

Molecular, structural, and immunologic relationships between different families of recombinant calcium-binding pollen allergens

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Background: Calcium-binding plant allergens can be grouped in different families according to the number of calcium-binding domains (EF hands).

Objective: We sought to identify pollens containing cross-reactive calcium-binding allergens and to investigate structural and immunologic similarities of members belonging to different families of calcium-binding allergens.

Methods: By means of multiple sequence alignment and molecular modeling, we searched for structural similarities among pollen allergens with 2 (Phl p 7, timothy grass; Aln g 4, alder), 3 (Bet v 3, birch) and 4 EF hands (Jun o 4, prickly juniper). Purified recombinant Aln g 4 and Jun o 4 were used to determine the prevalence of IgE recognition in 210 patients sensitized to different pollens and to search, by means of ELISA competition, for the presence of cross-reactive epitopes in pollens from 16 unrelated plant species. IgE cross-reactivity among the allergen families was studied with purified rPhl p 7, rAln g 4, rBet v 3, and rJun o 4 and 2 synthetic peptides comprising the N-terminal and C-terminal EF hands of Phl p 7 by means of ELISA competition.

Results: Structural similarities were found by using molecular modeling among the allergens with 2, 3, and 4 EF hands. Pollens from 16 unrelated plants contained Aln g 4- and Jun o 4-related epitopes. Twenty-two percent of the patients with multiple pollen sensitization reacted to at least one of the calcium-binding allergens. A hierarchy of IgE cross-reactivity (rPhl

p7 > rAln g 4 > rJun o 4 > rBet v 3) could be established that identified rPhl p 7 as the EF-hand allergen containing most IgE epitopes in the population studied.

Conclusion: The demonstration that members of different families of calcium-binding plant allergens share similarities suggests that it may be possible to use representative molecules for the diagnosis and therapy of allergies to EF-hand allergens. (*J Allergy Clin Immunol* 2002;109:314-20.)

Key words: Calcium-binding allergens, pollen allergy, cross-reactivity, recombinant allergens, molecular modeling

Detailed knowledge concerning the molecular nature of disease-eliciting allergens is relevant for the diagnosis, prevention, and therapy of type I allergy. The recent application of molecular biologic techniques to allergen characterization has revealed the primary structures of a great number of allergens and allowed for the production of recombinant allergens for diagnostic and therapeutic purposes.¹ Moreover, structural similarities found among allergens from diverse allergen sources represent the molecular and structural basis for immunologic cross-reactivity and thus explain why allergic patients can exhibit clinical symptoms and IgE reactivity to various unrelated allergen sources.²

Pollen from grasses, trees, and weeds are some of the most frequent allergen sources. During the last few years, calcium-binding allergens have been discovered to be highly cross-reactive in pollens from several plants.³ Calcium-binding proteins can be classified into 32 distinct subfamilies, which may contain from 2 to 8 calcium-binding domains (EF hands). The EF hand represents a highly conserved portion within calcium-binding proteins consisting of an α helix, a loop coordinating the Ca^{2+} ion, and a second α helix. Calcium ions are bound in the EF hand through 4 carboxylate or carboxamide groups and a single backbone carbonyl oxygen placed in the loop with a specific spacing. The calcium-binding plant allergens identified to date appear to be mainly expressed in pollen but not in other plant tissues^{4,5} and therefore may serve as markers for multiple pollen sensitization. According to the number of EF hands contained in their sequence, they can be classified into 3 groups. Two EF-hand allergens were described in pollens from birch (Bet v 4),^{4,6} alder (Aln g

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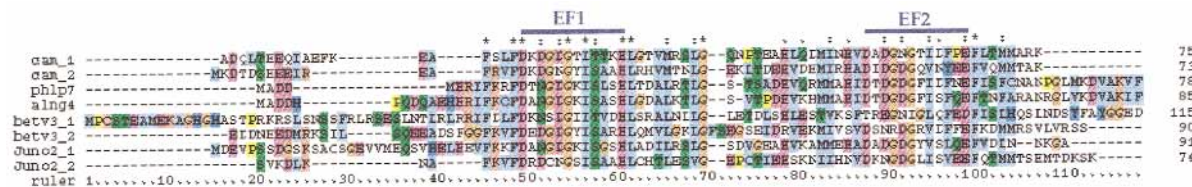


FIG 1. Multiple alignment of the amino acid sequences of the EF-hand globular domains of the 4 calcium-binding plant allergens (Phl p 7, Aln g 4, Bet v 3, and Jun o 4) with those of calmodulin. The EF-hand globular domains are designated on the left margin. Sequences were colored according to the chemical characteristics of the conserved amino acids (proline, yellow; glycine, orange; cysteine, pink; hydrophobic, blue; hydrophilic, green; acidic, purple; basic, red; hydrophobic tendency, dark blue). Hyphens in the sequences indicate gaps. The calcium-binding domains (EF1 and EF2) are indicated at the top. The numbering of amino acids is displayed in the ruler.

Abbreviation used

EF hand: Calcium-binding domain

4),⁷ olive (Ole e 3),⁸ Bermuda grass (Cyn d 7),^{9,10} timothy grass (Phl p 7),⁵ and rape (Bra r 1).¹¹ A pollen-specific allergen with 3 EF hands, Bet v 3, was cloned from birch pollen,¹² and allergens with 4 EF hands were isolated from prickly juniper (Jun o 4)¹³ and olive pollen (Ole e 8).¹⁴

No information is available concerning whether representatives of different families of calcium-binding allergens share IgE epitopes. In this study we used representative calcium-binding allergens from each family produced as pure, recombinant proteins to search for the presence of cross-reactive allergens in pollen from 16 different plants. Furthermore, we studied the prevalence of cosensitization to frequently recognized allergens with 2 and 4 EF hands, as exemplified by rAln g 4 and rJun o 4, in a population of 210 patients with pollen allergy.

Using purified recombinant allergens from alder (Aln g 4) and timothy grass (rPhl p 7) with 2 EF hands, Phl p 7-derived peptides, an allergen from birch with 3 EF hands (rBet v 3), and the allergen from juniper with 4 EF hands (rJun o 4), we studied the IgE and IgG₁₋₄ subclass recognition and the presence of cross-reactive IgE epitopes to establish a hierarchy of antibody cross-reactivity among the families of calcium-binding allergens. Possible relationships between structural features of calcium-binding pollen allergens and the presence of cross-reactive epitopes were also analyzed.

METHODS

Multiple sequence alignment and homology modeling

The amino acid sequences of calmodulin (pdb entry, 4cln.pdb), Phl p 7 (accession No. Y17835), Aln g 4 (accession No. Y17713), Bet v 3 (accession No. X79267), and Jun o 2 (now renamed Jun o 4, accession No. AF031471) were aligned by using CLUSTALX¹⁵ and, when necessary, adjusted by hand with the GDE sequence editor (S. Smith, Harvard University, Boston, Mass). Amino acids with related properties were colored with COLORMASK (J. Thompson, EMBL, Heidelberg, Germany). The EF-hand globular domains of

each protein were aligned. Globular domains consist of 2 EF-hand (helix-loop-helix) motifs. Therefore entire allergens with 2 EF hands were aligned with the globular domains of calmodulin (cam_1 and cam_2), Bet v 3 (betv3_1 and betv3_2), and Jun o 4 (juno2_1 and juno2_2).

Homology modeling of the calcium-binding allergens was achieved by using the program WHATIF,¹⁶ with the pdb entry 4cln.pdb (calcium-loaded calmodulin) as the template. The models are displayed as Corey, Pauling, Koltun representations. Hydrophobic residues, as well as positively and negatively charged residues, were colored differently. Within these models, the globular domains were not artificially linked because of the sequence differences between calmodulin and the allergens in this region.

Pollen allergen extracts, recombinant allergens, and peptides

Pollen extracts used in IgE ELISA competition experiments were supplied by Bayer (Spokane, Wash) and Stallergens (Fresnes, France) or prepared in house as previously described.¹⁷

The cDNA coding for Bet v 3 containing a C-terminal hexahistidine tag was obtained by means of PCR from the Bet v 3 cDNA by using the following primers: Bet v 3 F, 5' CGC GGA TCC CTG TTC CACAGA AGC CAT GGA A 3'; Bet v 3 RHIs, 5' CGC GGA TCC TAA TGA TGA TGA TGA GAG CTC CGC ACG AGA ACG CTA CG 3'. The PCR product was cut with *Bam*HI and subcloned into the *Bam*HI site of the pTM1 vector, a 4.8-kb derivative of pFastBac1 (Life Technologies, Mo).¹⁸ Recombinant Bet v 3 was expressed in baculovirus-infected Sf9 insect cells and purified by means of Nickel affinity chromatography (Twardosz and Valenta, unpublished data). Recombinant Aln g 4 and Phl p 7 were purified as previously described.^{5,7} Recombinant Jun o 4 was prepared as previously described.¹³ Phl p 7-derived peptides were synthesized by using a 9 fluorenyl methoxy carbonyl strategy with 2-(1H-Benzotriazol-1-yl)1,1,3,3-tetramethyluronium hexafluorophosphate activation on an Applied Biosystems peptide synthesizer Model 433A (ABI, Foster City, Calif), purified by means of HPLC, and checked by means of mass spectrometry (piChem, Graz, Austria).

Characterization of patients and sera

Sera were obtained from 210 patients with pollen allergy diagnosed by means of skin prick testing and specific IgE in vitro detection, as previously described.¹⁹ IgE ELISA competition studies and IgG subclass determinations were performed with sera containing IgE antibodies against calcium-binding allergens. Serum samples used as controls were collected from a nonatopic volunteer and from 2 atopic subjects without specific IgE to calcium-binding pollen allergens but with high levels of total and birch pollen-specific IgE.

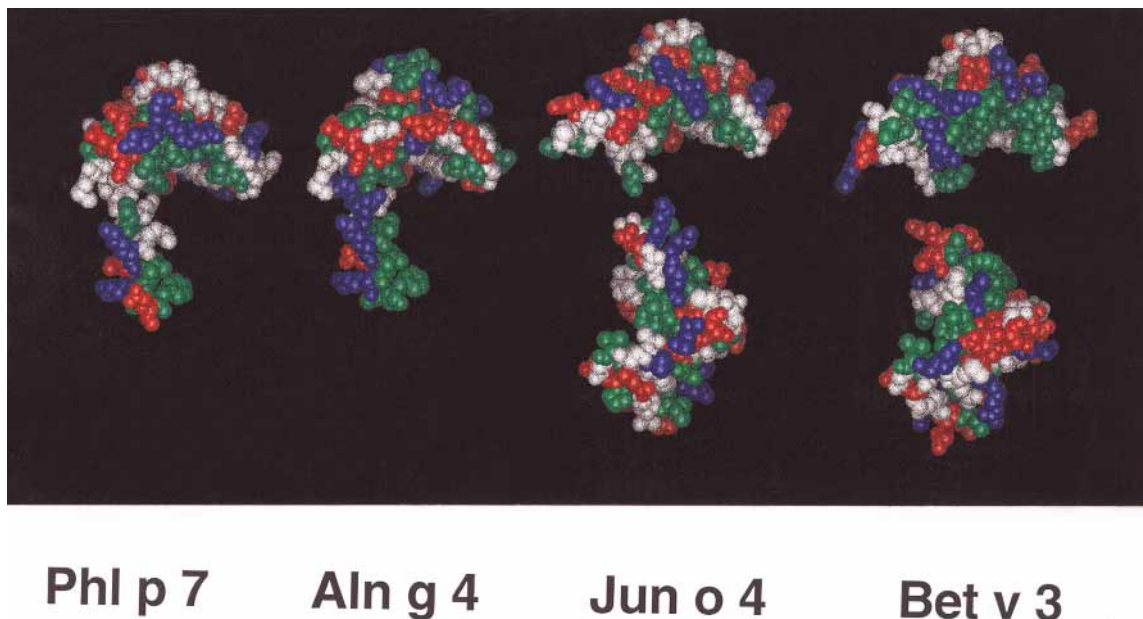


FIG 2. Corey, Pauling, Koltun representation of the models built for the 4 calcium-binding allergens (Phl p 7, Aln g 4, Jun o 4, and Bet v 3). The models are colored as follows: hydrophobic residues are colored in green; positively and negatively charged residues are colored in blue and red, respectively; and all other residues are colored in white. The N-terminus of all molecules is in the upper lobe on the left side. The C-terminus of the double globular-domain allergens (Jun o 4 and Bet v 3) is in the lower lobe behind the picture, and that for the single globular-domain allergens (Phl p 7 and Aln g 4) is on the right side of the lobe.

IgE immunoblotting and IgE immunoblot-inhibition experiments

Immunoblotting inhibitions were performed during overnight incubation of rAln g 4–positive and rJun o 4–positive pooled sera diluted 1:20 with 10-fold serial dilution of rAln g 4 or rJun o 4 (10, 1, and 0.1 µg/mL). IgE-allergen complexes were detected with iodine 125–labeled anti-human IgE (Bio-Allergy, Rome, Italy).

IgE and IgG₁₋₄ subclass ELISA and IgE ELISA competition experiments

Specific IgE for rAln g 4, rBet v 3, rPhl p 7, and rJun o 4 and for 2 synthetic peptides comprising the N-terminal and C-terminal EF hands of Phl p 7 (Pep 1 and Pep 2, respectively) were detected by means of ELISA, as previously described.²⁰ IgG subclass responses to recombinant allergens were determined by means of ELISA.²¹

ELISA inhibitions were performed according to the method of Barletta et al,²⁰ using the recombinant calcium-binding allergens and Pep 2 as antigens at a concentration of 5 µg/mL. In reciprocal inhibition, calcium-binding proteins were used as inhibitors in 10-fold dilutions, ranging from 0.001 to 30 µg/mL. ELISA inhibitions with recombinant proteins as antigens and 16 pollen extracts as inhibitors were performed with serum containing IgE antibodies to rAln g 4 and rJun o 4. Concentrations of pollen extracts ranged from 20 to 0.06 µg/mL in 2-fold dilutions.

RESULTS

Structural relationship of calcium-binding plant allergens

A multiple sequence alignment of the calcium-binding allergens and calmodulin is shown in Fig 1. Phl p 7 and

Aln g 4 contain 2 EF hands, each of them forming a globular domain. The other 2 allergens (Bet v 3 and Jun o 2, now renamed Jun o 4) both contain 4 EF hands, although the second EF hand of Bet v 3 might have lost calcium-binding activity (Fig 1).

The exposed surfaces of the 2 globular domains retain some common features (Fig 2). Strong differences are observed in the N- and C-termini and in the linkers between the globular domains. Bet v 3 contains a long and most likely unstructured N-terminus. The linker between the globular domains of Bet v 3 and Jun o 4 is longer than in calmodulin (Fig 1), possibly keeping the 2 globular domains spatially independent. The C-termini of the calcium-binding allergens are very different, although high similarity can be observed between Phl p 7 and Aln g 4. Their C-termini are highly hydrophobic (Fig 1). On the basis of the structural analysis of the calcium-binding allergens, common epitopes can be expected as a result of the similar architecture. However, a number of specific epitopes may be expected for the C-termini of Phl p 7 and Aln g 4, for the N-terminus of Bet v 3, and in the linker and possibly in the C-terminus of Jun o 4.

Pollen extracts from 16 plant species contain cross-reactive calcium-binding allergens

We found that pollen extracts from all 16 different plant species inhibited IgE binding to rAln g 4 and rJun o 4. The protein concentrations yielding a 50% inhibition

TABLE I. Pollen extracts from 16 plant species contain rJun o 4- and rAln g 4-related allergens

Allergen	Family	Inhibitor yielding 50% inhibition (μg/mL)	
		rJun o 4	rAln g 4
<i>Parietaria judaica</i> (pellitory)	Urticaceae	0.06	2.5
<i>Brassica rapa</i> (mustard)	Cruciferae	0.2	0.3
<i>Artemisia douglasiana</i> (mugwort)	Compositae	0.6	0.6
<i>Platanus occidentalis</i> (sycamore)	Platanaceae	0.15	0.6
<i>Tamarix gallica</i> (salt cedar)	Tamaricaceae	0.6	0.5
<i>Phoenix dactylifera</i> (palm)	Palmae	0.3	1.25
<i>Chenopodium album</i> (goosefoot)	Chenopodiaceae	1.25	0.15
<i>Tilia cordata</i> (lime)	Tiliaceae	1.25	0.07
<i>Plantago lanceolata</i> (plantain)	Plantaginaceae	10	0.6
<i>Ailanthus altissima</i> (tree of heaven)	Simaroubaceae	0.06	0.15
<i>Ambrosia elatior</i> (ragweed)	Compositae	0.03	10
<i>Eucalyptus globulus</i> (eucalyptus)	Myrtaceae	1.25	0.07
<i>Lolium perenne</i> (grass, rye)	Poaceae	0.015	0.03
<i>Olea europaea</i> (olive)	Oleaceae	10	0.025
<i>Betula verrucosa</i> (birch)	Betulaceae	2.5	10
<i>Robinia pseudacacia</i> (locust tree)	Leguminosae	0.15	10

A serum from a rJun o 4- and from a rAln g 4-reactive patient was preincubated with pollen extracts from the 16 plant species displayed. The protein concentration of the inhibitor yielding 50% inhibition of IgE binding is shown.

of IgE binding to the recombinant allergens are identified in Table I. In the case of rJun o 4, rye grass and ragweed pollen extract best inhibited IgE binding. The strongest inhibition of IgE binding to rAln g 4 was obtained with rye grass and olive pollen extracts (Table I).

IgE reactivity to calcium-binding plant allergens identifies a subset of allergic patients who are polysensitized to pollens from various plants

Of 210 patients with multiple pollen allergies, 32 (15.2%) patients showed IgE reactivity to rAln g 4 and rJun o 4, 14 (6.7%) sera contained IgE against rAln g 4 alone, and no patient reacted with rJun o 4 alone. Altogether, 46 (21.9%) sera showed IgE reactivity to at least one of the 2 calcium-binding allergens. All patients who had IgE antibodies against calcium-binding allergens were sensitized against pollens from several unrelated plant species (grass, tree, and weed pollen). The fact that no patient enrolled in the study group reacted with rJun o 4 alone indicated that allergens with 2 EF hands either cause more frequent sensitization than the rJun o 4 family of allergens or that they contain more cross-reactive IgE epitopes. Reciprocal IgE immunoblot-inhibition experiments were performed to address the latter question (Fig 3). rJun o 4 (10 μg/mL) only weakly inhibited IgE binding to blotted rAln g 4 (Fig 3, A, lane 4). However, rAln g 4 strongly inhibited IgE binding to blotted rJun o 4 down to a concentration of 0.1 μg/mL (Fig 3, B, lanes 1-3).

IgE and IgG subclass recognition of calcium-binding plant allergens and IgE cross-reactivity among different families of calcium-binding allergens

The analysis of a representative group of 5 patients allergic to calcium-binding allergens indicated that allergens with 2 EF hands (rPhl p 7 and rAln g 4) contained most IgE epitopes. When sera were analyzed for IgG subclass reactivity, we found that allergen-specific IgG subclass reactivity was not always associated with IgE reactivity and that nonatopic persons contained IgG antibodies to allergens (eg, high levels of IgG₃ antibodies to rJun o 4).

The presence of cross-reactive IgE epitopes on calcium-binding plant allergens from different families was studied by means of ELISA competition assay (Fig 4). As exemplified for the serum of one patient allergic to members of all 3 calcium-binding allergen families, we found that rJun o 4 yielded a dose-dependent inhibition of IgE binding to rJun o 4 itself, but the inhibition obtained with rAln g 4 and rPhl p 7 was far stronger (Fig 4, A). rBet v 3 and the Phl p 7-derived peptide did not significantly inhibit IgE binding to rJun o 4 (Fig 4, A). All calcium-binding allergens tested (rBet v 3, rAln g 4, rJun o 4, and rPhl p 7), but not the C-terminal Phl p 7-derived peptide, elicited a dose-dependent inhibition of IgE binding to rBet v 3 (Fig 4, B). IgE binding to plate-bound allergens with 2 EF hands (rAln g 4 and rPhl p 7) was inhibited most effectively with rPhl p 7 and rAln g 4 and, to a much lower degree, by rJun o 4 and the Phl p 7-derived C-terminal peptide (Fig 4, C and D). rPhl p 7 was the most potent competitor, whereas

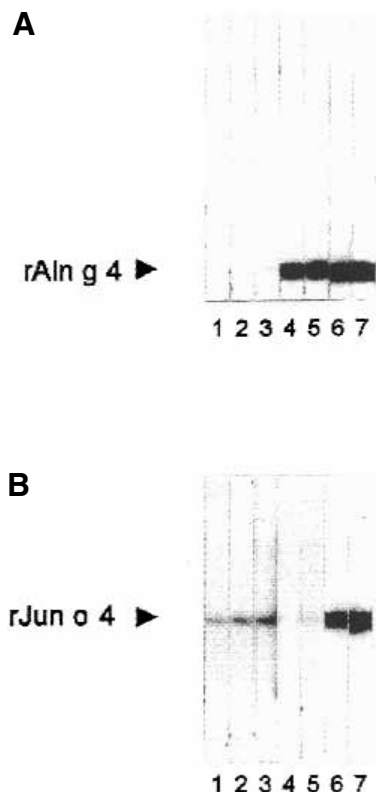


FIG 3. Cross-reactivity between rAln g 4 and rJun o 4, as demonstrated by immunoblotting-inhibition experiments. Inhibition of IgE binding to nitrocellulose-blotted rAln g 4 (A) or rJun o 4 (B) after preincubation of a Aln g 4/Jun o 4-reactive serum pool with rAln g 4 (lanes 1-3) or rJun o 4 (lanes 4-6) is shown. Concentrations of inhibitors added are 10 μ g/mL (lanes 1 and 4), 1 μ g/mL (lanes 2 and 5), 0.1 μ g/mL (lanes 3 and 6). Lane 7, No inhibitor added.

rBet v 3 did not significantly inhibit IgE binding to the allergens with 2 EF hands. Evidence for differences regarding the IgE epitopes between rPhl p 7 and rAln g 4, and in particular concerning their C-terminal portions, comes from the peptide-inhibition studies in Fig 4, E. IgE binding to the Phl p 7-derived C-terminal peptides was stronger after preadsorption of the serum with rPhl p 7 and the peptide but weaker with rAln g 4. rBet v 3 and rJun o 4 failed to inhibit IgE binding to the peptide (Fig 4, E). The results of the IgE competition studies thus document that members of the 3 families of calcium-binding plant allergens contain cross-reactive IgE epitopes. The result, that the allergens with 2 EF hands, particularly Phl p 7, contained most of the relevant IgE epitopes, could be confirmed by testing sera from 8 additional patients who were allergic to calcium-binding allergens (data not shown).

DISCUSSION

Calcium-binding allergens have been identified in pollens of various unrelated plant species. They can be grouped according to the number of EF hands within their sequence in different families.³ It has been demonstrated

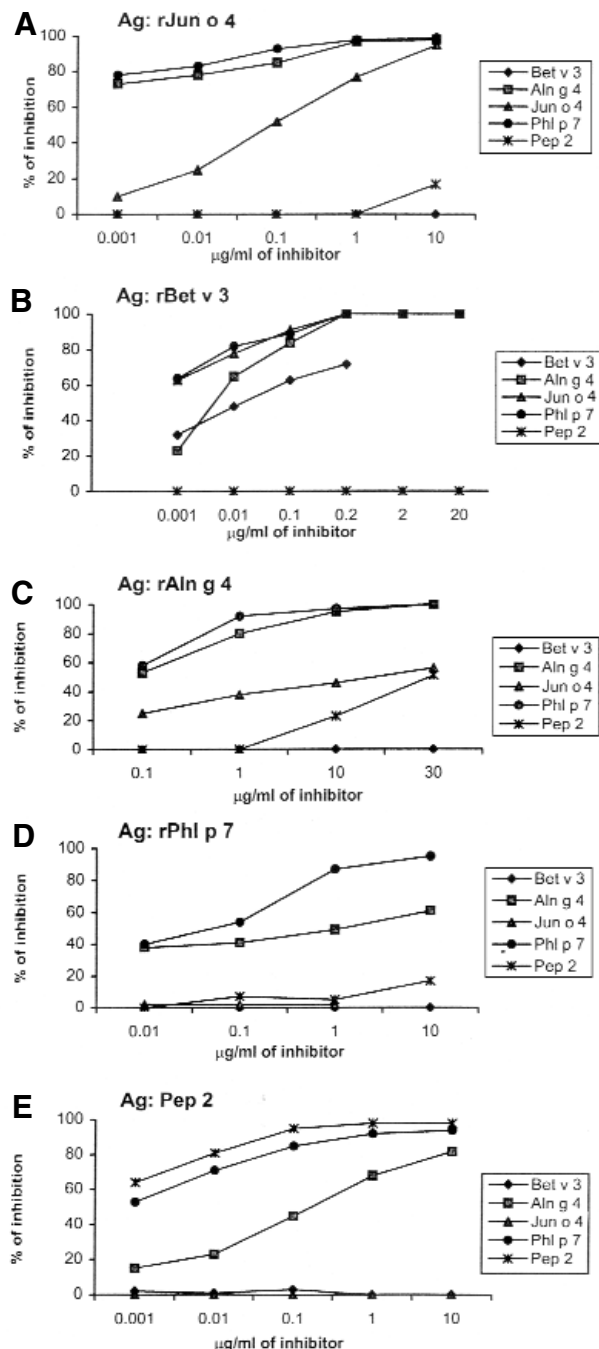


FIG 4. Specific IgE ELISA inhibition to rJun o 4 (A), rBet v 3 (B), rAln g 4 (C), rPhl p 7 (D), and synthetic rPhl p 7 peptide Pep 2 (E). Inhibitors were as follows: rJun o 4, triangles; rBet v 3, diamonds; rAln g 4, squares; rPhl p 7, circles; and Pep 2, asterisks. Ag, Antigen.

that members within a particular family of calcium-binding allergens share IgE epitopes,^{4,10} but no information was available as to whether different families of calcium-binding allergens contain cross-reactive IgE epitopes. Here we demonstrate that members of the allergen families with 2 (rPhl p 7 and rAln g 4), 3 (rBet v 3), and 4 (rJun o 4) EF hands exhibit structural similarities and share IgE epitopes.

Molecular modeling studies demonstrate a close similarity of the calcium-binding allergens from different families, as far as the globular domains of the allergens were concerned. In contrast, significant structural differences were found in the regions connecting the globular domains and in the termini of the molecules.

In agreement with the structural analysis, we could demonstrate that calcium-binding allergens belonging to the 3 different EF-hand families contained cross-reactive and non-cross-reactive IgE epitopes.

Allergens that exhibited IgE cross-reactivity with allergens with 2 and 4 EF hands could be detected in a broad variety of pollens from 16 unrelated plant species, suggesting that calcium-binding allergens are widely distributed in pollens from various plants. Those patients with IgE antibodies with specificity for calcium-binding allergens represented a subset of patients who were polysensitized against a broad variety of pollens from unrelated plant species (trees, grasses and weeds) but in most cases are also sensitized against various other non-cross-reactive allergens.

When we extended the analysis of antibody recognition of calcium-binding allergens to IgG subclass reactivities, we found that allergic, as well as nonatopic, individuals mounted a heterogeneous IgG subclass response against the purified calcium-binding allergens. This finding is in agreement with results obtained for other purified allergens and represents an example for the dissociation of the IgE and IgG subclass recognition of allergens, arguing against strictly sequential class switch to IgE in allergic patients.²²

IgE competition studies performed with purified recombinant calcium-binding allergens allowed us to establish a hierarchy of cross-reactivity among the members of the 3 allergen families. Using sera from our study group, which contained patients from a Middle European (Austria, Vienna) and a Mediterranean (Italy, Rome) population, we found that allergens with 2 EF hands, particularly Phl p 7, contained more IgE epitopes than members of the other EF-hand allergen families. There are several possibilities why certain of the EF-hand allergens exhibited stronger IgE reactivity than others. First, it is possible that patients are sensitized primarily against certain allergen sources (eg, grasses) and thus best recognize the allergens from the more prevalent allergen sources. Another possibility is that structural features may play a role regarding their IgE-binding capacity. This assumption is supported by the notion that those allergens containing more than 2 globular domains (eg, Bet v 3 and Jun o 4) were less potent IgE binders than those containing only 2 globular domains. It may thus be speculated that IgE recognition of calcium-binding allergens containing several globular domains is impaired by the close proximity of the domains in the larger molecules or perhaps because of reduced fold. The latter assumption would also be in agreement with the observation that the open version (ie, calcium-bound allergen version of a given calcium-binding allergen) was shown to expose more IgE epitopes than the closed version (ie, calcium-depleted version of the same molecule).³

The finding that certain calcium-binding pollen allergens (eg, rPhl p 7) contained most of the IgE epitopes present in other calcium-binding allergen families may have implications for diagnosis and therapy. Allergens such as rPhl p 7 may be used as diagnostic markers to identify allergic patients with multiple pollen sensitization caused by cross-reactivity to EF-hand allergens. A diagnostic test on the basis of one or a few calcium-binding allergens containing the majority of IgE epitopes may thus allow us to identify most patients sensitized to calcium-binding plant allergens. Furthermore, it may allow the development of specific immunotherapy strategies that are based on the administration of these molecules or hypoallergenic derivatives thereof.²³

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