteins shown in Fig. 2A were immunoblotted with anti-rDer f 34 pAb. The marker represents protein weight marker stained with

Coomassie Brilliant Blue. C, two-dimensional electrophoresis of *D. farinae* extract. *D. farinae* extract was separated by two-dimensional electrophoresis and proteins were visualized by silver staining. An arrow shows the corresponding spot for nDer f 34. D, a representative blot for two-dimensional immunoblotting stained with human plasma IgE from a HDM-allergic patient. Two-dimensional immunoblotting was carried out for 40 HDM-allergic patients' plasma, and the IgE-binding frequency was calculated for all positive spots. Molecular weights of positive spots were estimated from the molecular weight marker on another silver stained two-dimensional SDS-PAGE gel, which was electrophoresed simultaneously with the gel for immunoblotting. An arrow shows that corresponding positive signal for nDer f 34.

FIGURE 3. Imine deaminase activity of Der f 34. Imine deaminase activity of Der f 34 was analyzed using rTF-Der f 34, recombinant RidA protein from *S. enterica* as a positive control, mutant rTF-Der f 34, nonfunctional variant RidA protein (RidA^{R105A}) and rTF as a negative control, and Leu, Met, or Phe as a substrate. In the assay, L-amino acid oxidase dehydrated an amino acid to produce an imine, and the resultant imine reacts with semicarbazide to produce semicarbazone that has an absorbance at 248 nm. An imine deaminase accelerates the hydrolysis of substrate-derived imine intermediates to prevent the production of semicarbazone (26, 31). Semicarbazone formation at each time point from that of 0 min for vehicle (buffer, black diamonds), rTF-Der f 34 (red diamonds), mutant rTF-Der f 34 (purple diamonds), RidA protein (orange diamonds), variant RidA^{R105A} protein (green diamonds), and rTF (bright blue diamonds) are plotted on the Y-axis, and the incubation time for the reaction is shown on the X-axis. The data represents average $\pm$ SD for four independent experiments. Non-repeated ANOVA was applied to compare data among the groups and, when the differences was significant ($p < 0.01$), post hoc analysis using a Student-Newman-Keuls (SNK) test was performed. Significant differences as estimated by post hoc SNK test compared to rTF or vehicle as a negative control are shown as ** ($p < 0.01$) for rTF, or # ($p < 0.05$) or ## ($p < 0.01$) for vehicle, respectively.

FIGURE 4. IgE-binding potency of Der f 34. A, IgE-binding capacity of nDer f 34 in HDM-allergic patients. IgE-binding frequencies and IgE levels to nDer f 34 in HDM-allergic patients are shown for individuals. ELISA was performed using plasma from 19 HDM-allergic patients and 4 healthy volunteers. The average+3 $\times$ SD of IgE titer from 4 healthy volunteers was set as a threshold value for positive results (2.8 U/ml), and that threshold value is indicated by a horizontal broken line. An average value with SD of nDer f 34-specific IgE titer for 4 healthy volunteers is labeled with 'H'. An arbitrary unit (AU) is defined as an amount equivalent to 10 ng human IgE. B, cross-reactivity of Der f 34 with allergenic fungal extract.

The percent inhibition of binding between rTF-Der f 34 and IgG specific to Der f 34 in anti-HM2 fraction pAb by rTF-Der f 34, *D. farinae* feces extract indicated as Mite extract, crude fungal extract from *Aspergillus fumigatus* or *Alternaria alternata*, or rTF as a negative control are plotted on the Y-axis. Value for 0% inhibition was determined without inhibitor and 100% was set as saturated value for rTF-Der f 34 as a positive control. The data represents average $\pm$ SD for three individual experiments. Non-repeated ANOVA was applied to compare data among the groups and, when the differences was significant ($p < 0.01$), post hoc analysis using a Student-Newman-Keuls (SNK) test was performed. Significant differences as estimated by post hoc SNK test compared to rTF as a negative control: $**p < 0.01$.

FIGURE 1

A

102030405060

TGATTGATTGATTCAAATACACATAGCCAAACAATGAGTCCAAAACGTATTATATCAACA

M S† P† K† R† I† I† S† T†

708090100110120

CCGTTGGCACCGCAACCAATTGGACCATATTCACAAGCAGTTCAAGTTGGCAATACTGTT

P L A P Q P I G P Y S Q A V Q V G N T V

130140150160170180

TATCTTTCCGGACAAATTGGTATGAATGTACGTACTAATGAAATGGTTACCGGACCAATT

Y L S G Q I G M N V R T N E M V T G P I

190200210220230240

CGTGATGAAGCACAACAAGCATTTACGAATATGAAAGCCGTAGTTGAAGCAAGTGGTGCT

R D E A Q Q A F T N M K A V V E A S G A

250260270280290300

AAAATGTCCGATGTTGTTAAAGTGAATATTTTCATTTCGAAATTTCAATGATTTTCCTGCC

K M S D V V K V N I F I R N F N D F P A

310320330340350360

ATTAATGATGTGATGAAAGAATTTTTTCAATCACCATTTCCAGCACGATCAACAGTCGGT

I N D V M K E F F Q S P F P A R S T V G

370380390400410420

GTTGCTGAATTGCCGAAAAATGCTCGTGTTGAAATTGAAAGTATCGTTGTAATTGAATAA

V A E L P K N A R V E I E S I V V I E *

430440450460470480

ATGCGATGAGAAATTTGCATTTTCTCATTTTGTTTTTTTTTTAATTGACTGAAATGATCTG

490500510520

AATAAATGTCCCTTTTTTTTGGAAAAAAAAAAAAAAAAAAAAA

FIGURE 1

B

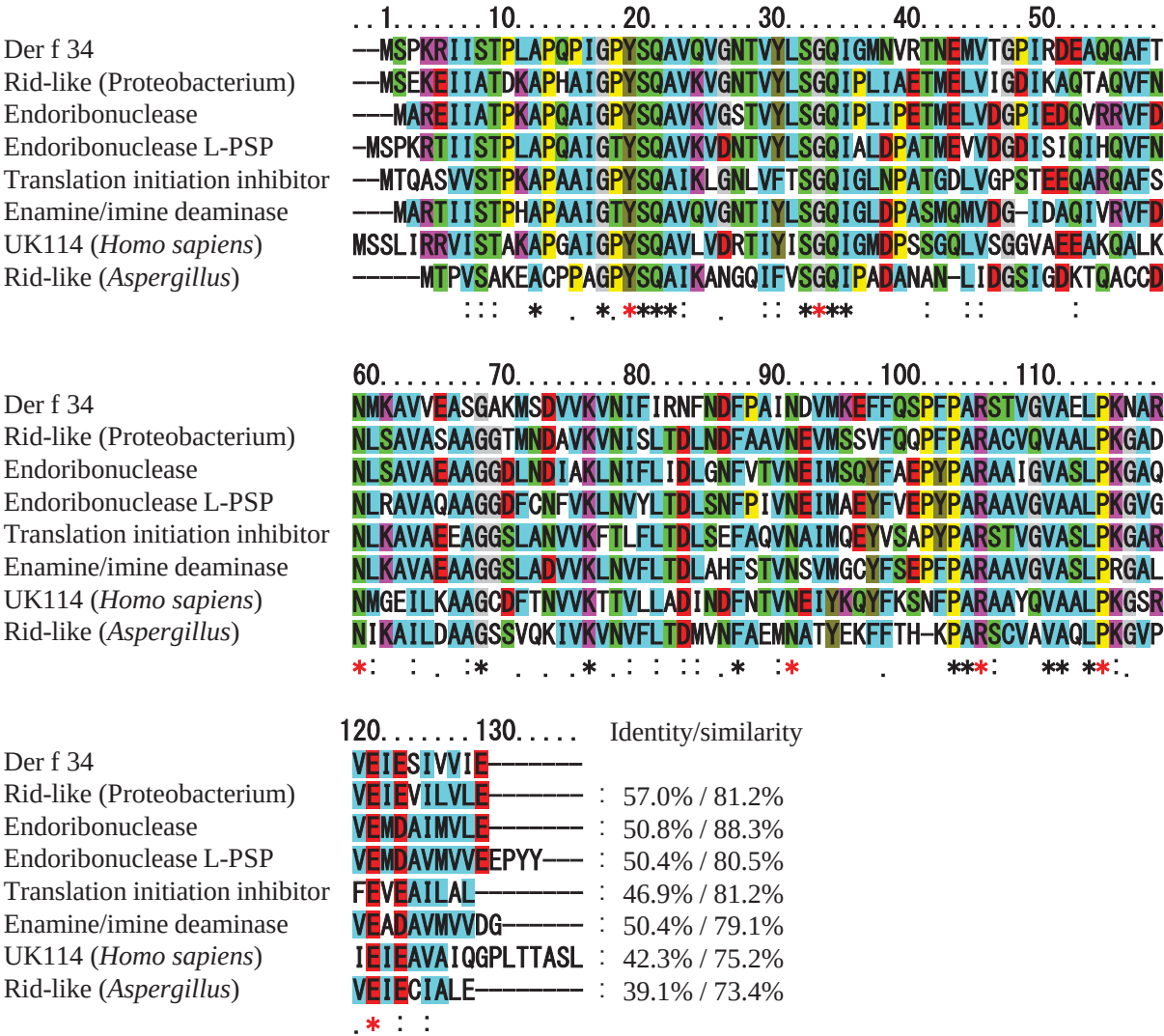


FIGURE 2

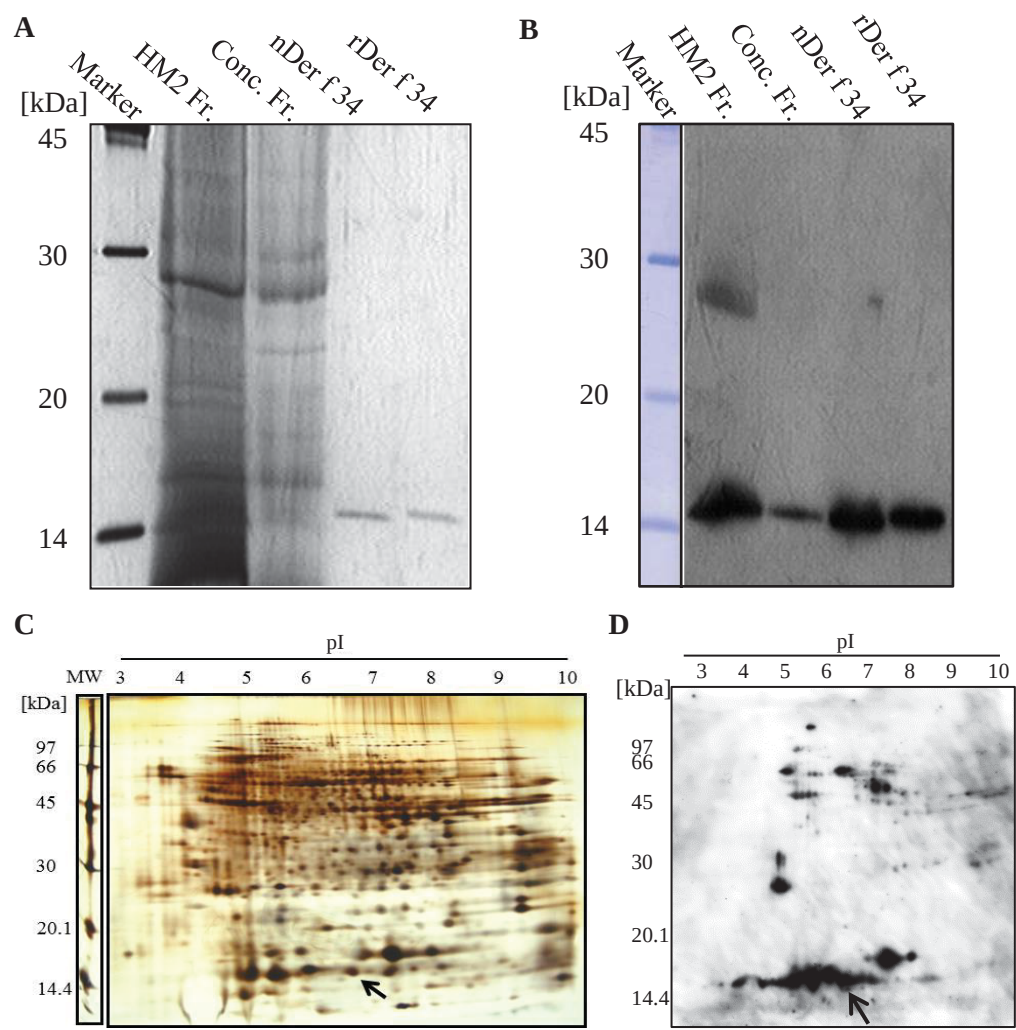


FIGURE 3

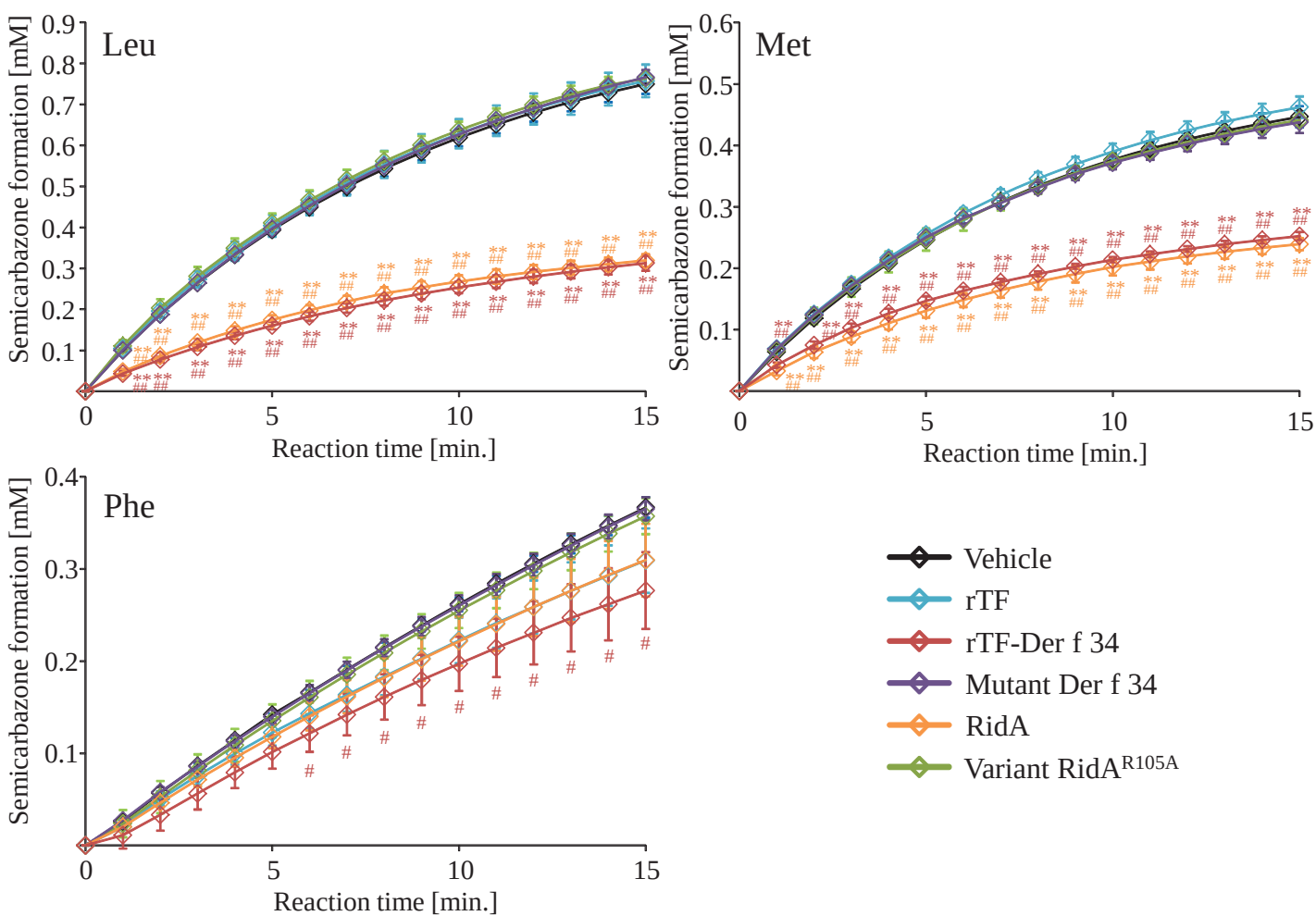
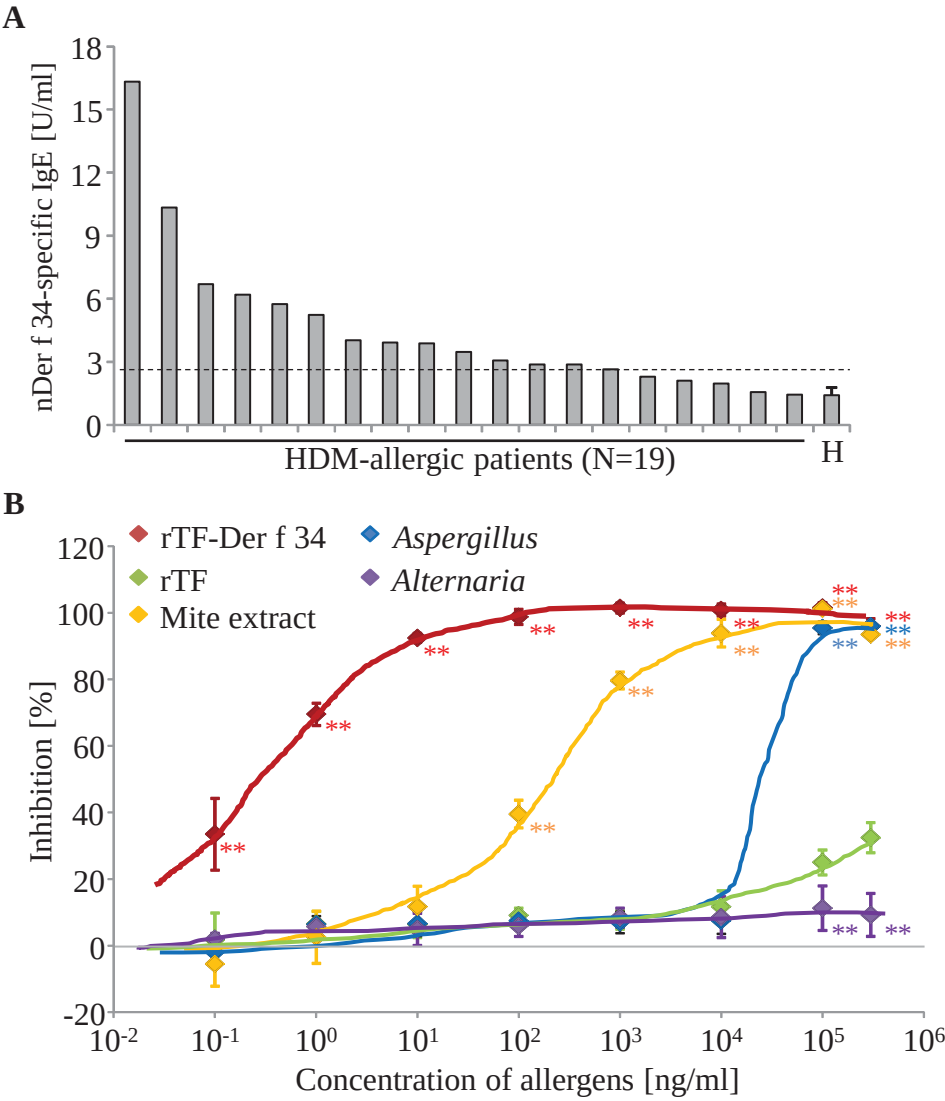


FIGURE 4



**Der f 34, a Novel Major House Dust Mite Allergen Belonging to a Highly Conserved
Rid/YjgF/YER057c/UK114 Family of Imine Deaminases**

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