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Molecular and Immunologic Characterization of a Highly Cross-Reactive Two EF-Hand Calcium-Binding Alder Pollen Allergen, Aln g 4: Structural Basis for Calcium-Modulated IgE Recognition¹

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Serum IgE was used to isolate a cDNA coding for a 9.4-kDa two EF-hand calcium-binding allergen, Aln g 4, from a λ gt11 expression cDNA library constructed from alder (*Alnus glutinosa*) pollen. rAln g 4 was overexpressed in *Escherichia coli* and purified to homogeneity. It reacted with serum IgE from 18% of pollen-allergic patients ($n = 122$); shared IgE epitopes with homologous allergens present in tree, grass, and weed pollens; and thus belongs to a family of highly cross-reactive pollen allergens. Exposure of two *E. coli*-expressed rAln g 4 fragments comprising amino acids 1–41 and 42–85 to patients' IgE Abs, as well as to a rabbit antiserum raised against purified rAln g 4, indicated that most of the B cell epitopes reside in the N-terminal portion of the protein. IgE recognition of Aln g 4 was strongly modulated by the presence or absence of calcium. Circular dichroism analysis of rAln g 4 revealed that the protein consisted mostly of α helical secondary structure and possessed a remarkable thermal stability and refolding capacity, a property that was greatly reduced after calcium depletion. Circular dichroism analysis of the calcium-bound and apo form of rAln g 4 indicated that calcium-induced modulation of IgE binding could be due to changes in the protein conformation. Purified rAln g 4 elicited dose-dependent basophil histamine release and immediate type skin reactions in sensitized patients. It may hence be useful for allergy diagnosis and for specific immunotherapy. *The Journal of Immunology*, 1998, 161: 7031–7039.

Type I allergy is a genetically determined hypersensitivity that is based on the production of IgE Abs against essentially innocuous Ags (i.e., allergens) (1, 2). The immediate type symptoms (e.g., allergic rhinitis, conjunctivitis, asthma, dermatitis, anaphylactic shock) result from allergen-induced cross-linking of effector cell-bound IgE Abs and the subsequent release of biologic mediators (e.g., histamine, leukotrienes) (3). Pollen from monocotyledonic (grasses) and dicotyledonic (trees, weeds) plants belongs to the most frequent and potent allergen sources (4). Pollen allergens represent low m.w. proteins or glycoproteins (5–70 kDa) that surprisingly are located mainly intracellularly in the pollen grains (5–8). They become rapidly eluted after pollen hydration on mucosal surfaces (9) or are liberated from small allergen-containing respirable particles (e.g., amyloplasts, P particles) (10).

Recently, the knowledge regarding the molecular nature of allergens has greatly increased due to the application of molecular cloning techniques for allergen characterization (11). The availability of cDNAs coding for allergens has facilitated the analysis of allergen structures (i.e., allergen sequences and three-dimensional structures) and the immunologic allergen characterization (T cell, B cell epitope mapping), and revealed the biologic functions of many allergens (12). Expression of allergen-encoding cDNAs provided us with a continuously increasing number of recombinant allergens for more precise and component-based allergy diagnosis and, perhaps, patient-tailored immunotherapy (11).

While certain plant allergens are rather selectively expressed in pollens of botanically related plant species (e.g., group 1 (13, 14), group 2 (15, 16), group 5 (17–19), and group 6 (20) grass pollen allergens in monocotyledonic plants), others represent proteins with important and, thus, conserved biologic functions. IgE recognition of the first type of allergens will cause clinical symptoms only after contact with a few allergen sources (e.g., grasses). By contrast, the production of IgE Abs to cross-reactive allergens will predispose an allergic patient to mount clinical symptoms on contact with a great variety of unrelated plants and plant-derived products (tree, grass, weed pollens (21), fruits and vegetables (22, 23), spices (24), or latex (25, 26)). Cross-reactivity thus represents the molecular basis for clinical symptoms on contact with various allergen sources and will perhaps help to identify a few relevant marker allergens that carry most of the relevant cross-reactive B and T cell epitopes for diagnostic and therapeutic purposes (27).

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³ Abbreviations used in this paper: CD, circular dichroism; aa, amino acid; IPTG, isopropyl- β -D-thiogalactopyranoside; RAST, radioallergosorbent test.

The highly cross-reactive plant allergens defined to date include: 1) profilins, a family of actin- and phosphatidylinositol-binding proteins with highly conserved three-dimensional structure that occur in all eukaryotic organisms (21, 28–30); 2) Bet v 1, the major birch pollen allergen and its homologues, which constitute a group of pathogenesis-related plant proteins with possible RNase activity (31, 32); Bet v 1-like allergens can be found as cross-reactive allergens in pollens of trees of the *Fagales* order (birch, alder, hazel, hornbeam, oak) (33) and in plant-derived food (fruits, vegetables, spices) (34); and 3) calcium-binding proteins, exemplified by Bet v 3 (35), a three EF-hand calcium-binding birch pollen allergen and a group of two EF-hand calcium-binding allergens recently described for birch (Bet v 4) (36, 37) and Bermuda grass (*Cyn d 7*) (38, 39).

In the present study, we report the isolation of a cDNA coding for a two EF-hand calcium-binding pollen allergen, Aln g 4, from an alder pollen expression cDNA library. Sequence comparisons indicate that Aln g 4 belongs to the family of two EF-hand allergens. rAln g 4 was expressed at high levels in *Escherichia coli* and purified to homogeneity to study its IgE-binding capacity, the prevalence of its IgE recognition, the share of IgE epitopes with homologues in various plant pollens, and its biologic activity (i.e., induction of basophil histamine release and immediate type skin reactions). Two rAln g 4 fragments comprising the N- and C-terminal portion of the allergen were expressed in *E. coli* to determine the Ab-binding sites of the allergen and the influence of calcium on the IgE recognition of Aln g 4. Conformations of calcium-bound and apo-rAln g 4 as well as their thermal stability and refolding propensity were analyzed by circular dichroism (CD)³ spectroscopy to investigate a possible structural basis for calcium-dependent IgE recognition.

Materials and Methods

Biologic materials, phage, E. coli strains, plasmids, patients sera, and rabbit antisera

Birch (*Betula verrucosa*), timothy grass (*Phleum pratense*), olive (*Olea europaea*), and Bermuda grass (*Cynodon dactylon*) pollen were purchased from Allergon (Vällinge, Sweden). *EcoRI* cut-dephosphorylated λ gt11 phage DNA was obtained from Amersham (Little Chalfont, U.K.). *E. coli* strain Y1090 (*hsd* ($r_k^- m_k^+$) *lac* U169, *Pro A*⁺, *Ion*⁻, *ara* D139, *Str A*, *Sup F trp* C22:Tn 10 (pMC9)) was purchased from Amersham; strain *E. coli* XL1-Blue *recA1 endA1 gyrA96 thi-1 hsdR17supE44 relA1 lac* (*F'* *pro AB lacI*^{ZDM15Tn19} (Tetr)) was from Stratagene (La Jolla, CA); and *E. coli* BL21 (DE3) (*F*⁻ *ompT* *r_B*⁻ *m_B*⁻ (DE3)) was from Novagen (Madison, WI). Plasmid pUC 18 was obtained from Boehringer Mannheim (Mannheim, Germany), and plasmid pET 17b from Novagen.

Birch ($n = 12$)-, grass ($n = 20$)-, maize ($n = 20$)-, olive ($n = 50$)-, and mugwort ($n = 20$)-allergic patients were characterized by positive case history and skin reactivity. The presence of serum IgE Abs specific for the pollen extracts was confirmed by RAST (radioallergosorbent test) analysis (Pharmacia, Uppsala, Sweden). The rabbit anti-rAln g 4 antiserum was generated by immunization of a rabbit with purified rAln g 4 using CFA (Charles River, Kisslegg, Germany). Preimmune serum samples were obtained from the same rabbit before immunization.

Protein extracts, Western blotting

Pollen protein extracts were prepared by homogenizing 1 g pollen in 50 ml of distilled water containing 5 mM PMSF with a mechanical homogenizer (Ultraturrax; IKA, Heidelberg, Germany) and extraction under continuous shaking at 4°C for 1 h. Extracts were then centrifuged at 20,000 × *g* for 30 min at 4°C to remove insoluble particles. Supernatants were frozen, lyophilized, and checked for protein quantity and quality by SDS-PAGE and Coomassie blue staining. Comparable amounts of each extract (200 μ g/cm) were separated by 12% SDS-PAGE (40) and blotted onto nitrocellulose (41).

IgE immunoscreening of the alder pollen cDNA library

A λ gt11 expression cDNA library was constructed from mature alder (*Alnus glutinosa*) pollen. Total pollen RNA was isolated using a phenol-SDS method (42). Poly(A)⁺ RNA was enriched by oligo(dT) cellulose chroma-

tography and reversely transcribed into cDNA using oligo(dT) primers and a cDNA synthesis system (Amersham). The resulting cDNA was methylated using *EcoRI* methylase (Promega, Madison, WI) and ligated to *EcoRI* 8-mer linkers (Boehringer Mannheim). Linkered cDNA was cut with *EcoRI*, unbound linkers were removed using a Nick column (Pharmacia), and the cDNA was ligated into dephosphorylated *EcoRI* cut λ gt11 arms. Recombinant λ gt11 DNA was packaged in vitro to yield an alder pollen expression cDNA library of 5×10^5 recombinant phage. *E. coli* Y1090 were infected with recombinant phage and plated onto Luria-Bertani plates containing 100 μ g/ml ampicillin, and synthesis of recombinant proteins was induced by overlay with nitrocellulose filters soaked in 10 mM IPTG after plaques became visible. Phage clones expressing allergens were isolated with serum IgE of a patient suffering from allergy to tree, grass, and weed pollens, as described (19). IgE-reactive phage clones were purified by two rounds of recloning.

Characterization of IgE-binding phage clones, plasmid subcloning, and DNA sequence analysis

The IgE immunoscreening of the alder pollen cDNA library yielded 23 IgE-reactive phage clones. Phage DNA from clones 1–23 was isolated using a plate lysate method (42). The phage DNA was cut with *KpnI/SacI* to obtain the allergen-encoding cDNA flanked on each side by approximately 1000 bp of λ gt11 DNA. The obtained DNA fragments were subcloned into the *KpnI/SacI* site of plasmid pUC 18, which was then transformed into *E. coli* XL1 Blue. Plasmid DNA was purified using Qiagen tips (Qiagen, Hilden, Germany), and both DNA strands were sequenced by the chain termination method (43) using λ gt11 forward, 5'-CGG GAT CCC GGT TTC CAT ATG GGG ATT GGT GGC-3', and λ gt11 reversed, 5'-CGC GGA TCC CGT TGA CAC CAG ACC AAC TGG TAA TG-3', and sequence-specific internal primers (MWG, Ebersberg, Germany), [³⁵S]dCTP (NEN, Stevenage, U.K.), and a T7 sequencing kit (Pharmacia). The sequences were analyzed with the McVector Program (Kodak, Rochester, NY). DNA and deduced amino acid sequences of the clones were compared with those submitted to GenBank using the program BlastN and BlastP of the BLAST server.

Expression in *E. coli* and purification of rAln g 4; MALDI-TOF (matrix-assisted laser desorption and ionization-time of flight) analysis of purified rAln g 4

The cDNA of one clone (clone 1) coding for Aln g 4 was PCR amplified from the λ gt11 phage using the primers: Aln g fwd, 5'-GG GAA TTC CAT ATG GCT GAC GAT CAT CCA CAG-3', and Aln g rev, 5'-CCG GAA TTC TTA GAA TAT CTT GGC AAC ATC CTT-3'. The *EcoRI* sites are printed in italics, and the *NdeI* site is underlined. The PCR product was cut with *NdeI* and *EcoRI*, gel purified, and subcloned into the *NdeI/EcoRI* site of plasmid pET 17b. pET 17b constructs were transformed in *E. coli* BL21 (DE3), and colonies expressing rAln g 4 were identified by screening nitrocellulose-blotted colonies with serum IgE from an Aln g 4-allergic patient, as described for IgE immunoscreening. Plasmid DNA was purified using Qiagen tips (Qiagen), and the DNA sequence of the expression construct was verified by sequence analysis. To yield high level expression of rAln g 4, the construct was transformed in *E. coli* BL 21 (DE 3). Cells were grown in liquid culture until an OD_{600 nm} of 0.4, and protein synthesis was induced by addition of IPTG to a final concentration of 0.5 mM and growth for additional 4 h at 30°C. Protein expression was analyzed in samples obtained before and after induction by SDS-PAGE and Coomassie blue staining. For the purification of rAln g 4, cell pellets obtained from 600 ml of liquid culture were homogenized in 15 ml of PBS containing 5 mM PMSF with an Ultraturrax (IKA). The homogenate was centrifuged in an SS34 rotor (RCS Sorvall; DuPont, Boston, MA) at 18,000 rpm, 4°C, for 30 min to remove insoluble materials. Increasing concentrations of ammonium sulfate (60, 70, 80% w/v) were added to the supernatant, which contained most of rAln g 4 to precipitate contaminating proteins at 4°C overnight. Precipitated material was removed by centrifugation in an SS34 rotor at 18,000 rpm, 4°C, for 30 min, and the rAln g 4-containing supernatant was dialyzed against water and lyophilized, resuspended in 50 ml of buffer I (25 mM imidazole, 1 mM β -mercaptoethanol, pH 7.4), and applied to a DEAE-anion exchange column (Pharmacia). rAln g 4 was eluted by a NaCl gradient (buffer I containing 500 mM NaCl) at approximately 200 mM NaCl. Fractions that, according to SDS-PAGE analysis, contained pure rAln g 4 were pooled, dialyzed against water, and lyophilized. As determined by centrifugation and SDS-PAGE analysis of supernatant and pellet fractions, rAln g 4 was completely soluble after reconstitution in water up to concentrations of 7 mg/ml.

Laser desorption mass spectra were acquired in a linear mode with a time-of-flight Compact MALDI II instrument (Kratos, Manchester, U.K.).

operating at 20 kV acceleration voltage, and equipped with a nitrogen UV laser (337 nm, pulse duration 3 ns) (piCHEM Research and Development, Graz, Austria). The m/z values were calibrated externally. Samples were dissolved in 10% acetonitrile (0.1% trifluoroacetic acid (TFA)). α -Cyano-4-hydroxycinnamic acid was used as a matrix dissolved in 60% acetonitrile (0.1% TFA). For sample preparation, a 1:1 mixture of protein solution and matrix solution was deposited onto the target and air dried.

Expression of rAln g 4 fragments in *E. coli*

cDNA fragments coding for two Aln g 4 fragments were generated by PCR amplification using the primers Aln g fwd and Eco rev, 5'-CCG GAA TTC TAC TAG GAG CCG GAT GTT TTC AA-3' for rAln g 4 aa 1–41, as well as Asn fwd, 5'-GTC GAG ATT AAT GGT CAC TCC GGA CGA GGT-3' and Aln g rev for rAln g 4 aa 42–85. The *AsnI* site is printed in italics, and the *EcoRI* site is underlined. PCR fragments containing the aa 1–41 and aa 42–85 encoding cDNA were cut with *NdeI/EcoRI* and *AsnI/EcoRI*, respectively, gel purified, and subcloned into the *NdeI/EcoRI* site of plasmid pET 17b. *E. coli* BL21 (DE3)-expressing rAln g 4 fragments were identified by colony screening with 1:1000 in buffer A-diluted rabbit anti-rAln g 4 antiserum and detection with a ^{125}I -labeled donkey anti-rabbit antiserum (Amersham). The sequences of the plasmid constructs (aa 1–41; aa 42–85) were verified by DNA sequencing. rAln g 4 fragments were expressed in *E. coli* BL21 (DE3) in liquid culture, as described for complete rAln g 4.

IgE inhibition experiments

The presence of cross-reactive IgE epitopes on rAln g 4 and Aln g 4-homologous allergens from timothy grass, olive, and Bermuda grass pollen was investigated by qualitative IgE immunoblot inhibition experiments (21). Sera from two patients who suffered from allergic symptoms after contact with tree, grass, and weed pollen, who exhibited positive skin reactions to the above three pollen extracts and contained rAln g 4-reactive IgE Abs and, for control purposes, serum from a nonatopic individual were studied. The sera were diluted 1/10 and preabsorbed overnight with *E. coli* extracts with and without rAln g 4, as described (21, 33). Preabsorbed sera were then exposed to nitrocellulose-blotted natural pollen extract, and bound IgE Abs were detected with ^{125}I -labeled anti-human IgE Abs (RAST; Pharmacia).

Calcium dependence of Ab binding to rAln g 4

Nitrocellulose-blotted purified rAln g 4 and *E. coli* extracts containing comparable amounts of rAln g 4 fragments (aa 1–41; aa 42–85) (approximately 5 $\mu\text{g}/\text{cm}$) were exposed to the rabbit anti-rAln g 4 antiserum, the rabbit preimmune serum, sera from four Aln g 4-allergic patients, serum from a nonallergic individual, and buffer A. Rabbit antisera were diluted 1/1000, and human sera were diluted 1/10 in buffer A containing 1 mM CaCl_2 or 10 mM EGTA, and incubated with identically prepared nitrocellulose strips. Bound human IgE Abs and rabbit Abs were detected with ^{125}I -labeled anti-human IgE Abs (RAST) and a ^{125}I -labeled donkey anti-rabbit antiserum, respectively, and visualized by autoradiography. Variations of IgE binding to the calcium-bound and apo forms of the proteins were also quantified by gamma counting of the strips in a gamma counter (Wallac, Turku, Finland).

CD measurements

CD spectra were recorded on a Jasco J-710 spectropolarimeter fitted with a Jasco PTC-348WI Peltier type temperature control system and interfaced with a Fisons HAAKE GH water bath. The instrument was calibrated with a 0.1% aqueous solution of D-10-camphorsulfonic acid. Results were expressed as the mean residue ellipticity (Θ) at a given wavelength. Far UV CD spectra were recorded at 20°C, 86°C, and 98°C in a 2-mm path-length quartz cuvette (Hellma, Forest Hills, NY), at a protein concentration of 10 μM . Spectra were recorded with 0.1 nm resolution and resulted from averaging 10 scans. The final spectra were corrected by subtracting the corresponding baseline spectrum obtained under identical conditions. All measurements were performed in MilliQ water, pH 7.2.

Thermal denaturation of rAln g 4 was monitored using a 2-mm cuvette (Hellma), by recording the ellipticity at a fixed wavelength while heating at 50°C/h with a computer-controlled circulating water bath. Fixed wavelengths were 221 and 222 nm for rAln g 4 in absence and presence of 5 mM EGTA, respectively. The reversibility of the unfolding process was checked by measuring the restoration of the CD signal upon cooling at 50°C/h to the starting temperature (20°C). Measurements were performed in MilliQ water, pH 7.2, at a protein concentration of 10 μM . Results were expressed as the mean residue ellipticity (Θ) at a given wavelength.

Histamine release experiments

Granulocytes were isolated from heparinized blood samples of pollen-allergic individuals containing rAln g 4-reactive IgE Abs ($n = 3$) and, for control purposes, of a nonatopic individual by dextran sedimentation (44). Cells were incubated with increasing concentrations (0.0001, 0.001, 0.01, 0.1, 1, and 10 $\mu\text{g}/\text{ml}$) of purified rAln g 4 or an anti-human IgE Ab. Histamine released into the supernatant was measured by RIA (Immuno-tech, Marseille, France). Total histamine was determined after freeze thawing of the cells. Results are expressed as mean values of triplicate determinations and represent the percentage of total histamine.

Skin-prick testing with rAln g 4

After informed consent was obtained, skin-prick tests were performed on the forearms of three pollen-allergic patients with, of one without rAln g 4-reactive IgE, and of a nonallergic individual. Twenty-microliter aliquots containing different concentrations of rAln g 4 (0.1, 1, 10, 20, and 40 $\mu\text{g}/\text{ml}$), birch pollen extract (70 $\mu\text{g}/\text{ml}$) (Soluprick; ALK, Horsholm, Sweden), histamine (1 mg/ml), and sodium chloride solutions (Soluprick; ALK) were pricked with sterile lancets (ALK). The skin reactions (wheals) were recorded 20 min after testing by photography and by transferring the ball point pen-surrounded wheal area with a Scotch Tape to paper. The mean wheal diameters (DMs) were determined as follows: $\text{DM} = (\text{D1} + \text{D2})/2$. D1 and D2 represent the maximal longitudinal and transversal diameters in mm, respectively (45).

Results

Isolation of cDNAs coding for Aln g 4, a two EF-hand calcium-binding alder pollen allergen with sequence homology to calcium-binding pollen allergens from trees, grasses, and weeds

A cDNA library constructed from mature alder pollen in phage $\lambda\text{gt}11$ was screened with serum IgE from a patient suffering from allergy to tree, grass, and weed pollens and plant-derived food. Sera with specificity for already known alder pollen allergens (i.e., the major alder pollen allergens, Aln g 1 (46), and alder pollen profilin) were used to select 23 phage clones containing cDNAs for novel allergens. All of these 23 clones contained cDNAs coding for a two EF-hand calcium-binding alder pollen allergen or isoforms thereof with significant sequence homology to a two EF-hand birch pollen allergen, Bet v 4 (36, 37) (Fig. 1A and B; data not shown). Therefore, the alder pollen allergen was designated Aln g 4 according to the Allergen Nomenclature Committee rules. On the basis of the first methionine-encoding ATG in clone 1, an open reading frame of 255 nucleotides was identified that coded for a protein of 9.4 kDa predicted m.w. From its sequence, Aln g 4 is an acidic protein with a predicted isoelectric point of 4.39 containing two copies of the most common calcium-binding motifs, the EF hand (47) (Fig. 1A, underlined). EF hands are helix-loop-helix motifs that usually pair together to bind to calcium through four carboxylate or carboxamide groups and a single backbone carbonyl oxygen placed in the loop with a specific spacing. Those amino acid residues that represent the calcium-binding domains in EF-hand proteins are found in Aln g 4, with no diversity from the consensus sequence. We can therefore confidently expect the protein to bind up to two calcium ions. No other sequence motifs or glycosylation sites could be predicted for the Aln g 4 sequence. A search to detect distal homology with other proteins in the SWISSPROT database shows that the top scores hit the N terminus of calmodulin as the closest EF-hand-containing protein. Aln g 4 shows 34% identity and 57% similarity with calmodulin from *Drosophila*. On this basis, we suggest that Aln g 4 could have similar ubiquitous regulation function.

Comparison of the Aln g 4-deduced amino acid sequence with other plant sequences revealed the highest degree of sequence identity with a family of two EF-hand pollen allergens recently described for birch (Bet v 4) (90.6%) (36, 37), rape (Bra n 1; Bra r 1) (74.7 and 75.9%, respectively) (48), and Bermuda grass (Cyn d 7) (66.3%) (38, 39) (Fig. 1B). Homology of the Aln g 4 N

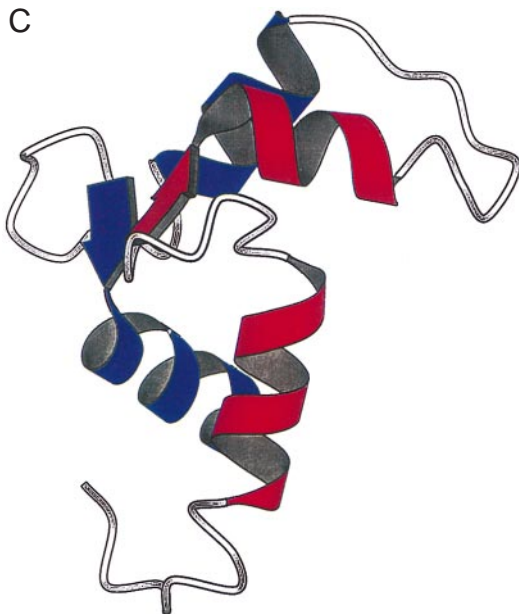
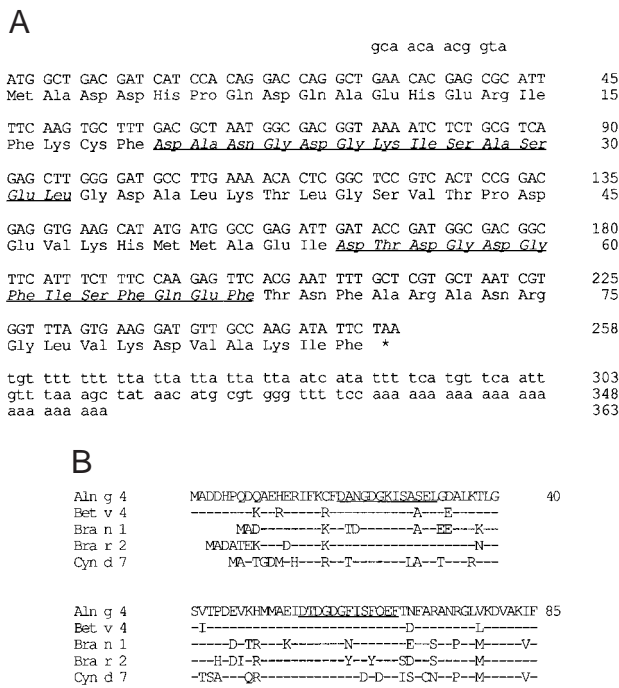


FIGURE 1. A, cDNA and deduced amino acid sequence of Aln g 4. The cDNA sequence of the noncoding regions is printed in lower and that of the coding region in upper case letters. The deduced amino acid sequence is printed below the nucleotide sequence. The two calcium-binding domains are underlined. The cDNA and deduced amino acid sequence of Aln g 4 have been submitted to the EMBL database under the accession number Y17713. B, Comparison of the Aln g 4 amino acid sequence with other two EF-hand plant allergens (birch, Bet v 4; rape, Bra n 1, Bra r 2; Bermuda grass, Cyn d 7). Identical amino acids are indicated by dashes. C, MOLSCRIPT representation of the Aln g 4 EF hands built according to *Drosophila* calmodulin. The secondary structure elements of the N terminus are in red, and those of the C terminus are in blue.

terminus was found with a partial N-terminal amino acid sequence determined for an olive pollen allergen, Ole e 3 (49).

A MOLSCRIPT representation of the model of the Aln g 4 EF hands was built on the coordinates of the calcium-bound form of *Drosophila* calmodulin (Fig. 1C) (50). The secondary structure

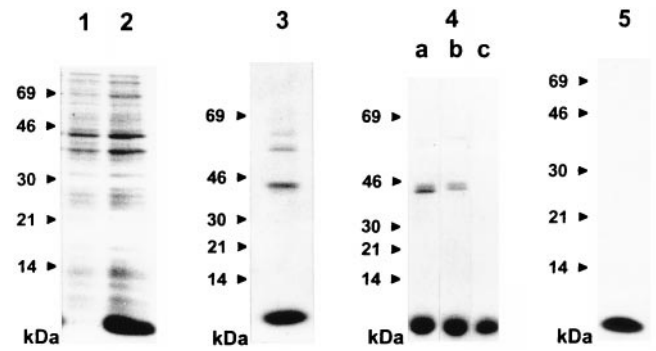


FIGURE 2. *E. coli* expression and purification of rAln g 4. Coomassie blue-stained SDS-PAGE containing *E. coli* extracts before (lane 1) and after (lane 2) induction of protein synthesis, soluble *E. coli* protein fraction without (lane 3) and with increasing amounts of ammonium sulfate (lane 4a, 60%; b, 70%; c, 80%), and DEAE-purified rAln g 4 (lane 5).

elements of the two EF hands are indicated in different colors, red for the N terminus and blue for the C terminus.

E. coli expression, purification, and mass-spectroscopic analysis of rAln g 4

rAln g 4 was expressed in *E. coli* BL21 (DE3) at high levels (10–20 mg/L *E. coli* culture). Based on SDS-PAGE analysis of *E. coli* extracts, rAln g 4 accounted for more than 25% of the total *E. coli* proteins (Fig. 2, lane 2). Cell fractionation revealed that rAln g 4 accumulated in the fraction of soluble *E. coli* proteins (Fig. 2, lane 3) and could be enriched in the soluble fraction up to 90% purity by precipitation of other proteins with ammonium sulfate (Fig. 2, lanes 4a–4c). An almost equal enrichment of rAln g 4 in the soluble protein fraction was obtained by boiling of the soluble *E. coli* extract, which led to precipitation of contaminating proteins (data not shown). Pure and completely soluble rAln g 4 was obtained by a final purification step via DEAE anion-exchange chromatography (Fig. 2, lane 5).

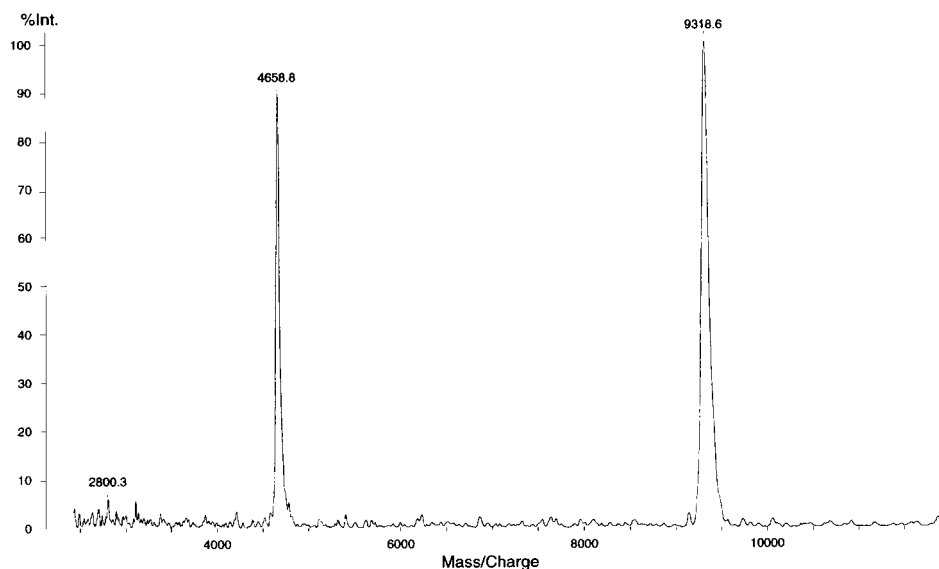
The analysis of purified rAln g 4 by mass spectroscopy revealed the presence of two peaks (9318.6 and 4658.8) that correspond to the MH⁺ and M2H2⁺ species of the same protein (Fig. 3). Results obtained by mass-spectroscopic analysis of the purified recombinant protein are in agreement with the predicted molecular mass (9361 Da), but differ from the apparent m.w. observed for rAln g 4 in denaturing SDS-PAGE (i.e., approximately 6–7 kDa). The latter is most likely due to the acidic character of the protein, which may cause an unusual migration in SDS-PAGE.

rAln g 4 represents a highly cross-reactive plant allergen

The frequency of IgE reactivity to rAln g 4 was determined for sera from olive ($n = 50$)-, grass ($n = 20$)-, birch ($n = 12$)-, and mugwort pollen ($n = 20$)-, and maize ($n = 20$)-allergic patients. A total of 22 of the 122 tested sera (18%) contained rAln g 4-specific IgE Abs (data not shown). The highest prevalence of rAln g 4 IgE recognition (24 and 20%) was found for the olive and mugwort pollen-allergic patients; 16.7% of the birch pollen-allergic patients and 10% of the grass pollen- and maize-allergic individuals displayed IgE reactivity to rAln g 4 (data not shown). The comparable prevalence of IgE reactivity to rAln g 4 in the different populations together with the sequence similarity of Aln g 4 with homologous two EF-hand allergens from tree, grass, and weed pollens (Fig. 1B) suggested that Aln g 4 shares IgE epitopes with homologous allergens in a great variety of pollen extracts.

Preabsorption of sera from two rAln g 4-reactive patients (Fig. 4, A and B) with *E. coli* extract containing rAln g 4 (Fig. 4, lanes

FIGURE 3. Mass-spectroscopic analysis of purified rAln g 4. The x-axis shows the mass/charge ratio, and the signal intensity is displayed on the y-axis as percentage of the most intensive signal obtained in the investigated mass range. The peaks at 9318.6 and 4658.8 correspond to the MH^+ and $M2H2^+$ species of rAln g 4.



), but not with *E. coli* alone (Fig. 4, lanes -) led to a complete or great reduction of IgE binding to moieties of 6–7 kDa in nitrocellulose-blotted timothy grass, olive, and Bermuda grass pollen extracts. IgE binding to higher m.w. components in the pollen extracts was not reduced after preabsorption of the sera with rAln g 4. The serum from a nonatopic individual showed no IgE reactivity to nitrocellulose-blotted pollen extracts (Fig. 4, lane N).

Purified rAln g 4 induces dose-dependent basophil histamine release and immediate type skin reactions in allergic patients

The biologic activity of purified rAln g 4 was evaluated by basophil degranulation experiments as well as by skin testing. As exemplified for one patient (Fig. 5A), purified rAln g 4 induced specific and dose-dependent histamine release from basophils of three pollen-allergic patients containing Aln g 4-reactive IgE Abs, but not from basophils of a nonatopic individual (N) (Fig. 5B). A significant histamine release was observed already at very low protein concentrations (0.0001–0.001 $\mu\text{g/ml}$). Incubation of basophils from all atopics as well as from the nonatopic individual with anti-human IgE mAbs yielded degranulation with anti-human IgE (Fig. 5, A and B).

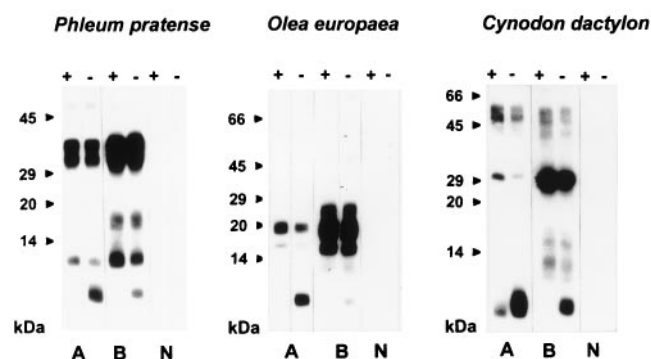


FIGURE 4. rAln g 4 shares IgE epitopes with homologous allergens in timothy grass, olive, and Bermuda grass pollen. Sera from two pollen-allergic patients (A, B) and from a nonatopic individual (N) were preabsorbed with rAln g 4 (+) or *E. coli* proteins (-) prior to exposure to nitrocellulose-blotted pollen extracts. Bound IgE Abs were detected with ^{125}I -labeled anti-human IgE antibodies and visualized by autoradiography.

The extremely high allergenic potential of rAln g 4 was confirmed by skin-prick test experiments. Purified rAln g 4 elicited strong immediate type skin reactions already at concentrations of

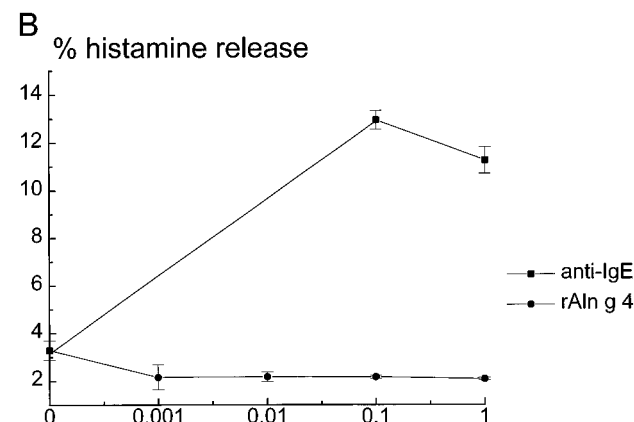
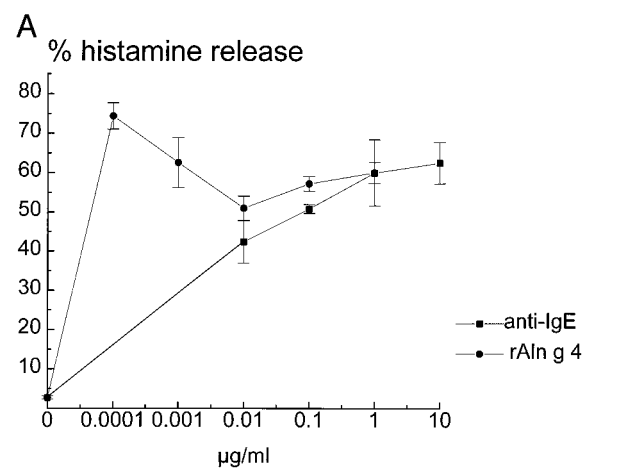


FIGURE 5. rAln g 4 induces basophil histamine release. Granulocytes from Aln g 4-reactive pollen-allergic patients (A) (panel A) and from a nonatopic individual (N) (panel B) were incubated with various concentrations (x-axis) of purified rAln g 4 (circles) or an anti-human IgE mAb (squares). The percentage of histamine release is displayed on the y-axis. Results represent the means ($\pm\text{SD}$) of triplicate determinations.

Table I. Induction of immediate type skin reactions with rAln g 4^a

Individual	rAln g 4							Birch	Histamine
	40 µg/ml	20 µg/ml	10 µg/ml	5 µg/ml	1 µg/ml	0.5 µg/ml	0.1 µg/ml		
A	ND	13	10.5	8	5.5	4.5	2	5	6
B	12.5	12	11	10.5	12	ND	ND	4	6
C	ND	13.5	11	10.5	9	ND	ND	11	7.5
D	ND	—	—	—	—	—	—	5	5.5
N	ND	—	—	—	—	—	—	—	5

^a Three allergic patients with (A, B, C) and one without (D) Aln g 4-specific IgE as well as a nonatopic individual (N) were pricked on their forearms with rAln g 4, birch pollen extract, histamine, and sodium chloride. Results are displayed as the mean wheal diameters (mm). ND, not determined. Dashes indicate lack of reactivity.

1 µg/ml in three pollen-allergic patients containing rAln g 4-reactive IgE Abs (A–C), but not in an allergic patient without rAln g 4-specific IgE (D) and a nonatopic person (N) (Table I). Birch pollen extract containing the Aln g 4-homologous allergen (Bet v 4) induced skin reactions in the allergic patients (A–D), all individuals showed skin reactivity to histamine, and no reaction to sodium chloride was observed in any of the tested persons (Table I).

IgE recognition of rAln g 4 is calcium dependent and involves primarily the N-terminal portion of the protein

Nitrocellulose-blotted complete rAln g 4 and two rAln g 4 fragments (aa 1–41, aa 42–85) were exposed to four sera from Aln g 4-reactive patients (A–D) and to a rabbit anti-Aln g 4 antiserum (R) in the presence (+) and absence (–) of calcium (Fig. 6). Results obtained showed that complete calcium-bound rAln g 4 bound IgE and rabbit Abs stronger than the apo form (Fig. 6, rAln g 4). rAln g 4 fragments, containing the N-terminal and C-terminal EF hand of Aln g 4, had a greatly reduced Ab-binding capacity. The N-terminal rAln g 4 fragment reacted with serum IgE from three of four patients and with rabbit anti-Aln g 4 Abs (Fig. 6, rAln g 4 aa 1–41). The C-terminal rAln g 4 fragments bound weakly the rabbit anti-rAln g 4 antiserum, but not serum IgE from the Aln g 4-allergic patients (Fig. 6, rAln g 4 aa 42–85). Both serum IgE and rabbit Ab reactivity to rAln g 4 fragments depended on protein-bound calcium.

CD analysis of the calcium-bound and apo form of rAln g 4

The far UV CD spectrum of purified rAln g 4 (Fig. 7A), recorded at 20°C, indicates that the protein contains a considerable amount of α helical secondary structure. The spectra of the protein in the absence and in the presence of calcium are similar in shape (two broad minima at 208 and 222 nm), but the presence of calcium seems to be consistent with an increase of helicity (Fig. 7A). Thermal unfolding of rAln g 4 was monitored as change in ellipticity at

221 nm (calcium-bound form) and 222 nm (5 mM EGTA; apo form) and expressed as molar ellipticity (Θ). The calcium-bound form shows a remarkable thermal stability, as most of the protein appeared to be folded up to 98°C, so that the transition is not completed below 100°C (Fig. 7, B and D). In the presence of 5 mM EGTA, the unfolding transition of rAln g 4 is monophasic and highly cooperative with a melting point of 51°C (Fig. 7C). At 86°C, the apo form assumes a random coil conformation with a typical minimum at about 200 nm (Fig. 7C). The unfolding transition of apo-rAln g 4 is monophasic and highly cooperative with a melting point of 51°C (Fig. 7D). rAln g 4 shows a high degree of folding reversibility, evident from the cooling curve profiles (Fig. 7D) and the far UV spectra recorded at 20°C, after cooling from 98°C and 86°C (Fig. 7, B and C), respectively, in absence or presence of EGTA. We must therefore conclude that the thermal stability and refolding behavior of the calcium-bound form of Aln g 4 is higher than that of the calcium-free form.

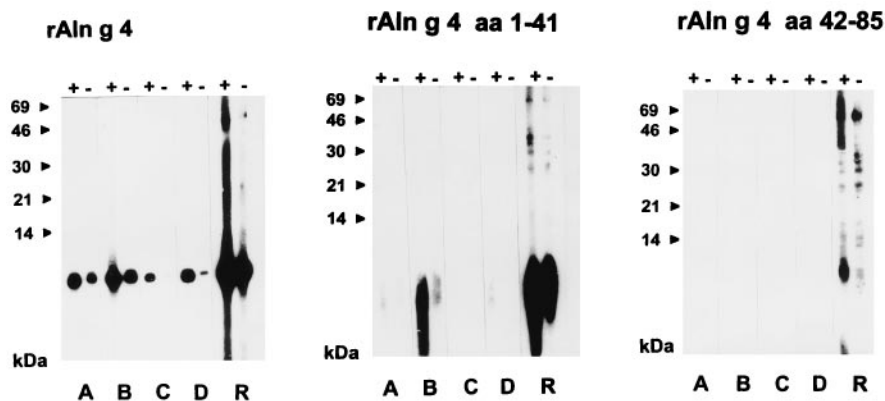
Changes of the Aln g 4 conformation in the presence and absence of protein-bound calcium predicted by molecular modeling

The CPK (Corey, Pauling, and Koltun) representation of Aln g 4 was built according to the coordinates of the calcium-bound and apo form of *Drosophila* calmodulin with the program InsightII (51) (Fig. 8). It gives a picture of the van der Waals surface of the atoms in the calcium-bound (left) and calcium-free form of the expected Aln g 4 structure. The conformational change upon calcium binding results in the exposure of hydrophobic patches (green) on the molecule surface, which are then able to interact with other molecules (e.g., Abs).

Discussion

We have isolated a cDNA coding for a 9.4-kDa two EF-hand calcium-binding allergen, Aln g 4, from an alder pollen expression

FIGURE 6. Calcium-dependent Ab recognition of complete rAln g 4, and rAln g 4 fragments (rAln g 4 aa 1–41, rAln g 4 aa 42–85). Nitrocellulose-blotted rAln g 4 and rAln g 4 fragments (aa 1–41; aa 42–85) were incubated with sera from Aln g 4-allergic patients (lanes A–D) and with a rabbit anti-rAln g 4 antiserum (lane R) in the presence (+) or absence (–) of calcium. Bound IgE or rabbit Abs were detected with ¹²⁵I-labeled anti-human IgE and ¹²⁵I-labeled donkey anti-rabbit Abs, respectively.



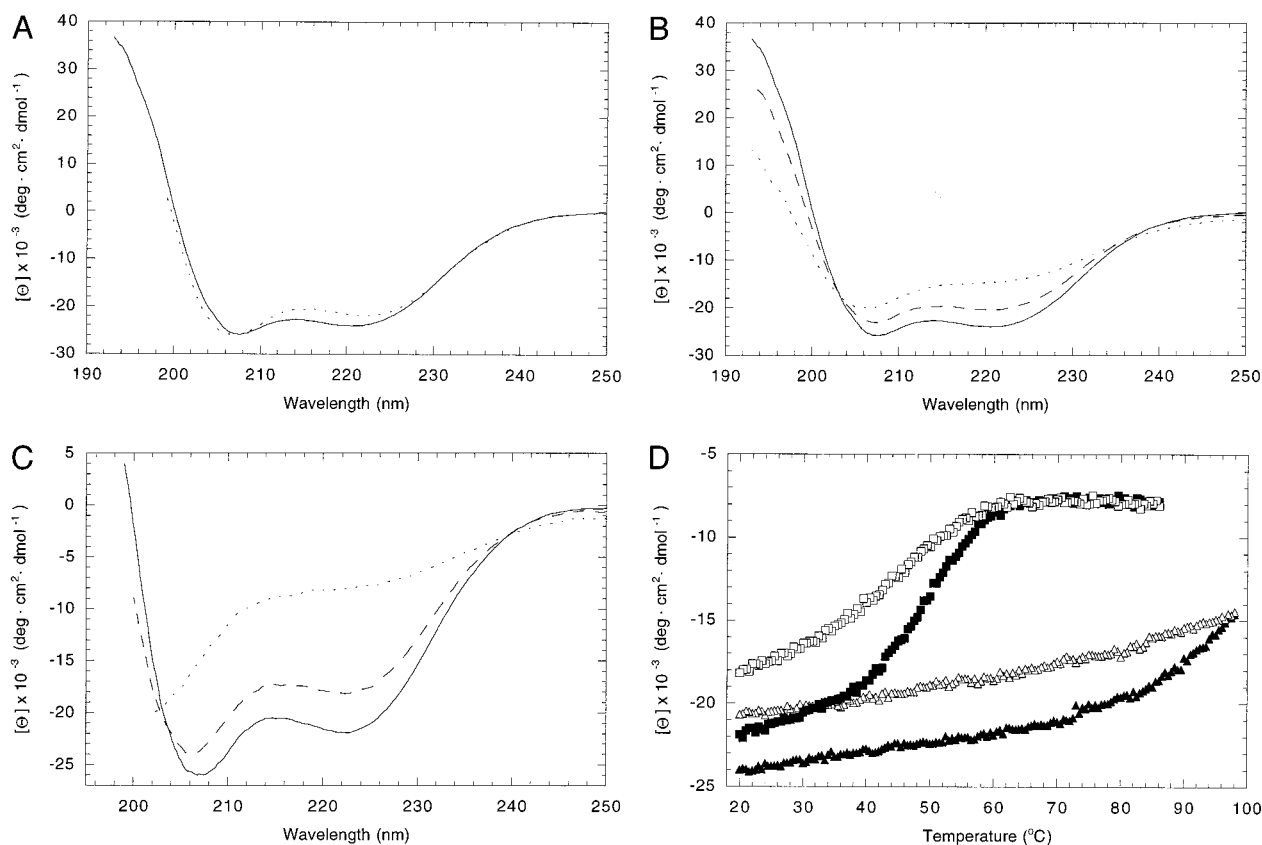
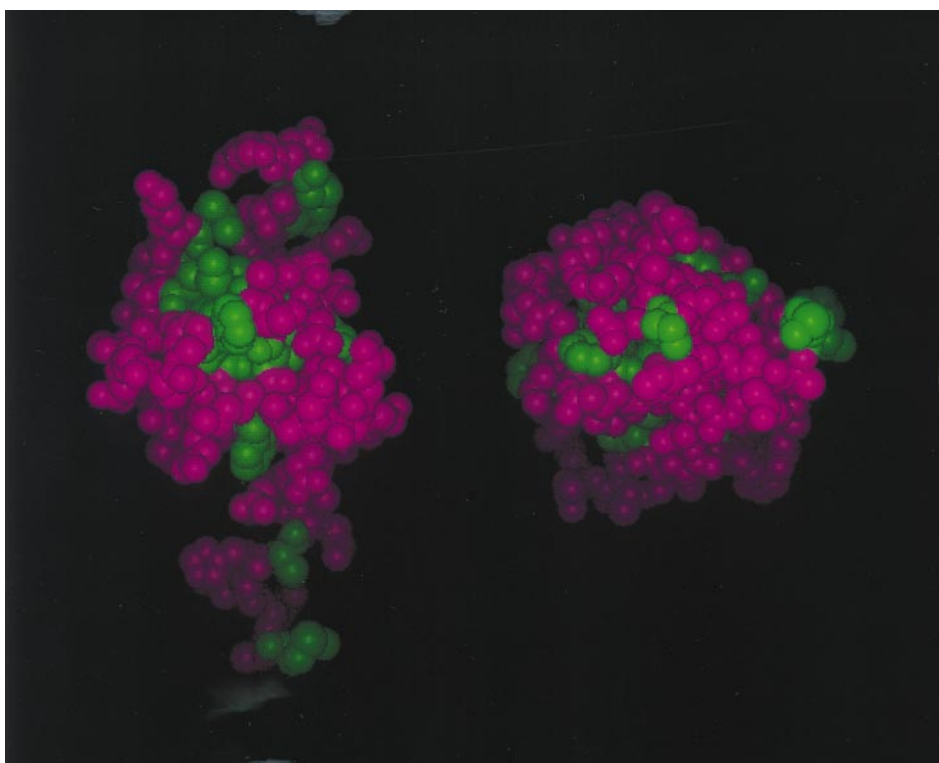


FIGURE 7. A, Far UV CD analysis of rAln g 4. CD analysis of calcium-bound rAln g 4 (continuous line) and rAln g 4 in presence of 5 mM EGTA (dotted line), at 20°C. B, Far UV CD analysis of calcium-bound rAln g 4 at 20°C (continuous line), 98°C (dotted line), and 20°C after cooling from 98°C (dashed line) as well as of C, rAln g 4 in the presence of 5 mM EGTA at 20°C (continuous line), 86°C (dotted line), and 20°C after cooling from 86°C (dashed line). All spectra are expressed as mean residue ellipticity (Θ ; y-axis) at a given wavelength (nm; x-axis). D, Thermal stability of rAln g 4 monitored by CD. Thermal denaturation (black triangles) and cooling (white triangles) of calcium-bound rAln g 4, monitored at 221 nm. Thermal denaturation (black squares) and cooling (white squares) of rAln g 4 in presence of 5 mM EGTA, monitored at 222 nm. Measurements were expressed as mean residue ellipticity (Θ ; y-axis) at a given temperature (°C; x-axis).

cDNA library using serum IgE from a patient suffering from tree, grass, and weed pollen allergy. Sequence analyses revealed that Aln g 4 belongs to a novel class of two EF-hand calcium-binding allergens recently described for birch (36, 37), Bermuda grass (38, 39), rape (48), olive (49), and timothy grass pollen (V. Niederberger and R. Valenta, unpublished data). The members of the two EF-hand allergen family share an average sequence identity of 77%, which is of comparable magnitude within and outside the calcium-binding domains, and thus represents a novel class of calcium-binding proteins. Exposure of rAln g 4 to sera from different plant-allergic populations (olive, grass, birch, mugwort, maize) revealed a comparable high prevalence of IgE recognition (average 18%) in the different groups. The high degree of sequence identity of Aln g 4 and its homologues in monocotyledonic (grasses) and dicotyledonic (trees, weeds) plants together with the comparable high prevalence of IgE recognition in different plant-allergic populations indicated that Aln g 4 may represent a highly cross-reactive pollen allergen. This assumption gained support by our demonstration that rAln g 4 shared IgE epitopes with allergens of comparable m.w. in natural pollen extracts from olive, timothy grass, and Bermuda grass. The biologic and clinical relevance of IgE Ab cross-reactivity were investigated by basophil histamine release experiments and skin-prick testing. For this purpose, rAln g 4 was overexpressed in *E. coli* and purified to homogeneity. *E. coli* expression and purification yielded milligram amounts of completely soluble and folded rAln g 4. Purified rAln g 4 induced

specific and dose-dependent basophil histamine release in patients who contained rAln g 4-reactive IgE Abs and suffered from allergy to pollens of a variety of plants (trees, grasses, weeds), thus confirming the clinical relevance of IgE cross-reactivity. Despite a rather low prevalence (18%) of IgE recognition of rAln g 4 among pollen-allergic individuals, the recombinant protein displayed an extremely high allergenic activity in the basophil histamine release experiments. Maximal histamine release was induced already at very low protein concentrations (0.0001–0.001 $\mu\text{g}/\text{ml}$), a finding that was confirmed by the skin-prick test experiments. Twenty-microliter aliquots of a 1 $\mu\text{g}/\text{ml}$ rAln g 4 solution elicited a histamine-equivalent skin reaction in patients containing rAln g 4-reactive IgE Abs. The latter finding is of note, considering that in previous skin-prick test studies performed with rBet v 1, the major birch pollen allergen, and with rBet v 2, birch profilin, best results were obtained at protein concentrations ranging between 10–20 $\mu\text{g}/\text{ml}$ (52, 53). Since in the tested individuals the IgE levels specific for rAln g 4 were not higher than those to other recombinant allergens (data not shown), we assume that the high allergenic activity of rAln g 4 is due to its native-like structure. CD-spectroscopic analysis of rAln g 4 indeed demonstrates that the recombinant protein is stably folded and consists mostly of α helical secondary structure. The CD measurements are in agreement with the helicity content expected from the sequence similarity with calmodulin, which predicts also in Aln g 4 two calcium-binding loops flanked by two α helices (Fig. 1C). Helix-loop-helix motifs,

FIGURE 8. CPK (Corey, Pauling, and Koltun) representation of the calcium-bound (*left*) and the calcium-free (*right*) form of the expected Aln g 4 structure. Hydrophilic and hydrophobic atoms are displayed in pink and green, respectively.



termed EF hands, are found in a variety of eukaryotic calcium-binding proteins, where they occur in side-to-side pairs of EF hands and form a globular unit (47, 54, 55). As previously noted for other calcium-binding allergens, we demonstrate that Ab recognition of rAln g 4 is strongly modulated by the presence or absence of protein-bound calcium (35–39, 56). Serum IgE and rabbit IgG binding to the calcium-depleted form (apo form) of rAln g 4 were greatly reduced. Exposure of two rAln g 4 fragments comprising the N- and C-terminal EF hand to serum IgE and to a rabbit anti-rAln g 4 antiserum indicates that the N-terminal EF hand contains most of the relevant Ab-binding sites. This result is in agreement with our previous finding that a recombinant fragment of the Aln g 4-homologous allergen from birch (Bet v 4) lacking the first 16 N-terminal amino acids had a greatly reduced IgE-binding capacity (36). On the basis of the Ab-binding experiments performed with the calcium-bound and apo form of rAln g 4, as well as with the two recombinant EF-hand domains, we would propose that Abs recognize mainly epitopes on the N-terminal EF-hand domain, which become accessible only in the calcium-bound conformation. CD-spectroscopic analysis of the calcium-bound and apo form of rAln g 4 revealed indeed significant conformational differences between the two forms. Moreover, we found a remarkable difference concerning the thermal stability and refolding capacity of the calcium-bound and apo form of rAln g 4. While a considerable proportion of calcium-bound rAln g 4 remained folded up to temperatures of 98°C, most of the apo form was denatured already at 55°C.

It is well known that EF-hand proteins undergo a major conformational change upon calcium binding from a closed to an open state allowing protein-protein interaction (47). Major changes are observed in the helix packing and in the exposure of hydrophobic residues upon calcium binding. In the apo form, the four helices would pack in pairs, forming a very compact structure (closed state), and upon calcium binding, the two helices of each EF-hand motif are pushed apart, inducing the exposure of hydrophobic residues (green) otherwise involved in helix-helix packing in the cal-

cium-free form (Fig. 8). The sequence similarity with calmodulin as well as the CD measurements conducted on the calcium-free and calcium-bound form of rAln g 4 are consistent with such a conformational transition. The immunologic data could therefore be explained by either suggesting the presence of epitopes that become accessible only in the calcium-bound form (open conformation), or suggesting that IgE recognition is only activated by the calcium-bound conformation. The latter would mean that many of the Aln g 4-allergic patients were preferentially sensitized against the calcium-bound conformation. The finding that most of the patients displayed reduced IgE binding to the apo form of Aln g 4 may have significant clinical relevance inasmuch as stably engineered apo forms with reduced IgE-binding capacity may be used for immunotherapy with reduced risk of anaphylactic side effects.

In conclusion, rAln g 4 represents an important target structure for cross-reactive IgE Abs of patients suffering from allergy to pollens of many unrelated plant species. It may therefore be used for diagnostic purposes to explain and predict symptoms in individuals suffering from allergy to pollens of botanically unrelated plants. Based on the knowledge of conformation-dependent IgE recognition of Aln g 4 and the epitopic areas involved in Ab binding, it may be possible to generate recombinant hypoallergenic Aln g 4 variants for specific immunotherapy.

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