Analysis of the structure and allergenicity of recombinant pro- and mature Der p 1 and Der f 1: Major conformational IgE epitopes blocked by prodomains

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Background: The major house dust mite group 1 allergens Der p 1 and Der f 1, which belong to the papain-like cysteine protease family, are the most potent of indoor allergens. However, little information is available on the location of IgE epitopes.

Objective: We investigated the allergenicities of recombinant proforms and mature forms of Der p 1 and Der f 1 to compare them with natural Der p 1 and Der f 1 and to obtain information on the conformational IgE-binding epitopes.

Methods: Secreted pro-Der p 1 and pro-Der f 1 and their mutants without hyperglycosylation expressed in yeast were converted to mature forms. We purified the proforms and mature forms and analyzed their apparent molecular sizes and secondary structures by means of gel-filtration and circular dichroism analysis and their allergenicities by means of assays for IgE binding, IgE-binding inhibition, and basophil histamine release. The tertiary structure of pro-Der f 1 was predicted by molecular modeling.

Results: The recombinant mature forms exhibited similar molecular sizes, secondary structures, and allergenicities as their natural types. On the other hand, their proforms exhibited different secondary structures and less allergenicities than the mature forms in all sera and volunteers tested. Molecular modeling revealed that the prosegment is anchored at the prosegment-binding loop and the substrate-binding cleft on the surface of the mature portion.

Conclusions: Our studies indicate that the prodomains of Der p 1 and Der f 1 reduce allergenicity and that the major conformational IgE epitopes commonly found in a broad population of patients exist within the 2 regions blocked by the prosegments. Recombinant Der p 1 and Der f 1 and the findings in the present study will be the basis for allergen standardization and the design of safer and more effective allergen vaccines. (J Allergy Clin Immunol 2005;115:555-63.)

Key words: *House dust mite, Der p 1, Der f 1, recombinant allergen, conformational IgE epitopes, prodomain, proform, mature form, cysteine protease, molecular modeling*

House dust mites of 2 species of *Dermatophagoides* (*Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*) associated with allergic diseases, such as bronchial asthma, rhinitis, and atopic dermatitis, are the most important causative factor among indoor allergens.[1] Group 1 and group 2 allergens are major allergens derived from house dust mites on the basis of the frequency of patients sensitized, amount of specific IgE, and content in mite extract.[2] Group 1 allergens (Der p 1 and Der f 1) are the most clinically relevant allergen proteins because they exist in abundance in mite fecal pellets and account for more than 50% of IgE antibodies against total mite extract. They belong to the papain-like cysteine protease family.[3,4] The enzymatic activity of Der p 1 has been suggested to be involved in the pathogenesis of allergy.[5-9]

The cDNAs for Der p 1 and Der f 1 were isolated when cDNA cloning of allergens began.[3,4] They encode signal peptides of 18 amino acid residues responsible for secretion, 80 residue propeptides, and 222 residue mature portions for Der p 1 and 223 residues for Der f 1. Mature Der p 1 and Der f 1 have 82% sequence homology with each other. Although molecular modeling of mature Der p 1 on the basis of the tertiary structures of plant cysteine proteases has been performed,[10] its structure has not been determined by means of x-ray crystallography.

Molecular analysis of the IgE-binding epitopes of Der p 1 and Der f 1 using correctly folded and fully active recombinant proteins has not been reported. Synthetic peptides and recombinant fragments expressed in *Escherichia coli* show little or no IgE-binding activity compared with that of natural allergens,[11-14] and therefore little information is available on conformational IgE-binding epitopes. We and others reported on efficient

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Abbreviation used
CD: Circular dichroism

systems that can be used to produce recombinant mature Der f 1[15-18] and Der p 1.[15,19] Although they were modified with hyperglycosylation when expressed in yeast,[15,16,18,19] we successfully produced mutants without the hyperglycosylation and with IgE binding and proteolytic activities by means of acid treatment of the proforms.[15-17] These systems enable the analysis of IgE epitopes and pathogenic proteolytic activity, which are dependent on the tertiary structure of the allergens. They can also be a basis for allergen standardization and the design of safer and more effective allergen vaccines for allergen-specific immunotherapy.[20,21]

Prosegments of papain-like cysteine proteases block enzymatic activity and assist in the folding of mature segments. Recently, a research group analyzed allergenicity of pro-Der p 1, but it was hyperglycosylated and not spontaneously converted to the active mature form, and therefore it was compared with natural Der p 1 but not with its appropriate counterpart, recombinant mature Der p 1.[22] We also reported on decreased allergenicity of pro-Der f 1 in a patient, but this contained unfolded forms, aggregated forms, or both.[16,17] In the present study we investigated the allergenicities of highly purified and correctly folded recombinant pro- and mature Der p 1 and Der f 1. We found that the prodomains of these proteases function to reduce allergenicity in a broad population of patients. We also defined the locations of major conformational IgE-binding epitopes blocked by the prosegments by interpreting our experimental results on the basis of the tertiary structure of pro-Der f 1 predicted from the crystal structures of the proforms and mature forms of homologous cysteine proteases.

METHODS

Preparation of recombinant proforms and mature forms of Der p 1 and Der f 1

A method described previously was followed, with some modifications.[15,16] Briefly, recombinant proforms were secreted into the culture supernatant of yeast *Pichia pastoris* transfectant cells and converted into the prosequence-removed mature forms by means of dialysis against an acidic buffer. The mature forms were purified by anion-exchange and size-exclusion column chromatography. The proforms were purified by means of the same methods as the mature forms but without activation by dialysis against the acidic buffer. The purities were checked by using SDS-PAGE, and protein concentrations were determined by means of the Bradford procedure with a protein assay kit (Bio Rad, Richmond, Calif), with bovine IgG (Bio-Rad) as the standard.

Natural Der p 1 and Der f 1

Natural Der p 1 and Der f 1 used for gel-filtration, IgE-binding, inhibition, and histamine-releasing assays were purified from whole culture extracts of the house dust mites *D pteronyssinus* and *D farinae*, respectively, as previously described by Yasueda et al.[23] For circular dichroism (CD) analysis, commercially available natural Der p 1 (Indoor Biotechnologies, Charlottesville, Va) and Der f 1 (Asahi Breweries, Ltd, Tokyo, Japan) were used.

Gel-filtration analysis

Purified recombinant and natural Der p 1 and Der f 1 (0.1 mL, 0.5 mg/mL) were subjected to gel-filtration chromatography on Protein-Pak 125 (0.78 cm × 30 cm; Waters, Milford, Mass) at 0.5 mL/min with 0.1 M potassium phosphate buffer (pH 6.5) containing 0.01% sodium azide, and the absorbances at 280 nm were monitored. Molecular weight standards for gel filtration used for the estimation of the apparent molecular sizes were as follows: carbonic anhydrase, 25 kd (Wako, Osaka, Japan); bovine pancreas chymotrypsinogen A, 25 kd; hen egg ovalbumin, 43 kd; and BSA, 67 kd (Amersham Biosciences, Uppsala, Sweden).

CD

CD spectra were measured on a JASCO J-820 spectropolarimeter (Japan Spectroscopic Co, Ltd, Tokyo, Japan) by using a 0.1-cm cell at 25°C over 190 to 260 nm. Samples at 0.1 mg/mL were dialysed against 10 mM potassium phosphate buffer (pH 7.5). The experimental parameters were as follows: bandwidth of 1.0 nm, except for natural and recombinant mature Der f 1 (0.5 nm); sensitivity of 100 millidegrees; 0.5 nm/datum step resolution; 50 nm/min scan speed; and 10 scans. The protein concentrations were determined on the basis of their absorbances at 280 nm or the Bradford procedure by using the protein assay kit, with BSA (Bio-Rad) as the standard. The fractions of helical structures were estimated by using the mean residual ellipticity at 222 nm.[24]

RAST–enzyme immunoassay

Sera were obtained from mite-sensitive atopic patients with bronchial asthma, allergic rhinitis, and/or atopic dermatitis who had positive skin prick test responses. IgE reactivities were measured by means of RAST–enzyme immunoassay, as previously described.[25] Briefly, the diluted serum of each allergic patient was incubated with paper discs coupled with the allergens. Then IgE that bound to the disc was detected with β-galactosidase–conjugated anti-human IgE antibodies by measuring the fluorescence. The values calculated by subtracting the background of the paper discs without the coupling of allergen from the original values were used as the IgE reactivities.

Inhibition assay

The method used was previously described.[26] Briefly, the diluted serum of each allergic patient was incubated with inhibitors, and then the solution was added to paper discs coupled with natural Der p 1 and Der f 1. IgE that bound to the discs was detected with β-galactosidase–conjugated anti-human IgE antibodies. The percentage of inhibition was expressed as the relative reduction of the fluorescence intensity in each sample to that when no inhibitors were added.

Histamine release

The method used was as described in the literature[27] with an HRT-Shionogi (Shionogi and Co, Ltd, Osaka, Japan), with some modifications. The percentage of released histamine was calculated as the relative amount of histamine in the supernatant to the total histamine content. The amount of released histamine after incubation with 0.2 mg/mL digitonin was used as the total histamine content for the calculation.

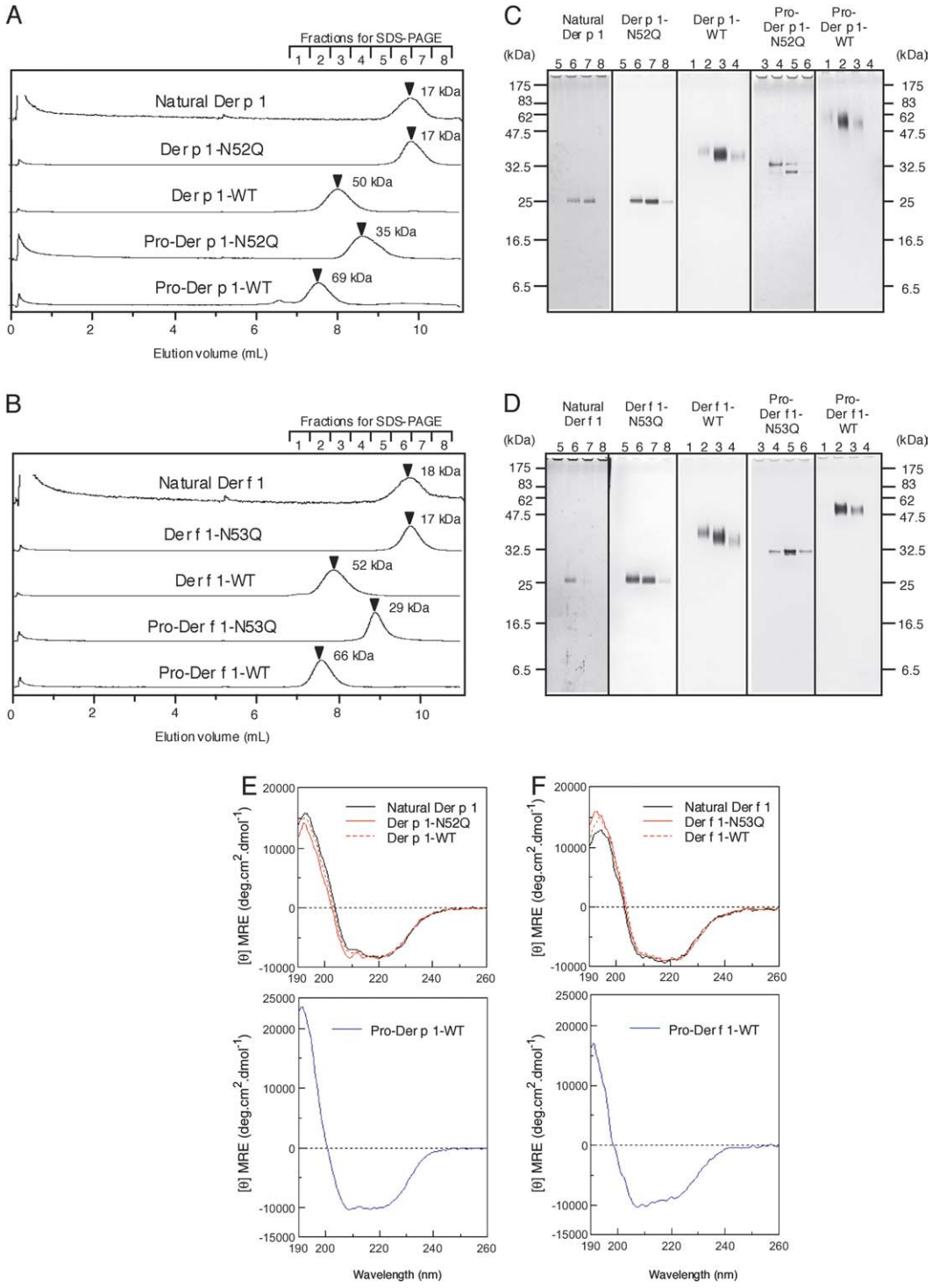

FIG 1. Molecular sizes and secondary structures of purified recombinant proforms and mature forms and natural types of Der p 1 (**A**, **C**, and **E**) and Der f 1 (**B**, **D**, and **F**). Fig 1, *A-D*, Gel-filtration analysis. The fractions shown as 1 through 8 in Fig 1, *A* and *B*, were subjected to SDS-PAGE (15% to 25%) under nonreducing conditions and silver stained and are shown in Fig 1, *C* and *D*, respectively. Fig 1, *E* and *F*, CD spectra.

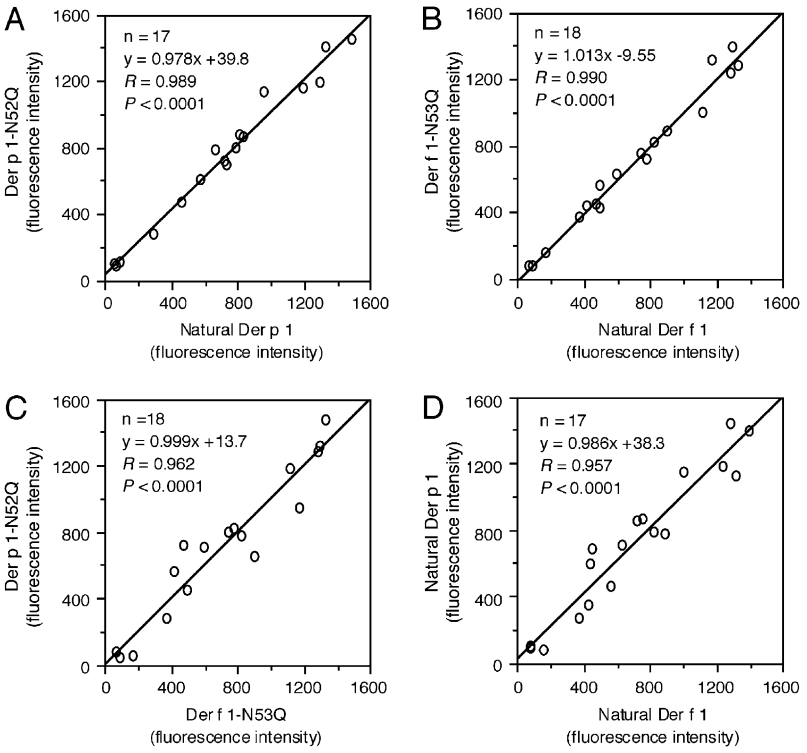

FIG 2. Comparison of direct IgE-binding capacity among recombinant and natural Der p 1 and Der f 1. The serum dilution was 1:8. The concentration of allergens in the immobilization step was 50 nM.

Homology modeling

The tertiary structure of pro-Der f 1 was modeled on the basis of the crystal structures of 3 homologues, procaricain (1pciC),[28] human procathepsin K (7pckC),[29] and mature cruzain (1aim_),[30] by using the automated comparative protein modeling server SWISS-MODEL.[31]

RESULTS

Expression and purification of recombinant Der p 1 and Der f 1

Der p 1 and Der f 1 have an *N*-glycosylation motif in their mature amino acid sequences, and therefore recombinant forms designated Der p 1-WT and Der f 1-WT with wild-type sequences are modified with yeast-derived hyperglycosylation. To obtain recombinant proteins without hyperglycosylation, we generated mutants designated Der p 1-N52Q and Der f 1-N53Q, in which the *N*-glycosylation motif within the mature sequence was disrupted. Although productivity of pro-Der p 1-N52Q and pro-Der f 1-N53Q was lower than that of pro-Der p 1-WT and pro-Der f 1-WT, we optimized culture conditions and achieved preparation of the highly purified proforms and mature forms. The sizes of the recombinant mature forms without *N*-glycosylation and the natural types, which have a theoretic molecular weight of 25 kd, were identical to each other and estimated to be 17 to 18 kd (Fig 1, *A* and *B*).

On SDS-PAGE for each fraction, 2 bands were detected for pro-Der p 1-N52Q, and the one with a lower molecular weight showed similar mobility with pro-Der f 1-N53Q, revealing that the slight broadness of the gel-filtration peak for pro-Der p 1-N52Q was attributed to differential modifications of the *N*-glycosylation motif in the Der p 1 propeptide (Fig 1, *C*). Bands associated with the recombinant forms were smeared when the mature portions were glycosylated, suggesting heterogeneity in the size of the sugar chain (Fig 1, *C* and *D*). The results of gel filtration and SDS-PAGE showed that all recombinant Der p 1 and Der f 1 was monomeric and highly purified and that the molecular sizes of Der p 1-N52Q and Der f 1-N53Q were identical to those of natural types purified from the mite cultures.

Secondary structures of recombinant Der p 1 and Der f 1

CD spectra of recombinant mature Der p 1 and Der f 1 were identical to those of the natural types, showing that their secondary structures were identical to each other (Fig 1, *E* and *F*, upper panels). *N*-glycosylation of the mature forms did not affect the spectra. The proforms showed small but obvious differences from the mature forms (Fig 1, *E* and *F*, lower panels). The calculated values of the helical content are as follows: recombinant mature forms and natural types, 19% to 21%; pro-Der p 1-WT, 25%; and pro-Der f 1-WT, 21%.

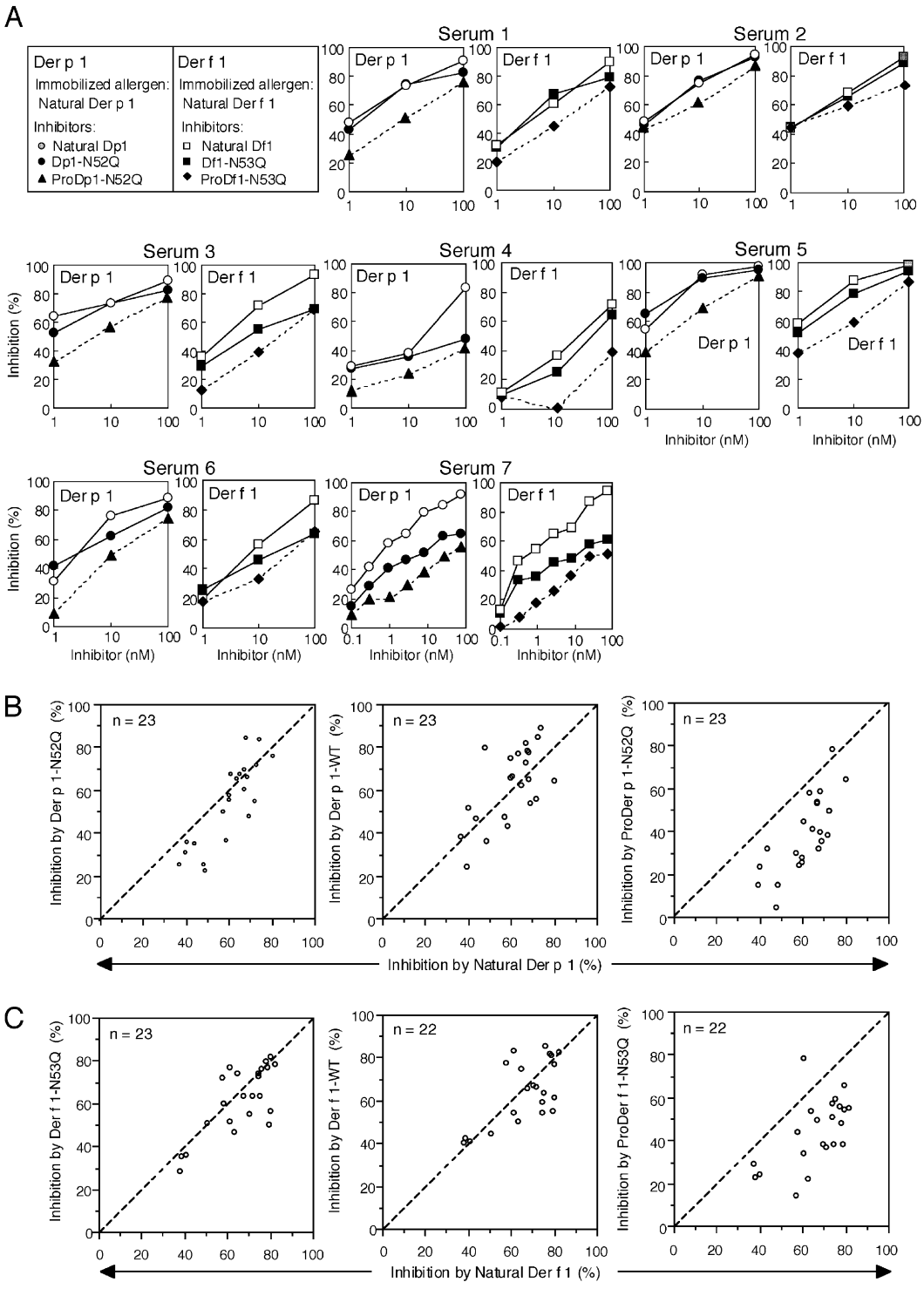

FIG 3. Inhibition of IgE binding to immobilized natural Der p 1 and Der f 1 by means of soluble recombinant proforms and mature forms and natural types. The concentration of the natural allergens in the immobilization step was 50 nM. **A**, The serum dilution was 1:8 for sera 1 through 4 and 7, 1:4 for serum 5, and 1:100 for serum 6. *Dp1*, Der p 1; *Df1*, Der f 1. **B** and **C**, The serum dilution was 1:8.

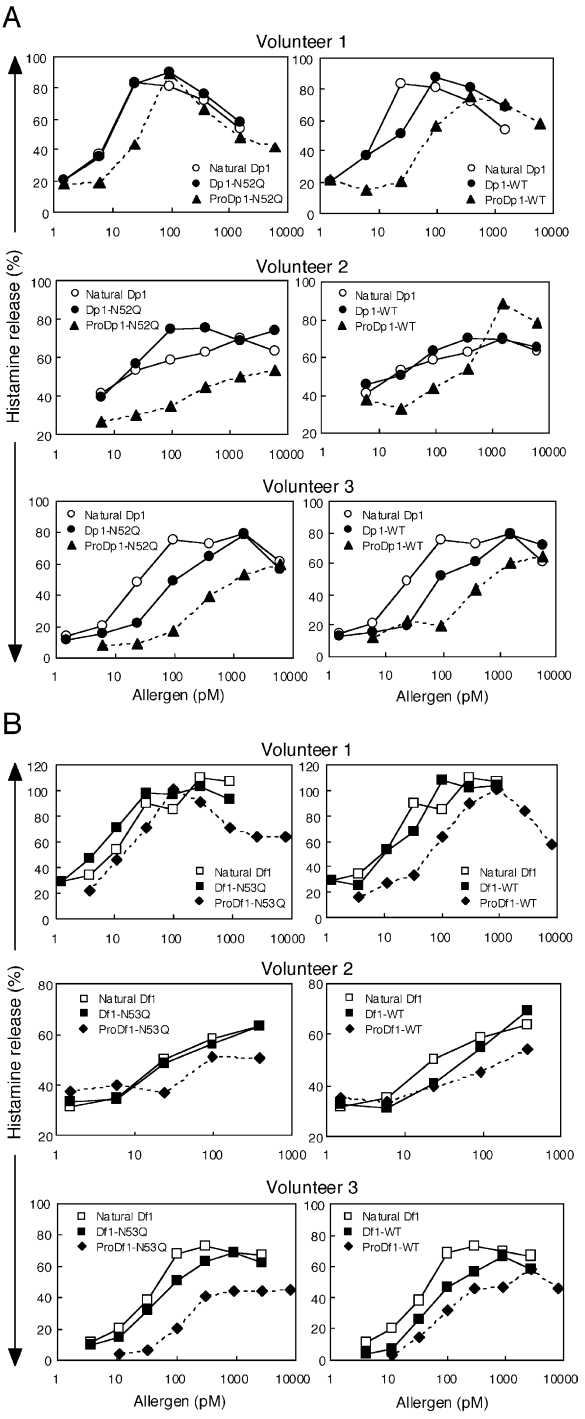

FIG 4. Histamine release from basophils stimulated with recombinant proforms and mature forms and natural types of Der p 1 **(A)** and Der f 1 **(B)**. Spontaneous histamine release for volunteers 1, 2, and 3 were 19%, 27%, and 7% in Fig 4, *A*, and 19%, 30%, and 4% in Fig 4, *B*, respectively. The results shown are representative of 2 or 3 independent experiments.

Direct IgE-binding capacity of recombinant and natural Der p 1 and Der f 1

Seventeen or 18 individual sera from patients with mite allergy were tested to compare direct IgE binding to immobilized Der p 1-N52Q, Der f 1-N53Q, and the natural types (Fig 2). Close correlations of IgE binding between the recombinant mature forms and natural types (Fig 2, *A* and *B*) and between Der p 1 and Der f 1 (Fig 2, *C* and *D*) were observed.

Activities of recombinant Der p 1 and Der f 1 for the inhibition of IgE binding to immobilized natural allergens

For further analysis of IgE-binding capacity, the activities of the recombinant proforms and mature forms responsible for competitive inhibition of IgE binding to immobilized natural allergens were analyzed (Fig 3, *A*). For 5 of the 7 sera, the efficiency of inhibition by the recombinant mature forms was similar to that of the natural types. However, for sera 4 and 7 for Der p 1 and sera 3 and 7 for Der f 1, inhibition by the recombinant mature forms was lower than that of the natural types. Inhibition by the proforms was lower than that of the recombinant mature forms and the natural types for all tested sera.

Furthermore, we examined 22 or 23 sera for similar analysis at an inhibitor concentration of 10 nM. On average, inhibition by the recombinant mature forms with or without yeast-derived *N*-glycosylation of the mature portions was almost equivalent to that of the natural types, although some individual sera showed greater or lesser inhibition for the recombinant mature forms than for the natural types (Fig. 3, *B* and *C*, left and middle panels). On the other hand, proforms exhibited less inhibition than the recombinant mature forms and the natural types for almost all sera (Fig 3, *B* and *C*, right panels). A paired *t* test showed significant differences between the proforms and recombinant mature forms and between the proforms and natural types ($P < .0001$).

Biologic activity of recombinant Der p 1 and Der f 1 in stimulating histamine release by basophils

To evaluate the allergenicity of recombinant proforms and mature forms at the cellular level, their biologic activities in inducing the release of histamine from allergic volunteers' basophils were assessed (Fig 4). For 3 volunteers, the proforms exhibited lower biologic activity than the recombinant mature forms. Three to 10 times concentrations of the proforms relative to the recombinant mature forms were needed to exhibit the same histamine release response. The decreased activity of the proforms in inhibiting IgE binding to immobilized natural allergens and in stimulating histamine release by basophils indicated that the propeptides linked to the N-terminal of mature Der p 1 and Der f 1 blocked conformational IgE-binding sites on the surface of the mature portion.

The biologic activities of the recombinant mature forms were equivalent to or slightly higher than those of the natural types for volunteers 1 and 2 and lower for volunteer 3. Sera 5, 6, and 7 in Fig 3, *A*, were from volunteers 1, 2, and 3 in Fig 4, respectively. The lower histamine-releasing activity of recombinant mature forms

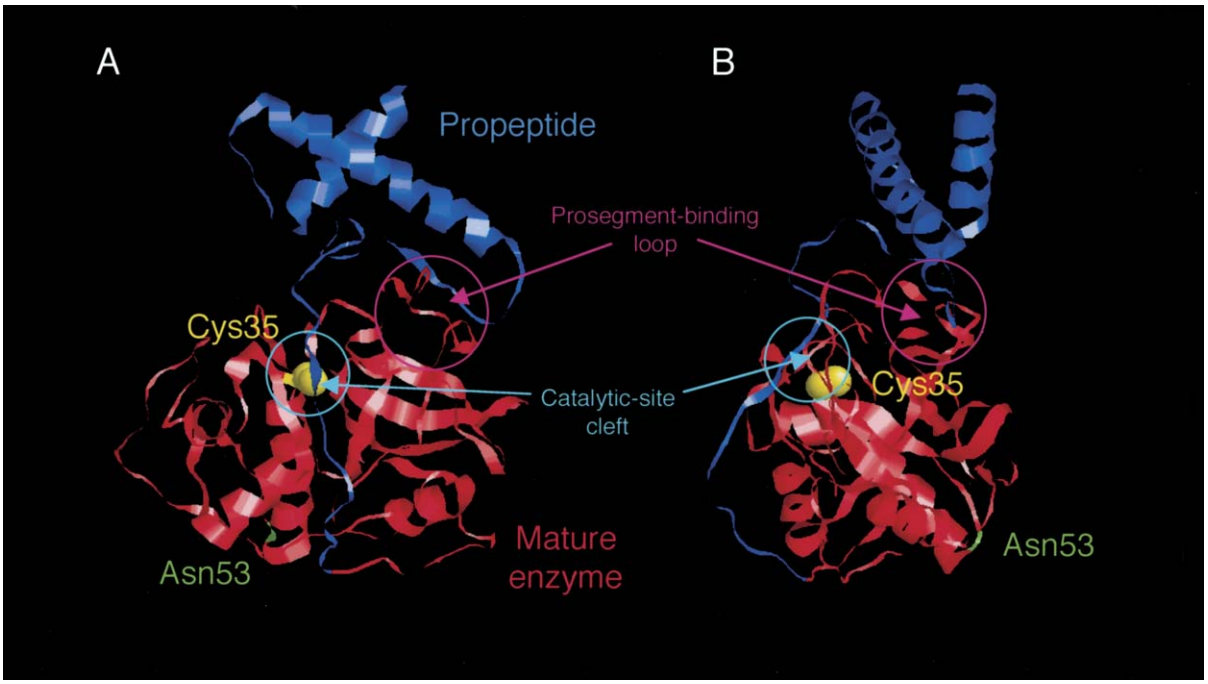

FIG 5. Homology modeling of the tertiary structure of pro-Der f 1. **A**, Front view. **B**, View from the right side of Fig 5, *A*. The prosegment, mature portion, and the N-glycosylation site Asn53 are shown in blue, red, and green, respectively. The catalytic center, Cys35, is shown as a space-filling model in yellow. These figures were produced by using the RasMol program.[32]

from volunteer 3 (Fig 4) was consistent with the significantly lower inhibitory activity for IgE binding for the corresponding serum 7 (Fig 3, *A*).

Molecular modeling of the proform of Der f 1

Molecular modeling of pro-Der f 1 was performed on the basis of the crystal structures of other cysteine proteases to obtain information on the location of IgE-binding sites blocked by the propeptides. In the predicted tertiary structure (Fig 5),[32] the Der f 1 prosegment consisted of an N-terminal domain-structure followed by a C-terminal extended loop.[28,29,33] The prosegment was tightly anchored at 2 regions of the mature enzyme, the prosegment binding loop and the substrate binding cleft surrounding the catalytic residue Cys35. Asn53, the potential *N*-glycosylation site in mature Der f 1, was located on the surface distant from the catalytic site and the prosegment.

The predicted structure has 19% α-helix character for the mature portion and 26% for the whole proform. These values were almost identical to the values for recombinant and natural Der p 1 and Der f 1 calculated from their CD spectra (Fig 1, *E* and *F*), except for pro-Der f 1-WT. The N-terminal sequence of the purified pro-Der f 1-WT was determined to start from −80 just after the putative signal-cleavage site and from −59, which was at the N-terminal of the second helix (α2) in the predicted structure, and we determined that approximately 75% of pro-Der f 1-WT was composed of the truncated proform starting from −59.[15] The helical content of the α1-truncated pro-Der f 1 was 22% in the modeled structure. This explains why the helical content of pro-Der f 1-WT (21%) calculated from the CD spectrum was less than that for the model of full-length pro-Der f 1 (26%).

DISCUSSION

We showed that the allergenicities of the proforms were less than those of the mature forms in a broad population of patients and that recombinant mature Der p 1 and Der f 1 are similar to the natural types in their structures and allergenicities and retain proteolytic activities. Furthermore, we disclosed how the prosegments inhibit the allergenicity by using a predicted 3-dimensional model of pro-Der f 1. Less allergenicity of the proforms than the mature forms indicates the importance of the 2 regions on the molecular surface of the mature portion blocked by the prosegments as major conformational IgE epitopes.

Equivalency for direct IgE binding suggested equivalency of the IgE epitopes between the recombinant and natural types. Equivalency for IgE binding between Der p 1 and Der f 1 correlated with reports on the high cross-reactivity between Der p 1 and Der f 1.[34-36] We assume that the results of the competitive inhibition assay reflect differences in affinity for IgE between the recombinant mature forms and natural types rather than differences in the numbers of IgE epitopes because of the equivalency seen for direct IgE binding. Such differences in affinity might result from sequence polymorphisms in

natural Der p 1 and Der f 1. Because at least 12 isoforms with different amino acid sequences for Der p 1[37] and 2 for Der f 1[4,14] have been identified, patients could be sensitized to different isoforms by the environment. Differences in the composition of isoforms of these allergens because of the regions where the patients live could explain the nonassociation of IgE-binding inhibition activity between the natural allergens composed of several isoforms and the cloned recombinant one in these patients.

We indicated that the prodomains of Der p 1 and Der f 1 reduce allergenicity in addition to functioning as prodomains of papain-like cysteine proteases, which block proteolytic activity and assist in the correct folding of the mature portions. Proforms of Der p 1 and Der f 1 showed less allergenicities than the recombinant mature forms for almost all tested sera and volunteers. We observed that direct IgE binding to immobilized proforms was higher than or equivalent to binding to the mature forms (unpublished observation), suggesting that the proforms were more efficiently immobilized than the mature forms, that IgE epitopes blocked by the prosegments in solution structure of the proforms were exposed when immobilized and could be recognized by IgE, or both. In the 3-dimensional model of pro-Der f 1, the prosegment interacts with the mature protein at 2 regions. This suggests that these regions are major conformational IgE epitopes of Der p 1 and Der f 1. The C-terminal extended loop region of the prosegment, which is followed by the N-terminal of the mature portion, might also contribute to the inhibition of IgE binding by means of steric hindrance. *N*-glycosylation of Asn52 and Asn53 in recombinant Der p 1 and Der f 1, respectively, caused no significant effect on their allergenicities. This suggests that regions near these residues do not contain major IgE epitopes. It was reported that phage peptides screened with an inhibitory anti-Der p 1 mouse mAb had a sequential motif in common with a Der p 1 sequence 147-160.[38] Their results do not conflict with our conclusion because the corresponding sequence 148-161 in Der f 1 is located at the prosegment binding loop of our pro-Der f 1 model.

In summary, we characterized the allergenicities of structurally folded and highly purified recombinant proforms and mature forms of Der p 1 and Der f 1 and interpreted the results on the basis of their secondary and predicted 3-dimensional structures for the first time. Recombinant mature Der p 1 and Der f 1 retained overall identity to their natural types in terms of structure and allergenicity and showed proteolytic activities. On the other hand, the allergenicities of the proforms were less than those of the mature forms. Molecular modeling of pro-Der f 1 suggested that the 2 regions in which the prosegment is anchored are major IgE-binding epitopes. Recombinant Der p 1 and Der f 1, and the findings regarding the conformational IgE epitopes in the present study, will be the basis for allergen standardization and the design of safer and more effective allergen vaccines. For example, determination of tertiary structure and analysis by site-directed mutagenesis would give detailed information of the structure and function of these allergens in future studies.

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