Cross-reactivity studies of a new group 2 allergen from the dust mite *Glycyphagus domesticus*, Gly d 2, and group 2 allergens from *Dermatophagoides pteronyssinus*, *Lepidoglyphus destructor*, and *Tyrophagus putrescentiae* with recombinant allergens

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Background: Dust mites are important inducers of allergic disease. Group 2 allergens are recognized as major allergens in several mite species, including *Dermatophagoides pteronyssinus*, *Lepidoglyphus destructor*, and *Tyrophagus putrescentiae*. No allergens have thus far been characterized on the molecular level from the dust mite *Glycyphagus domesticus*.
Objective: We sought to examine the cross-reactivity among group 2 allergens of *G domesticus*, *L destructor*, *T putrescentiae*, and *D pteronyssinus*.
Methods: A group 2 allergen from *G domesticus*, Gly d 2, was cloned and expressed as a recombinant protein. Cross-reactivity between Gly d 2 and 3 other group 2 allergens, Lep d 2, Tyr p 2, and Der p 2, was studied by using individual sera and a serum pool RAST-positive to *G domesticus*, *L destructor*, *T putrescentiae*, and *D pteronyssinus*. Recombinant allergens were used as inhibitors of IgE binding in immunoblotting experiments. Molecular modeling on the basis of the Der p 2 structure was carried out for Gly d 2, Lep d 2, and Tyr p 2.
Results: Two cDNAs encoding isoforms of Gly d 2 were isolated, but only the Gly d 2.02 isoform was used in this study. Sixteen of 17 subjects had IgE to Gly d 2. The protein sequence of Gly d 2 revealed 79% identity to Lep d 2 and 46% and 41% identity to Tyr p 2 and Der p 2, respectively. Extensive cross-reactivity was demonstrated among Gly d 2, Lep d 2, and Tyr p 2, but little cross-reactivity was found between these allergens and Der p 2. According to the tertiary structure of Der p 2 and 3-dimensional models of Gly d 2, Lep d 2, and Tyr p 2, differences reside mainly in surface-exposed residues.
Conclusion: Gly d 2 showed high sequence homology to Lep d 2. Cross-reactivity was observed between Gly d 2, Lep d 2, and Tyr p 2, but only limited cross-reactivity was demonstrated between these 3 allergens and Der p 2. (J Allergy Clin Immunol 2001;107:511-8.)

Key words: *Cross-reactivity, Der p 2, Glycyphagus domesticus, Gly d 2, Lep d 2, mite, recombinant allergen, Tyr p 2*

House dust mites belonging to the Pyroglyphidae family (eg, *Dermatophagoides* species) have been recognized as a major cause of allergic diseases, such as asthma and rhinitis.[1,2] Exposure to these mites is common in indoor environments.

Nonpyroglyphid mites, belonging to the Glycyphagidae (eg, *Lepidoglyphus destructor* and *Glycyphagus domesticus*) and Acaridae (eg, *Tyrophagus putrescentiae*) families have been reported to cause occupational allergy among farmers, bakers, and grain workers.[3-8] Apart from handling of hay, grain, and straw, the prevalence of sensitization to nonpyroglyphid mites seems to be associated with damp housing conditions.[9,10] Several investigators have also reported sensitization to these species in urban populations.[11-15]

Studies of cross-reactivity between *Dermatophagoides* species and nonpyroglyphid mites have been carried out with conflicting results.[12,14,16-22] One obvious reason for the discrepancy between different cross-reactivity studies is that sera of different origins are

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Abbreviations used
MW: Molecular weight
PVDF: Polyvinylidene fluoride
RACE: Rapid amplification of cDNA ends

compared. A second possible cause is that, with few exceptions,[23-25] whole mite extracts have been used for cross-inhibition. The allergen extracts are difficult to standardize, and different allergenic components in the extracts cross-react to various extents. In a few cases in which separate components in extracts of different mite species have been studied, an allergenic component with an approximate molecular weight (MW) of 15 kd has been shown to cross-react between different mite species.[18-19,22,26-27] The 15-kd components probably correspond to group 2 mite allergens, which have been recognized as major allergens in a number of mite species.[28] Several of these allergens have been cloned and expressed as recombinant proteins, among them Der p 2 from *Dermatophagoides pteronyssinus*,[29] Lep d 2 from *L destructor*,[30] and Tyr p 2 from *T putrescentiae*.[31] The group 2 allergens are all members of the same family of related proteins and show various degrees of homology. When aligned, Lep d 2 and Tyr p 2 share 43% identical residues, Lep d 2 and Der p 2 share 37% identical residues, and Tyr p 2 and Der p 2 share 40% identical residues. The tertiary structures of Der p 2[32] and Der f 2 from the related dust mite *Dermatophagoides farinae*[33] have been determined by means of nuclear magnetic resonance, and the results show that the group 2 mite allergens are not homologous to any other known protein family. The biologic function of these proteins is also unknown.

L destructor and *G domesticus* are 2 phylogenetically closely related mite species, and a high degree of cross-inhibition between *L destructor* and *G domesticus* extracts has been reported.[16] No allergens from *G domesticus* have been characterized on the molecular level. We here report the cloning and expression in *Escherichia coli* of the group 2 allergen from *G domesticus*, named Gly d 2.* Gly d 2 shows a much higher sequence homology to Lep d 2 (79%) than to other characterized group 2 allergens. It is therefore of interest to study the cross-reactivity between Gly d 2 and Lep d 2, as well as that between Gly d 2 and other group 2 allergens, including the major indoor dust mite allergen Der p 2. This is also relevant considering that these allergens exist in the same, as well as separate, environments.

*The group 2 allergen from *Glycyphagus domesticus* was named Gly d 2 according to the nomenclature of the World Health Organization/IUS Allergen Nomenclature Subcommittee, World Health Organization, Geneva, Switzerland.[36] Nucleotide sequences were submitted to the EMBL nucleotide sequence data base (accession numbers AJ249864 [Gly d 2.01] and AJ272216 [Gly d 2.02]).

METHODS

Preparation of mite extracts, purification, and protein sequencing of Gly d 2

Mite extracts were prepared from whole mite cultures obtained from Allergon AB (Ängelholm, Sweden), as previously described.[19] *G domesticus* extract was made 60% saturated with ammonium sulphate, the desalted soluble fraction was run on a 15% SDS-PAGE, and the separated proteins were electroblotted to a polyvinylidene fluoride (PVDF) membrane (Immobilon-PSQ; Millipore, Bedford, Mass). An approximately 15-kd Coomassie Brilliant Blue R250–stained band was cut out and directly subjected to N-terminal protein sequence analysis in a Perkin Elmer ABI 494 Procice cLC protein sequencer (Perkin Elmer, Foster City, Calif).

Isolation of mRNA and PCR amplification

G domesticus mRNA was isolated from 1 g of living mites (Allergon AB) by using Straight A's mRNA isolation system (Novagen Inc, Madison, Wis). cDNA synthesis, second-strand synthesis, adaptor ligation, and rapid amplification of cDNA ends (RACE) reactions were carried out by using the Marathon cDNA Amplification kit (Clontech, Palo Alto, Calif). 3'RACE was performed with a degenerate primer corresponding to the N-terminal amino acid sequence of the 15 kd protein from *G domesticus* extract, GGCAAGATGAACTT(C/T)AA(A/G)GA(C/T)TG(C/T)GG, and the AP1 primer (Clontech) corresponding to the adaptor-sequence. 5'RACE was performed with a gene-specific primer, CGCATGCCATGACACCGTGGTCAC, and the AP1 primer (Clontech). The entire coding sequence of Gly d 2 was amplified in a PCR+1 reaction.[34,35] In the PCR+1 method the DNA template is asymmetrically amplified, and a third primer containing a 5' restriction site is added to a last cycle of amplification. Freshly prepared adapter-ligated cDNA (Advantage 2 PCR-kit, Clontech) was amplified with a 5' primer, GCGGTCGTTTCGGCCGGCAAGAT (the underlined GGC codon corresponds to the N-terminal glycine residue in the mature *G domesticus* protein), and a 3' primer in 5-fold excess over the 5' primer, ATTGATTTATTTTCGACTTGTCC (underlined is the stop codon). In a final PCR cycle, a PCR+1 primer, AGATCTGCGGTCGTTTCGGCCGGCAAGAT (underlined is a *Bgl*II-restriction site), was added. The PCR product was blunt-end cloned into pT7 Blue vector by using the Perfectly Blunt Cloning kit (Novagen), and PCR+1 clones were identified by cleavage of plasmid DNA with *Bgl*II and *Eco*RI (all restriction endonucleases from New England Biolabs Inc, Beverly, Mass), and analysis of restriction fragments were identified by agarose electrophoresis.

DNA sequencing

Plasmid DNA was sequenced in an ABI 377 sequencer (Perkin Elmer) by using the ABI PRISM dRhodamine Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer) with a T7-promoter and T7-terminator primer (Novagen) for sequencing of pET-clones and a T7-promoter and M13-primer (Perkin Elmer) for pT7-Blue clones. Sequence alignments were obtained by using the DNASTAR MegAlign program (DNASTAR Inc, Madison, Wis).

Cloning into pET 17b and expression in *E coli*

A PCR+1 clone was used as the template to PCR amplify DNA encoding the mature Gly d 2 protein. The 5' primer, TCTAGA-**CATATG**GGCAAGATGAAGTTTAAGGAT, introduces a start codon (underlined) 5' of the GGC encoding the N-terminal glycine in the mature protein, and the 3' primer, GGATCC**AAGCTT**AGTG-GTGATGGTGATGGTGGTCGACTTGCCGGCCGAA, includes 6 histidine codons before the stop codon (underlined). The primers also included restriction enzyme sites (bold), *Nde*I in the 5' primer and

*Hin*dIII in the 3' primer. The *Nde*I-*Hin*dIII–digested PCR product was cloned into the expression vector pET 17 b (Novagen) at the *Nde*I and *Hin*dIII sites. The pET vector containing the Gly d 2 insert was initially transformed into a nonexpressing *E coli* strain, XL-2 Blue, and thereafter to the expression strain BL21 (DE3) pLysS (Novagen). Expression was performed according to the protocol for the pET expression system (Novagen). The expressed recombinant protein was mainly recovered as inclusion bodies, which were solubilized in 20 mmol/L TRIS-HCl (pH 8.0), 100 mmol/L NaCl, and 6 mol/L guanidinium hydrochloride. His-tagged recombinant protein was affinity purified by using immobilized metal affinity chromatography (Talon, Clontech) and eluted from the resin with 100 mmol/L imidazol, 20 mmol/L TRIS-HCl (pH 8), and 100 mmol/L NaCl. The recombinant protein was subsequently dialyzed against PBS.

Recombinant allergens

Recombinant Lep d 2.01 (equal to isoform Lep d 1.0101[30] but later renamed Lep d 2.01 according to the allergen nomenclature[36]), rTyr p 2, and rDer p 2.0101 (D1S) were expressed with 6 histidine tags at their C-termini and purified as described elsewhere.[31,37,38] Each recombinant group 2 allergen inhibited, in a dose-dependent manner, IgE binding to 14- to 16-kd (approximate MW) components in the corresponding extracts. Recombinant Gly d 2 was prepared as described in the previous section.

Analysis of rGly d 2 by means of size-exclusion chromatography

G domesticus extract (0.5 mg) and rGly d 2 (0.5 mg) were subjected to size-exclusion chromatography on a Superose-12 FPLC column (Amersham Pharmacia Biotech, Uppsala, Sweden) in PBS. The column was calibrated with BSA (67 kd), ovalbumin (43 kd), chymotrypsinogen A (25 kd), and RNase A (13.7 kd). Fractions were collected and analyzed by using ELISA or immunoblotting with a polyclonal rabbit antiserum raised against rLep d 2, which also recognizes a 15-kd component in *G domesticus* extract and rGly d 2.

Serum samples

Sera were obtained from farmers who had previously participated in an epidemiologic investigation of respiratory diseases.[39] A serum pool was prepared containing equal amounts of 7 individual sera. Each serum in the pool was positive to *G domesticus*, *L destructor*, *T putrescentiae*, and *D pteronyssinus*, with IgE ranging from 1.0 to 25.4 k_AU/L (Pharmacia CAP System RAST FEIA; Pharmacia and Upjohn Diagnostics AB, Uppsala, Sweden), and also shown by immunoblotting to react with an IgE-binding component of 15 kd (approximate MW) in *G domesticus*, *L destructor*, *T putrescentiae*, and *D pteronyssinus* extracts. A serum with high RAST values for several pollen allergens but negative to mite allergens was used as negative control.

SDS-PAGE and Western blot analysis

SDS-PAGE was performed under nonreducing conditions by using 15% SDS polyacrylamide gels. For extract, 15 μg/mm and for recombinant allergens, 0.2 μg/mm was loaded onto the gel. Separated proteins were blotted onto Immobilon-P PVDF membranes (Millipore). Immunodetection with human sera was performed as described earlier[19] with the serum pool or individual sera at a dilution of 1:4, affinity-purified rabbit anti-human IgE (Miab, Uppsala, Sweden), and alkaline phosphatase–conjugated goat anti-rabbit IgG (Dako A/S, Glostrup, Denmark). Finally, the IgE-binding proteins were visualized by using an Alkaline Phosphatase Conjugate Substrate Kit (Bio-Rad, Richmond, Va).

Blotting inhibition

Recombinant Gly d 2, rTyr p 2, rLep d 2, and rDer p 2 were used as inhibitors in the cross-reactivity experiments. The serum pool or individual sera (final dilution, 1:4) were preincubated for 1 hour at room temperature with 1 or 10 μg of inhibitor. The mixtures were then incubated with membrane strips, and the allergenic components were visualized as described in the previous section.

Calculation of inhibition results

The PVDF membrane strips were evaluated by means of densitometry scanning with a Bio-Rad Model GS-670 Imaging Densitometer and the Molecular Analyst Software version 2.1. The OD value for each recombinant allergen incubated with serum only (without inhibitor) was taken as 100% binding of IgE (A). Background values (0% IgE binding) were obtained from strips with recombinant allergen incubated with control serum (B). Inhibition was calculated by using the following formula:

$$\% \text{ inhibition} = 100 - 100 \times (X - B)/(A - B),$$

where X is the OD value obtained for PVDF strips with recombinant allergen incubated with serum and inhibitor and A and B are as above.

Molecular modeling

Models were generated by using the rDer p 2.0101 (D1S) x-ray crystal structure as a template. The crystallography results will be discussed elsewhere; however, there is good agreement between the tertiary structure determined by means of x-ray crystallography and the nuclear magnetic resonance structure reported by Mueller et al.[32] Modeling was conducted by using standard homology modeling methods. SYBYL (Tripos, Inc, St Louis, Mo) was used for insertions or deletions of 1 to 3 amino acid residues, local minimization, sequence alignment, and viewing of the resulting structures. The molecular modeling program CONGEN (Congenomics Inc, Princeton, NJ) was used for amino acid substitutions, side chain reconstitution, energy minimization, and molecular dynamics. In all cases the lowest energy conformer was selected for further consideration. The final figures were produced by using MIDAS software (UCSF Computer Graphics Laboratory, San Francisco, Calif).

RESULTS

Isolation, cloning, and expression of Gly d 2

An N-terminal amino acid sequence was obtained from an IgE-binding protein of approximately 15 kd in *G domesticus* extract: GKMNFKDXGKGEVKELDI. At position 8, no amino acid residue was detected. The reason for this is probably that there is a cysteine residue at this position, and nonmodified cysteines could not be detected in the analysis. A degenerate primer was designed from this N-terminal protein sequence for use in RT-PCR, with *G domesticus* cDNA as a template. A DNA sequence was obtained with an open-reading frame encoding 141 amino acids, including a typical signal sequence corresponding to the 16 N-terminal amino acids in the translated sequence. Variations were found in the 3'RACE and 5'RACE products, and when compared with the N-terminal protein sequence, polymorphisms were found at codon position 4 (N in amino acid sequence/K or N in deduced sequence), position 6 (K/K or T), position 10 (K/K or H), position 11 (G/G or N), position 13 (V/V or I), position 14 (K/T or K), position 17 (D/D or S), and position 18 (I/I or V). This indicated that several isoforms of the protein exist. Two different PCR+1 clones were isolated, sequenced, and identified as group 2 allergens, and therefore, according to the nomenclature,[36] named Gly d

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Gly d 2  GKMKFKDCGKGEVTELDITDCSGDF-CVIHRGKPLTLEAKFAAN 43
Lep d 2  ...T.....H.........G....T-......EKM......... 43
Tyr p 2  .QV..T....K.IASVAVDG.E..L-....KS..VHVI.E.T.. 43
Der p 2  DQVDV...ANH.IKKVLVPG.H.SEP.I.....FQ...V.E.. 44

Gly d 2  QDT--TKATIKVLAKVAGTPIQVPGLETDGCKFVKCPIKKGDPI 85
Lep d 2  .....A.V..........T..............I...V...EAL 85
Tyr p 2  .....S.IEV..TGQLN.LEVPI..I......VL...L...TKY 85
Der p 2  .N.KTA.IE..--.SID.LEVD...IDPNA.HYM...LV..QQY 86

Gly d 2  DFKYTTTVPAILPKVKAEV-TAELVGDHGVLACG-RFGRQVE    125
Lep d 2  ..I.SG.I...T.....D.-....I.....M...-TVHG...    125
Tyr p 2  TMN.SVN..SVV.NI.TV.-KLLAT.E.......-AVNTD.KP   126
Der p 2  .I...WN..K.A..SENV.V.VKVM..D.....AIATHAKIRD   129
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FIG 1. Alignment of group 2 allergens: Gly d 2, Lep d 2, Tyr p 2, and Der p 2. Sequences of the mature proteins without signal sequences are shown. The pairwise amino acid sequence identities are as follows: Gly d 2-Lep d 2, 79%; Gly d 2-Tyr p 2, 45%; Gly d 2-Der p 2, 42%; Lep d 2-Tyr p 2, 43%; Lep d 2-Der p2, 37%; and Tyr p 2-Der p 2, 40%. Residues identical to the Gly d 2 sequence are shown as *dots*, and gaps introduced to optimize the alignment are shown as *dashes*.

TABLE I. Inhibition of IgE binding to rGly d 2 by recombinant allergens

Serum	rGly d 2	rLep d 2	rTyr p 2	rDer p 2
1	**85**	79	75	58
2	**99**	98	100	88
3	**98**	100	96	62
4	**98**	99	78	11
5	**100**	99	82	68
6	**96**	94	79	37
7	**100**	97	62	3
8	**99**	96	54	7
9	**100**	100	88	2
10	**100**	99	88	26

Inhibition of IgE binding in 10 individual *G domesticus*–positive sera to rGly d 2. Ten micrograms of each of the following inhibitors was used: rGly d 2, rLep d 2, rTyr p 2, and rDer p 2. Data are obtained from densitometry scanning of immunoblots and expressed as percentage of inhibition, as described in the "Methods" section. The homologous inhibition with rGly d 2 is shown in bold.

TABLE II. Cross-inhibition of recombinant group 2 allergens

Inhibitor	rGly d 2	rLep d 2	rTyr p 2	rDer p 2
1 μg rGly d 2	**82**	46	45	14
10 μg rGly d 2	**90**	74	66	10
1 μg rLep d 2	78	**90**	100	0
10 μg rLep d 2	92	**92**	97	0
1 μg rTyr p 2	37	37	**95**	0
10 μg rTyr p 2	57	46	**85**	0
1 μg rDer p 2	6	0	0	**89**
10 μg rDer p 2	10	2	20	**96**

Homologous and cross-inhibition of IgE binding in the serum pool to rGly d 2, rLep d 2, rTyr p 2, and rDer p 2. Each recombinant allergen was inhibited with 1 and 10 μg inhibitor. Data are obtained from densitometry scanning of immunoblots and expressed as percentage of inhibition, as described in the "Methods" section. Homologous inhibition is shown in bold.

2.01 and Gly d 2.02. The sequences were submitted to the EMBL nucleotide sequence data base, accession numbers AJ249864 (Gly d 2.01) and AJ272216 (Gly d 2.02). Both clones were expressed in *E coli* as recombinant proteins with C-terminal 6 histidine tags. Only Gly d 2.02 (referred to as Gly d 2 in this report) was subsequently used in this study because it inhibited IgE binding of the serum pool to *G domesticus* extract, whereas Gly d 2.01 caused only minor inhibition of IgE binding to *G domesticus* extract (data not shown). The sequence of the mature Gly d 2 protein is shown in Fig 1. Aligned to other group 2 allergens, it shows the highest sequence homology to Lep d 2 (79% identity).

IgE binding to native and recombinant Gly d 2

Seventeen sera RAST-positive to *G domesticus* were tested in immunoblotting experiments, and 16 sera recognized a protein with an approximate MW of 15 kd in *G domesticus* extract (Fig 2, *A*). The same 16 sera exhibited IgE binding to rGly d 2 (Fig 2, *B*), and 7 of these sera were included in the serum pool later used in cross-inhibition experiments. The recombinant Gly d 2 inhibited in a dose-dependent manner (0.01, 0.1, 1, and 10 μg inhibitor) the IgE binding of the serum pool to a 15-kd (approximate MW) component in the *G domesticus* extract (not shown). At the highest dose tested (ie, 10 μg), the inhibition was 77%. The higher MW bands present on the rGly d 2 immunoblot strips (Fig 2, *B*) probably correspond to multimers of the allergen because IgE binding to these bands was inhibited to the same extent as to the monomers in the inhibition experiments (not shown). When rGly d 2 was run in size-exclusion chromatography, rGly d 2 eluted as a broad peak corresponding to approximate MWs between 15 and 30 kd, whereas native Gly d 2 in *G domesticus* extract eluted as a more distinct peak corresponding to 15 kd. However, on SDS-PAGE, the eluted fractions were shown to contain mainly 15-kd components and less than 10% 30-kd components. This was also confirmed by protein staining of blotting membranes with rGly d 2.

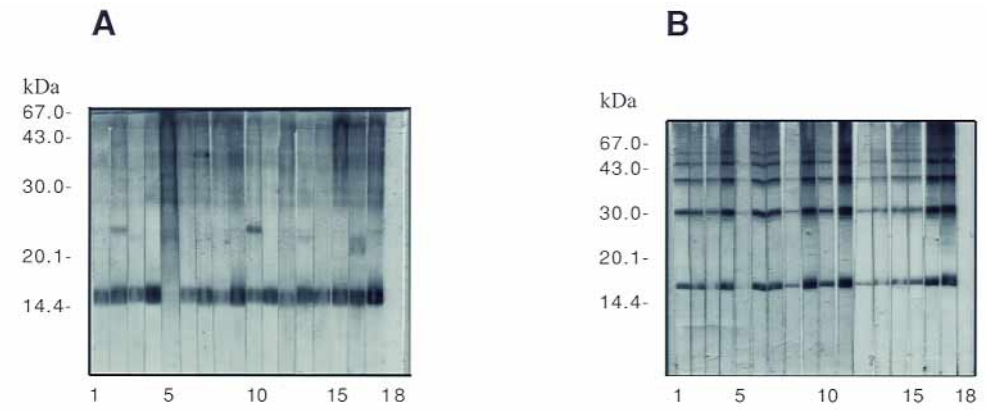

FIG 2. IgE binding to *G domesticus* extract **(A)** and rGly d 2 **(B)** in individual *G domesticus* RAST-positive sera *(strips 1-17)* and a control serum RAST-negative to mite allergens *(strip 18)*. The bands corresponding to higher molecular mass (≥~30 kd) present on the blotting membrane in **B** are probably multimers of rGly d 2.

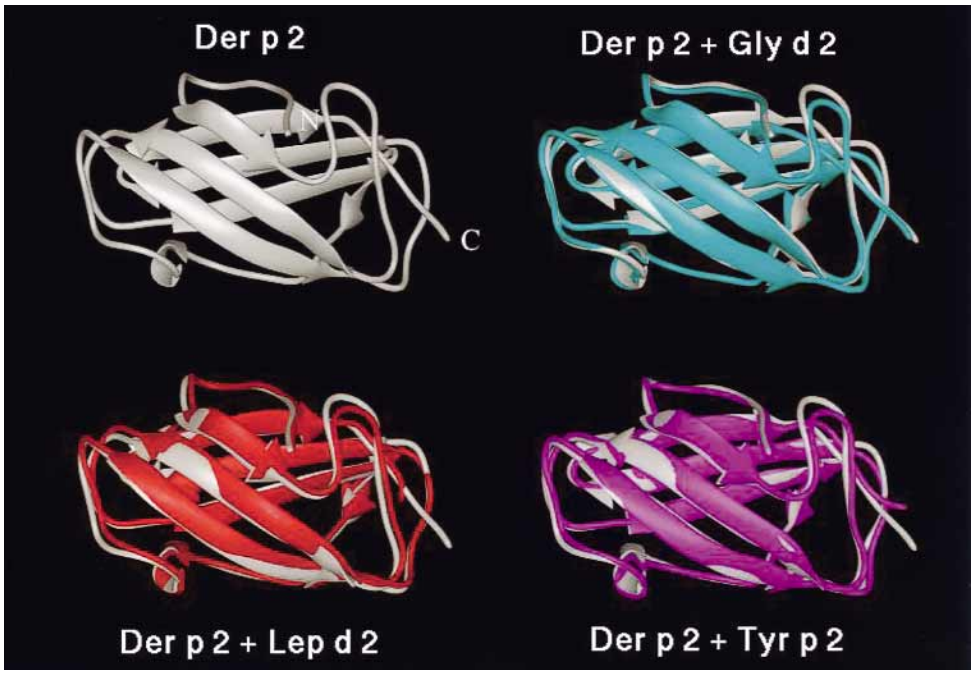

FIG 3. Crystal structure of Der p 2 and molecular models of Gly d 2, Lep d 2, and Tyr p 2 superimposed on the Der p 2 structure. N- and C-termini are indicated in the Der p 2 structure.

Cross-inhibition of IgE binding to rGly d 2 in individual sera

Ten *G domesticus*–positive sera were used in inhibition experiments to examine the cross-reactivity of Gly d 2 to other group 2 allergens. As shown in Table I, IgE binding to rGly d 2 was completely inhibited by rLep d 2 in all 10 sera. Extensive inhibition was also obtained with rTyr p 2 in all sera, whereas rDer p 2 inhibited IgE binding to rGly d 2 in some sera but not in others (Table I).

Cross-inhibition of IgE binding to rGly d 2, rLep d 2, rTyr p 2, and rDer p 2

Cross-inhibition of IgE binding to the recombinant group 2 allergens was studied in the serum pool. In Table II the numerical values of the inhibition, as calculated from densitometry scanning of immunoblots of rGly d 2, rLep d 2, rTyr p 2, and rDer p 2, are given. In all cases the homologous inhibition of recombinant allergens is 90% or greater. Furthermore, rLep d 2 almost complete-

ly blocked IgE binding of the serum pool to rGly d 2 and rTyr p 2. Recombinant Gly d 2 inhibited 74% of the IgE binding to rLep d 2 and 66% to rTyr p 2 at the highest concentration tested. Inhibition was also observed with rTyr p 2 as inhibitor of IgE binding to rGly d 2 and rLep d 2. In contrast, there was essentially no cross-inhibition between rDer p 2 and the 3 other mite allergens. The control serum did not show IgE reactivity to any of the 4 recombinant mite allergens.

Molecular modeling of Gly d 2, Lep d 2, and Tyr p 2 and comparison with the tertiary structure of Der p 2

Fig 3 shows models of the nonpyroglyphid mite group 2 allergens superimposed on the crystal structure of rDer p 2. The models show essentially identical secondary and tertiary structures. Minor differences occur in loop positions and at the carboxyl termini. The core residues are either maintained or substituted with conservative amino acids. The majority of sequence differences are located on the solvent-exposed surface.

DISCUSSION

In this study we examined the specific cross-inhibition capacity among group 2 allergens of *G domesticus*, *L destructor*, *T putrescentiae*, and *D pteronyssinus* by using pure recombinant allergens. The group 2 allergen of *G domesticus*, Gly d 2, has not been characterized before, and we here report on the cloning and expression of this new allergen. During the cloning procedure, we observed that several isoforms of Gly d 2 exist, which is in agreement with what has been reported for group 2 allergens.[30-31,40] We have earlier applied the PCR+1 method for cloning of isoforms of Lep d 2[30] and Tyr p 2.[31] This method was developed to circumvent problems with artifactual DNA sequences arising when polymorphic DNA is amplified and subcloned into bacteria, which will repair mismatches randomly during replication.[34] Two isoforms of Gly d 2 were isolated, but only one of these isoforms was able to inhibit IgE binding to native Gly d 2 in *G domesticus* extract, indicating that it is a more commonly occurring isoform in the *G domesticus* extract. Probably more than these 2 isoforms exist because none of them was identical to the deduced N-terminal protein sequence. When Gly d 2 was expressed in *E coli*, the resulting recombinant protein seems to form both monomers and multimers, as shown on immunoblots of rGly d 2. The multimers may be a result of improper folding and disulphide bridge formation in the bacteria or incorrect refolding and aggregation after solubilization of inclusion bodies. However, size-exclusion chromatography of rGly d 2 showed that the majority of rGly d 2 molecules are found as monomers.

We conclude that Gly d 2 is a major allergen of *G domesticus* because 16 of 17 *G domesticus* RAST–positive subjects had IgE that bound both to a 15-kd component in *G domesticus* extract and to rGly d 2. Recombinant Gly d 2 was also able to inhibit IgE binding to a native 15-kd component in *G domesticus* extract. However, the inhibition was not complete, which might be due to the presence of multiple Gly d 2 isoforms or other allergens in the same MW range in the extract. For example, Der p 5 has an apparent MW of 14 kd,[41,42] and a 13-kd allergen homologous to fatty acid–binding proteins and denoted as group 13 mite allergen has been identified in the nonpyroglyphid mites *Blomia tropicalis* and *Acarus siro*.[43,44] We have also recently identified 2 new allergens in *L destructor* that are homologous to group 5 and group 13 allergens (manuscript in press).

The protein sequence identity between group 2 allergens from different dust mite species ranges from around 35% to over 85% (the latter figure for species within the same genus, ie, *D pteronyssinus* and *D farinae*). Gly d 2 is 79% identical to Lep d 2, which is in agreement with the close phylogenetic relationship between *G domesticus* and *L destructor*, but only around 40% identical to Tyr p 2 and Der p 2.

Our results showed extensive cross-reactivity among Gly d 2, Lep d 2, and Tyr p 2. The fact that rLep d 2 caused almost complete inhibition of IgE binding to rGly d 2 and rTyr p 2 may reflect that the sera used in this study originate from a farming population that is heavily exposed to *L destructor*.[45] Similar observations have been made in studies in which sera from populations primarily sensitized to *Dermatophagoides* species have been used. Only *Dermatophagoides* extracts were able to completely inhibit IgE binding of these sera to other mite extracts.[12,21,22] We observed only limited cross-inhibition between Der p 2 and the 3 nonpyroglyphid mite allergens. This observation is in agreement with some studies but not others. No or only limited cross-inhibition could be demonstrated between *D pteronyssinus* and *L destructor* or *T putrescentiae* in 2 studies.[12,16] Other studies have shown that *D pteronyssinus* extract can inhibit IgE binding to *L destructor* and *T putrescentiae* extracts, but *L destructor* or *T putrescentiae* extracts did not inhibit IgE binding to *D pteronyssinus*.[14,21] Park et al[22] reported extensive cross-reactivity among *D pteronyssinus*, *D farinae*, and *T putrescentiae,* and a 16-kd component in the *D pteronyssinus* extract, identified as Der p 2, was shown to be a cross-reacting component. Johansson et al[18,19] also demonstrated limited cross-inhibition between 15- and 16-kd components in *D pteronyssinus*, *L destructor*, and *T putrescentiae* extracts. The conflicting results obtained in studies on cross-reactivity probably reflect the different origins of sera used. The mite exposure varies widely in populations, and the degree of exposure may influence how many and to which epitopes in a single allergen the IgE response is raised. The IgE epitopes of group 2 allergens might either be common to several mite species or specific to a single allergen. Therefore exposure to different compositions of mite fauna probably affects how allergens from different mite species cross-react.

The extensive cross-inhibition among rGly d 2, rLep d 2, and rTyr p 2 compared with the limited cross-inhibition between these allergens and rDer p 2 is interesting

from a structural point of view. Although the amino acid sequence identity between Der p 2 and the nonpyroglyphid mite allergens is only approximately 40%, the structure models generated in this study predict that the alpha carbon backbone tertiary structures of the nonpyroglyphid mite allergens are essentially identical to the Der p 2 structure. The majority of the sequence differences correspond to the amino acid side chains that reside on the solvent-accessible surface. The differences in antibody binding can thus be attributed to changes in surface topology because of these substitutions and not global changes in the 3-dimensional structure.

The observation on the lack of cross-reactivity between Der p 2 and the 3 nonpyroglyphid mite group 2 allergens is of clinical importance when diagnostic methods on the basis of recombinant allergens are developed. Thus allergens from different mite species, as well as allergens other than the group 2 allergens, have to be taken into account to obtain reliable tools for diagnosis and treatment of dust mite allergy.

Protein sequence analysis was carried out at the Protein Analysis Centre, Karolinska Institutet, Stockholm, Sweden.

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