

A Human Monoclonal IgE Antibody Defines a Highly Allergenic Fragment of the Major Timothy Grass Pollen Allergen, Phl p 5: Molecular, Immunological, and Structural Characterization of the Epitope-Containing Domain¹

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Almost 90% of grass pollen-allergic patients are sensitized against group 5 grass pollen allergens. We isolated a monoclonal human IgE Fab out of a combinatorial library prepared from lymphocytes of a grass pollen-allergic patient and studied its interaction with group 5 allergens. The IgE Fab cross-reacted with group 5A isoallergens from several grass and corn species. By allergen gene fragmentation we mapped the binding site of the IgE Fab to a 11.2-kDa N-terminal fragment of the major timothy grass pollen allergen Phl p 5A. The IgE Fab-defined Phl p 5A fragment was expressed in *Escherichia coli* and purified to homogeneity. Circular dichroism analysis revealed that the rPhl p 5A domain, as well as complete rPhl p 5A, assumed a folded conformation consisting predominantly of an α helical secondary structure, and exhibited a remarkable refolding capacity. It reacted with serum IgE from 76% of grass pollen-allergic patients and revealed an extremely high allergenic activity in basophil histamine release as well as skin test experiments. Thus, the rPhl p 5A domain represents an important allergen domain containing several IgE epitopes in a configuration optimal for efficient effector cell activation. We suggest the rPhl p 5A fragment and the corresponding IgE Fab as paradigmatic tools to explore the structural requirements for highly efficient effector cell activation and, perhaps later, for the development of generally applicable allergen-specific therapy strategies. *The Journal of Immunology*, 2000, 165: 3849–3859.

Type I allergy, a genetically determined hypersensitivity disease that is based on the formation of IgE Abs against harmless Ags (allergens) affects almost 500 million people worldwide (1). Allergic patients exhibit immediate type symptoms (allergic rhinitis, conjunctivitis, asthma, dermatitis, and anaphylactic shock) when allergens cross-link effector cell (mast cell, basophil)-bound IgE Abs and thus induce the release of biologically active mediators (histamine, leukotrienes) (2). To behave as an allergen, an Ag must contain at least two binding sites for IgE Abs (IgE epitopes) (3).

With the implementation of molecular biological techniques to the field of allergen characterization, the sequence, nature, and three-dimensional structure of several important allergens have been revealed, and we begin to understand how IgE Abs recognize

allergens at a molecular level (4–6). As for B cell epitopes in general, basically two types of IgE epitopes have been identified. Epitopes that consist of a stretch of few contiguous amino acids are termed “continuous epitopes” (7). Epitopes composed of at least two nonadjacent domains of the molecule that are brought into close proximity within the folded molecule are named “discontinuous epitopes” (7). IgE epitopes of several relevant allergens, e.g., the major birch pollen allergen Bet v 1 (8), the calcium-binding pollen allergens, Bet v 3 (9), Bet v 4 (10, 11), Phl p 7 (12), Aln g 4 (13), Bra r 1 (14), and parvalbumin (15), belong to the discontinuous type. Continuous IgE epitopes were identified on several grass pollen allergens, e.g., major rye grass pollen allergens Lol p 1 (16) and Lol p 5 (17), major timothy grass pollen allergen Phl p 1 (18, 19), and velvet grass allergen Hol l 1 (20).

Investigations on the three-dimensional structure and IgE epitopes of major allergens have recently gained great attention as they may open new avenues to reduce the allergenic activity of allergens by genetic engineering or peptide chemistry (5, 21). Although there seems to be no common structural theme that would determine the allergenic activity of a given protein, it turned out that proper folding and high propensity to refold after denaturation are frequent features of potent allergens (12, 13, 15, 22–25).

To obtain monoclonal IgE Abs with specificity for major allergens we have constructed an IgE combinatorial library from lymphocytes of a grass pollen-allergic patient (26). Here we study the interaction of a recombinant monoclonal human IgE Fab with one of the most prominent and potent environmental allergens, the major timothy grass pollen allergen Phl p 5A (27). Phl p 5A represents a major grass pollen allergen that is recognized by >80% of grass pollen-allergic patients. In sensitized patients it accounts for

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up to 60% of grass pollen-specific IgE, it induces strong IgE responses in experimental animal systems, and exhibits a surprising potency to activate allergic effector cells and to elicit immediate type skin and nasal reactions (27–30; V. Niederberger and R. Valenta, unpublished data). By allergen gene fragmentation we identified a Phl p 5A domain that contains the binding site for the human monoclonal IgE Fab. The IgE Fab-defined Phl p 5A fragment was expressed in *Escherichia coli*, purified to homogeneity, and characterized regarding its secondary structure contents, thermal stability, and refolding capacity by circular dichroism (CD)³ analysis. IgE binding, basophil histamine release, and skin prick testing revealed that the fragment represents an important allergen domain for most grass pollen-allergic patients and exhibits high allergenic activity. We suggest the three-dimensional structure analysis of the interaction of the rPhl p 5A domain and its corresponding IgE Ab for the elucidation of the structural requirements for highly efficient effector cell activation.

Materials and Methods

Materials, patients' sera, and Abs

Pollen from timothy grass, sweet vernal grass, oat, Bermuda grass, rye grass, common reed, Kentucky Bluegrass, rye, wheat, and maize were purchased from Allergon (Välinge, Sweden). Sera were collected from grass pollen-allergic patients who were characterized by case history, skin prick testing, ¹²⁵I-labeled anti-human IgE Abs (RAST), and by testing with recombinant grass pollen allergens as described (31, 32). Sera from allergic patients without grass pollen allergy and from nonallergic individuals were included as negative controls. A rabbit anti-rPhl p 5A antiserum was obtained by immunization of a rabbit with purified rPhl p 5A using CFA (Charles River, Kisslegg, Germany). A ¹²⁵I-labeled donkey anti-rabbit Ig antiserum was purchased from Amersham (Buckinghamshire, U.K.). Mouse mAbs BG6 and Bo9 with specificity for Phl p 5A and Phl p 5B, respectively, are described (33). An alkaline phosphatase (AP)-coupled rabbit anti-mouse IgG+IgM antiserum was purchased from Jackson ImmunoResearch (West Grove, Pa.). RAST were obtained from Pharmacia (Pharmacia Diagnostics, Uppsala, Sweden).

Preparation of pollen extracts, recombinant allergens, and allergen fragments

One hundred milligrams of natural pollen of each grass and corn species were separately resuspended in 5 ml SDS-sample buffer and homogenized with an ultraturax (IKA, Stauffen, Germany) for 1 min, boiled for 5 min at 95°C, and centrifuged at 14,500 rpm in a bench fuge for 5 min to remove insoluble materials (34). Comparable amounts of extracted proteins (~100 µg/cm) were separated by a preparative 14% SDS-PAGE under reducing conditions and blotted onto nitrocellulose (35–37). Recombinant Phl p 5A and rPhl p 6 were expressed in *E. coli* BL21 (DE3) and purified as described (25, 28). Recombinant Phl p 5B and a recombinant fragment comprising the 136 C-terminal amino acids of Phl p 5B were expressed in *E. coli* using plasmid pMalc and purified as described (38).

Expression of a recombinant human IgE Fab and a complete Fab-derived recombinant human IgG1 with specificity for Phl p 5A

A human IgE Fab (clone 5) with specificity for the major timothy grass pollen allergen, Phl p 5A, was obtained from an IgE combinatorial library constructed from lymphocytes of a grass pollen-allergic patient (26). cDNAs coding for IgE Fds and light chains were obtained by RT-PCR from lymphocyte-derived RNA and recombined in plasmid pComb3H. The phage expressing a human Phl p 5A-specific IgE Fab was isolated via panning to ELISA plate-bound Phl p 5A. Soluble Phl p 5A-specific recombinant IgE Fabs were produced by removing the *SpeI* and *NheI* fragment from the original pComb3H construct. The resulting plasmid construct was transformed into *E. coli* XL-1 Blue and expressed in liquid culture (LB medium containing 50 mg/L ampicillin) after addition of isopropyl-β-thiogalactopyranoside (IPTG) to a final concentration of 1.5 mM

as described (39). For control purposes, *E. coli* cultures were transformed with a construct expressing an IgE Fab with specificity for a Phl p 5A-unrelated allergen. Fab containing *E. coli* extracts were prepared as described (39).

Complete recombinant IgG1 Abs containing the Fab-derived variable regions were obtained by expression in mammalian cells. cDNAs coding for the heavy and light chain variable region of the IgE Fab were amplified from the IgE Fab-expressing pComb3H plasmid using the VH (5' VH primer: CGG GAT CCG TGC ATT CCG AGG TGC AGC TGC TCG AG; 3' VH primer: CGG GAT CCG ACG TAC GAC TCA CCT GAA GAG ACG GTG ACC AT) and VK (5' VK primer: CGG AAT TCG TGC ATT CCG ACA TCC AGA TGA CCC AGT CTC CAT CTT CC; 3' VK primer: CGG AAT TCA CGT ACG TTC TAC TCA CGT TTG ATT TCC ACC TT) primer pairs, respectively. These primer pairs contained *BsmI* (underlined) and *BsiWI* (italics) restriction sites to allow subcloning of the VH products into plasmid pLNOH2 and of the VK product into plasmid pLNOK (40). The correct insertions of the Fab-derived cDNAs coding for heavy chain and light chain variable regions into the plasmids were confirmed by sequencing of the constructs. Plasmids pLNOH2 and pLNOK allowed transient coexpression of the Fab-derived variable regions as IgG1 heavy chain and κ light chain, respectively, after cotransfection into COS-7 cells (40). Supernatants of transfected COS-7 cells were checked for the presence of human IgG1 Abs with specificity for the recombinant Phl p 5A fragment by ELISA as described (28).

The concentrations of Phl p 5A fragment-reactive IgE Fabs and the complete IgG1 were determined by ELISA in duplicate determinations. Recombinant ELISA plate-bound Phl p 5A fragment was exposed to serial 1:2 dilutions of *E. coli* extracts containing the IgE Fab to culture supernatants with the complete IgG1 Ab and, to establish reference curves, to different concentrations of purified reagents purified via affinity to the allergen (26). Bound Fabs and IgG1 Abs were detected with an AP-conjugated goat anti-human Fab antiserum (Pierce, Rockford, IL). The concentrations of Fab and IgG1 in the extracts were calculated according to the reference curves established with the affinity-purified materials.

Mapping of the binding site of the human IgE Fab using recombinant allergen fragments

A random fragment expression library prepared from sonicated Lol p 5A and Lol p 5B cDNAs in phage λ gt11 was screened with serum IgE from grass pollen-allergic patients to identify IgE-reactive allergen fragments (17). λ gt11 clones expressing IgE-reactive fragments of the major rye grass pollen allergens Lol p 5A and Lol p 5B, and for control purposes, phage containing unrelated cDNAs (clones 29, 31, and 87) or empty wild-type phage (clone 0) were probed for reactivity with the recombinant human IgE Fab. In brief, *E. coli* Y1090 were grown overnight in LB medium containing 0.4% w/v maltose and 50 µg/ml ampicillin, harvested by centrifugation (3000 rpm, 10 min, 4°C), and resuspended in 1:10 vol of 10 mM MgSO₄. *E. coli* were then dissolved in 0.6% w/v agarose and plated as confluent lawn onto LB plates containing 50 mg/L ampicillin. One-microliter aliquots of phage lysates containing >10⁵ PFUs were dotted onto the plates. Plates were incubated at 43°C until plaques became visible and protein synthesis was induced by overlay with 10 mM IPTG-soaked nitrocellulose filters for an additional 3–4 h at 37°C. The phage clone that reacted with the IgE Fab was identified by immunoscreening, and the corresponding phage DNA was isolated (41). The cDNA encoding the Fab-reactive Lol p 5B fragment was PCR amplified from phage DNA using λgt forward: 5'-CGG GAT CCC GGT TTC CAT ATG GGG ATT GGT GGC-3' and λgt11 reverse: 5'-CGC GGA TCC CGT TGA CAC CAG ACC AAC TGG TAA TG-3' primers containing internal *Bam*HI sites (underlined). The PCR product was cut with *Bam*HI, gel-purified, subcloned into plasmid pUC18, and sequenced (41, 42).

Multiple sequence alignment and secondary structure prediction of Phl p 5A-homologous allergens

Search between the deduced amino acid sequence of the rLol p 5B fragment and protein databases was made using the FASTA program of the GCG package (43). Multiple sequence alignment was produced with CLUSTAL W (44) and, if necessary, edited by hand. Protein secondary structure and solvent accessibility predictions were made using the PHD program on the EMBL PredictProtein server (45, 46).

Two-dimensional PAGE and immunoblotting

Two-dimensional PAGE was performed according to Görg et al. (47). The proteins were immediately blotted onto nitrocellulose membranes. The determination of pI and m.w. was achieved by the use of a marker protein test mix 9 (Serva, Heidelberg, Germany) and low range SDS-PAGE standards

³ Abbreviations used in this paper: CD, circular dichroism; AP, alkaline phosphatase; IPTG, isopropyl-β-thiogalactopyranoside; RAST, ¹²⁵I-labeled anti-human IgE Abs.

(Bio-Rad, Richmond, CA), respectively. Nitrocellulose sheets containing timothy grass pollen extract separated by two-dimensional electrophoresis were blocked twice for 5 min and once for 30 min with TBST (50 mM Tris, 150 mM NaCl, pH 7.5, containing 0.1% v/v Tween 20) containing 0.5% w/v BSA at room temperature. The membranes were then probed with serum IgE (diluted 1:10 in TBST/0.5% w/v BSA) from the grass pollen-allergic patient used for the construction of the IgE combinatorial library (26), with the recombinant human IgE Fab (bacterial periplasmic extract diluted 1:1 in TBST/0.5% w/v BSA) and with culture supernatants from hybridomas secreting mouse mAbs with specificity for Phl p 5A (BG6) or Phl p 5B (Bo9) diluted 1:10 in TBST/0.5% w/v BSA overnight at 4°C. Membranes were washed two times for 15 min and once for 30 min with TBST/0.5% w/v BSA. Bound human IgE Abs were detected with RAST (Pharmacia) diluted 1:10 in buffer A (50 mM sodium phosphate buffer, pH 7.4, containing 0.5% w/v BSA, 0.5% v/v Tween 20, and 0.05% w/v NaN₃) overnight. Bound IgE Fabs were visualized with an AP-coupled goat anti-human Fab antiserum (Pierce) diluted 1:5000 in TBST/0.5% w/v BSA, and bound mouse Abs were detected with an AP-conjugated rabbit anti-mouse Ig antiserum (The Jackson Laboratory, Bar Harbor, ME) diluted 1:2000 in TBST/0.5% w/v BSA.

Reactivity of serum IgE and the recombinant monoclonal human IgE Fab with rPhl p 5 isoforms/fragments

Nitrocellulose membranes containing comparable amounts (5 µg/cm preparative SDS-PAGE) of blotted recombinant allergens (rPhl p 5A, rPhl p 5B, the N-terminal Phl p 5A fragment, and a C-terminal Phl p 5B fragment) were blocked in buffer A and incubated with 1:5 in buffer A-diluted sera from grass pollen-allergic patients, and for control purposes, with serum from an allergic patient without grass pollen allergy and serum from a nonatopic individual overnight at 4°C. In addition, blots were incubated with bacterial extracts with and without the recombinant IgE Fab. After washing, bound human IgE Abs were detected with ¹²⁵I-labeled anti-human IgE Abs (Pharmacia) and bound IgE Fabs with an AP-coupled goat anti-human Fab antiserum (Pierce).

Expression and purification of the rPhl p 5A allergen fragment

A recombinant rPhl p 5A fragment equivalent to the Fab-reactive Lol p 5B fragment (Fig. 1C) was obtained by inserting the cDNA coding for amino acid 56-165 of Phl p 5A into plasmid pET17b and expressing the allergen domain in *E. coli* BL 21(DE3). Transformed bacteria were grown in liquid culture to an OD_{600 nm} of 0.6-0.8, and protein synthesis was induced by the addition of IPTG to a final concentration of 0.5 mM. Cells were harvested by centrifugation, suspended in 25 mM imidazole, pH 7.4, 0.1% Triton X-100, lysed for 30 min at room temperature by the addition of lysozyme (20 µg/g cells), and by three to four cycles of freeze-thawing. DNA in the bacterial extract was digested by the addition of DNase I (0.1 mg/g cells) and incubation for 20 min at room temperature. The extract was then centrifuged for 20 min at 18,000 rpm to remove insoluble materials. Addition of ammonium sulfate (40-60% w/v) to the supernatant enriched the rPhl p 5A fragment in the protein precipitate. The pellet was resuspended in 5 ml 10 mM sodium phosphate buffer, pH 7, and desalted via Sephadex PD 10 columns (Pharmacia). The eluate was applied to a Sp-Sephacrose column (Pharmacia) in 10 mM sodium phosphate buffer, pH 7, and eluted with a 0-0.5 M NaCl gradient. Fractions containing >80% pure rPhl p 5A fragment were dialysed against 10 mM Tris, pH 10, and loaded onto a DEAE cellulose-Sepharose column (Pharmacia). rPhl p 5A fragment was purified to homogeneity by elution with a 0-0.5 M NaCl gradient. Fractions containing a homogenous rPhl p 5A fragment preparation were lyophilized, resuspended in Aqua bidest. and desalted using Sephadex PD 10 columns. The protein concentration was determined using the protein extinction coefficient according to (48). Mass spectroscopic analysis was performed on a triple quadrupole mass spectrometer (API III; PE-SCIEX, Ontario, Canada).

CD analysis

CD spectra were recorded on a Jasco J-710 spectropolarimeter fitted with a thermostatted cell holder and interfaced with a Neslab RTE-110 water bath. The instrument was calibrated with a 0.1% aqueous solution of d-10-camphor-sulfonic acid. Results were expressed as mean residue ellipticity (Θ) ($\times 10^{-3}$ deg $\times$ cm² $\times$ dmol⁻¹), at a given wavelength. Far-UV CD spectra were recorded in quartz cuvettes (Hellma; Mullheim, Baden, Germany). Spectra were recorded with 0.1-nm resolution and resulted from averaging 10 scans. 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 stability followed by CD

Thermal denaturation of the rPhl p 5A fragment was monitored by recording the ellipticity at 222 nm while heating at 50°C/h with a computer-controlled circulating water bath. 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). The fraction of folded protein was calculated as $F = 1 - U$, where $U = (\Theta_{222} - \Theta_N)/(\Theta_U - \Theta_N)$, Θ_N is the ellipticity of the protein in the native state, and Θ_U is that of the denatured protein. For rPhl p 5A fragment, Θ_U was assumed equal to Θ_{222} at 90°C and Θ_{222} at 20°C.

IgE binding experiments

Serum IgE with specificity for complete rPhl p 5A and the rPhl p 5A fragment was quantified by RAST-based experiments. To ensure Ag excess, 8-µg aliquots of each protein were immobilized to nitrocellulose under nondenaturing conditions by dot blotting. Nitrocellulose-bound proteins were exposed in duplicate to two different serum dilutions (1:4, 1:8) prepared in buffer A as described for two-dimensional blots. Bound IgE Abs were detected with RAST (Pharmacia) and quantified by gamma counting in a gamma counter (1277 Gammamaster; LKB, Wallac, Gaithersburg, MD). Results represent means of duplicate determinations with variations of <10%, and the percentage of Phl p 5A-specific IgE bound by the rPhl p 5A fragment is displayed (Table I).

ELISA competition experiments

The influence of the human IgE Fab, a corresponding bivalent IgG1 Ab, and a polyclonal rabbit anti-rPhl p 5A antiserum on the binding of patients IgE Abs to the rPhl p 5A fragment was investigated by ELISA competition experiments. Purified rPhl p 5A fragment was coated to ELISA plates (Greiner, Kremsmünster, Austria) in 0.1 M sodium bicarbonate pH 9.6 (1 µg/ml) overnight at 4°C. Plates were washed twice with TBST containing 0.5% w/v BSA and blocked with TBST containing 3% w/v BSA at 37°C for 3 h. ELISA plate-bound rPhl p 5A fragment was preincubated overnight at 4°C with in TBST-diluted bacterial lysate containing 1.5 µg/ml of the recombinant human Phl p 5-specific IgE Fab or, for control purposes, with bacterial lysates containing a recombinant IgE Fab specific for a Phl p 5A-unrelated allergen. In similar experiments, ELISA plate-bound rPhl p 5A fragment was preincubated with culture medium without (control) or with 1.5 µg/ml of a recombinant bivalent Phl p 5A-specific IgG1, a rabbit anti-rPhl p 5A antiserum or, for control purposes, with the preimmune serum of the rabbit, diluted 1:100 in TBST. After preincubations, plates were washed five times in TBST containing 0.5% w/v BSA and 100-µl aliquots of patients' sera (diluted 1:5 in TBST containing 0.5% w/v BSA) were added to the wells overnight at 4°C. Plates were then washed five times with TBST/0.5% w/v BSA and incubated with an AP-coupled mouse anti-human IgE mAb (PharMingen, San Diego, CA) diluted 1:1000 in TBST/0.5% w/v BSA at 37°C for 2 h. Plates were washed five times and bound anti-human IgE Abs were visualized by color reaction using AP substrate (Sigma, St. Louis, MO). Optical densities corresponding to the amounts of bound IgE Abs were measured in an ELISA reader at a 405-nm wavelength (Dynatech, Denkendorf, Germany). To avoid plate-to-plate variabilities, experiments were performed for the serum of each in duplicate on the same plate, and results were recorded at the same time point. The percentage of inhibition of IgE binding was calculated from the mean OD values measured with or without addition of competitor according to the following formula: % inhibition = $100 - OD_{inhibitor} \times 100/OD_{control}$ (Table II).

Basophil histamine release assays

Heparinized blood samples were obtained by venipuncture from six grass pollen-allergic patients and, for control purposes, from a nonatopic individual after informed consent was given. Granulocytes were isolated by dextran sedimentation and histamine release experiments were performed as described (49). In brief, enriched basophils were exposed to different concentrations of purified rPhl p 5A, the recombinant Phl p 5A fragment, rPhl p 6, or a monoclonal anti-human IgE Ab for 30 min at 37°C. Then, cells were centrifuged at 4°C and the cell-free supernatants were recovered. Histamine released in the cell-free supernatants was determined by RIA (Immunotech, Marseille, France) and is expressed as the percentage of total histamine determined after cell lysis (49).

Skin prick testing

After informed consent was obtained from three grass pollen-allergic patients and one nonallergic individual, skin prick tests were performed on

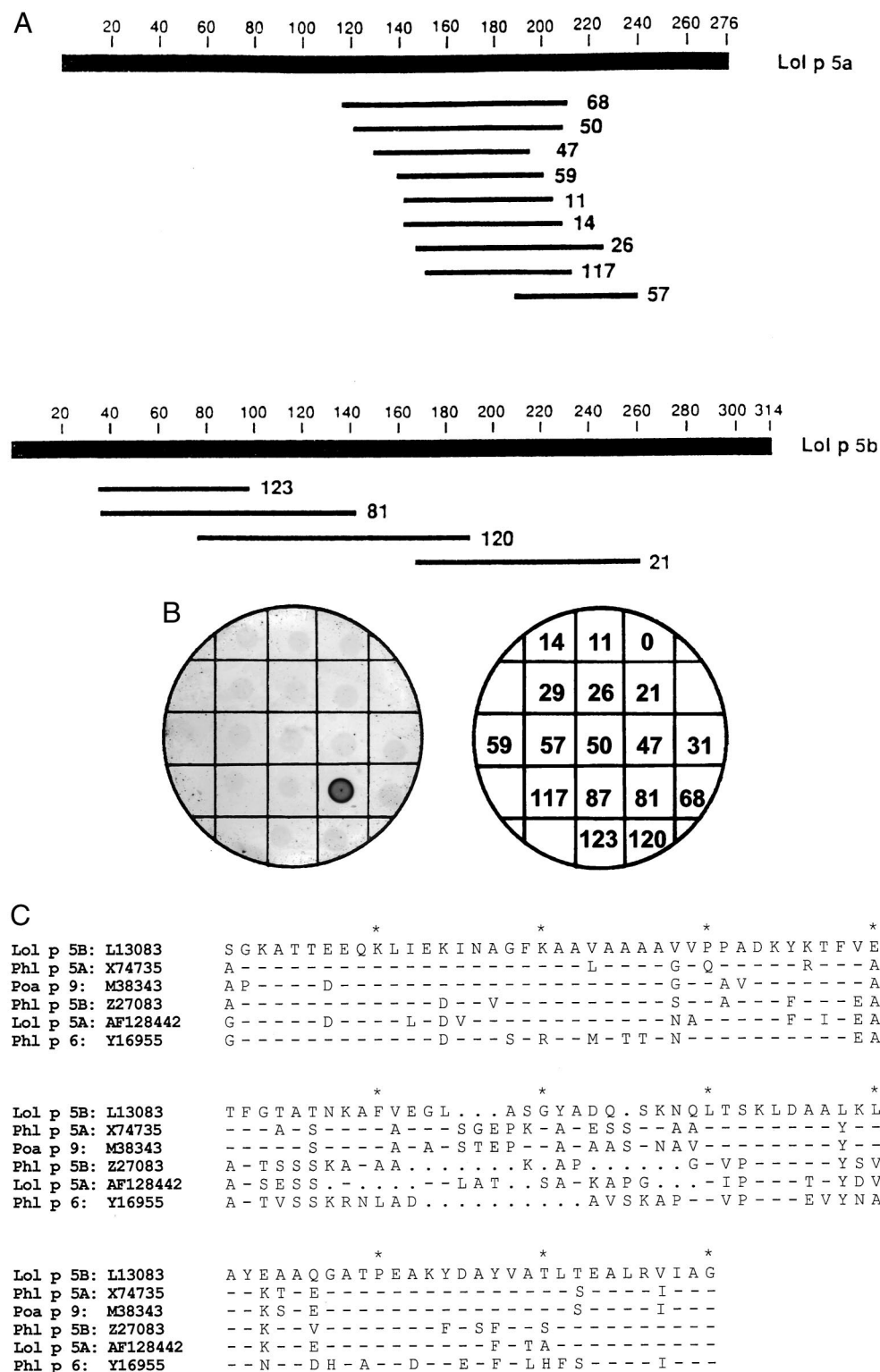


FIGURE 1. A, Localization of clones representing IgE-reactive fragments of Lol p 5A and Lol p 5B (clones 11–123). B, Lol p 5A- and Lol p 5B-derived clones, Lol p 5-unrelated (clones 29, 31, 87) and a phage clone without insert (clone 0) were immobilized to a nitrocellulose filter in the order given and probed with the recombinant human IgE Fab. C, Alignment of the amino acid sequence of the Fab-reactive domain from Lol p 5B (clone 81) with the sequences of homologous allergens from timothy grass (*Phleum pratense*) and Kentucky Bluegrass (*Poa pratensis*). The allergen designations and gene bank accession numbers are displayed on the left side. Dashes indicate identical amino acids and dots represent gaps.

the forearms of the patients as described (8). Individuals were pricked with 20-μl aliquots of solutions containing different concentrations (125, 25, 5, 1, and 0.2 μg/ml) of purified rPhl p 5A fragment and rPhl p 5A diluted in 0.9% sodium chloride. For control purposes, histamine, NaCl, timothy grass, and birch pollen extract (ALK, Horsholm, Denmark) were used. The

skin reactions were recorded 20 min after testing by photography and by transferring the ballpoint pen-surrounded wheal area with a scotch tape to paper. The mean wheal diameters displayed in Table III were determined as follows: $DM = (D1 + D2)/2$. D1 and D2 represent the maximal longitudinal and transversal diameters, respectively, in millimeters.

Table I. *IgE binding capacity of complete rPhl p 5A and the rPhl p 5A fragment*

Patient No.	cpm rPhl p 5A	cpm rPhl p 5A fragment	%
1	607.4	111.6	18.4
2	1933.2	284.0	14.7
3	435.2	169.5	38.9
4	1635.5	347.2	21.2
5	2550.4	242.5	9.5
6	790.0	318.6	40.3
7	4240.9	1379.3	32.5
8	724.9	108.0	14.9
9	7399.1	1616.9	21.9
10	1044.9	109.6	10.5
11	2712.4	440.5	16.2
12	604.7	268.5	44.4
13	939.6	129.0	13.7
14	1287.8	487.0	37.8
15	1485.8	492.0	33.1
16	455.0	172.9	38.0
17	3710.7	785.1	21.2
18	1734.0	136.0	7.8
19	1018.3	197.9	19.4
20	1507.8	443.5	29.4
21	660.0	157.8	23.9
22	3583.3	1083.5	30.2
23	3642.5	948.6	26.1
24	1144.9	584.9	51.1
25	499.7	184.7	37.0
26	7105.0	561.0	7.9
Mean	1734.3	378.4	24.0
Nonallergics			
27	19.8	24.2	
28	14.6	22.6	

Results

Mapping of the binding site of a recombinant human IgE Fab specific for group 5 grass pollen allergens

IgE-reactive phages were isolated from an available cDNA library expressing recombinant Lol p 5 fragments as described (17). These phage clones (Fig. 1A, clones 11–123) and for control purposes, phage clones containing cDNAs unrelated to Lol p 5 (Fig. 1B, clones 29, 31, and 87) or wild-type phage (clone 0), were tested for reactivity with the recombinant human IgE Fab (Fig. 1, A and B). One of the Lol p 5-derived fragment clones (Fig. 1B, clone 81) reacted with the IgE Fab. Clone 81 represented an N-terminal fragment comprising amino acid 36–141 of the mature Lol p 5B (Fig. 1C, top line). Notably, two other Lol p 5B-derived clones (clone 120: FGTATNKAFFVEGLASGYADQSKNQLTSKLDAAALKLA YEAAQGATPEAKYDAYVATLLEALRVIAAGTLEHVHAVKPA AEEVKVGAIPAAEVQLIDKVDAAAYRTAATAANAAPAND, aa 77–189; clone 123: SGKATTEEQKLEKINAGFKAAVAAAAVV PPADKYKTFVETFGTATNKAFFVE GLASGYADQS, aa 36–97), which together covered the entire sequence defined by clone 81, did not react with the IgE Fab. This finding would indicate that the Fab recognizes a conformational epitope that requires most of the clone 81-defined sequence. The clone 81-defined Lol p 5B fragment corresponded to an N-terminal fragment of Phl p 5 (timothy grass-*Phleum pratense*), which is highly homologous to group 5 allergens from velvet grass (*Holcus lanatus*), Kentucky Bluegrass (*Poa pratensis*), Canary grass (*Phaseolus aquaticus*), wheat (*Triticum vulgare*), and group 6 allergens from timothy grass. The secondary structure prediction of the clone 81-defined Lol p 5B fragment indicated that it consists almost exclusively of an α helical secondary structure, which is composed by four α -helices (data not shown). Within the clone 81-defined fragment, Phl p 5A,

Table II. *Inhibition of patients' IgE binding to rPhl p 5 fragment*

Patient No.	% Inhibition of IgE binding		
	IgE-Fab α Phl p 5	IgG1 gaPhl p 5	Rabbit garPhl p 5
1	3.7	24.0	73.9
2	12.8	25.6	83.5
3	4.3	14.6	87.2
4	10.4	36.5	85.3
5	20.6	29.4	79.7
6	17.3	36.8	87.6
7	15.1	16.4	79.8
8	4.4	0.4	88.7
Mean	11.1	22.9	83.2

Lol p 5B, and Poa p 5 isoforms exhibited the highest degree of sequence homology, whereas Phl p 5B, Lol p 5A, and Phl p 6 lacked several amino acids (Fig. 1C). Therefore, we used the clone 81-defined Lol p 5B sequence to produce the corresponding domain of the timothy grass pollen allergen Phl p 5A as recombinant protein (Fig. 1C, second line).

The recombinant human IgE Fab reacts with an N-terminal fragment of Phl p 5A

The cDNA coding for the Phl p 5A fragment corresponding to the clone 81-defined Lol p 5B fragment (Fig. 1C, second line) was PCR-amplified from the Phl p 5A-encoding cDNA (27) and inserted into plasmid pET17b. The N-terminal rPhl p 5A fragment was produced in the cytoplasm of *E. coli* as soluble protein and could be purified to homogeneity. In SDS-PAGE purified rPhl p 5A fragment migrated at 11 kDa, and the matrix-assisted laser desorption and ionization time-of-flight analysis of the purified rPhl p 5A fragment revealed the presence of a major peak at 11.2 kDa (data not shown).

Next we tested sera from grass pollen-allergic individuals and the recombinant IgE Fab for reactivity to nitrocellulose-blotted rPhl p 5A, rPhl p 5B, the N-terminal rPhl p 5A fragment comprising the clone 81-defined portion (Fig. 1C), and a 136-aa-long C-terminal recombinant fragment of rPhl p 5B. Sera from all seven grass pollen-allergic patients displayed IgE reactivity to rPhl p 5A and B (Fig. 2, A and B, lanes 1–7) as well as to the recombinant

Table III. *Immediate type skin reactions induced with rPhl p 5A and the rPhl p 5A fragment*

	Grass Pollen Allergic Patients			Nonallergic Individual
	A	B	C	D
rPhl p 5 fragment (μ g/ml)				
125	9.5	12.5	ND	0
25	5.5	12.0	12.5	0
5	5.0	11.0	5.5	0
1	4.5	7.0	5.0	0
0.2	2.5	8.0	4.5	0
rPhl p 5 (μ g/ml)				
125	9.5	9.5	ND	0
25	5.5	10.5	12.0	0
5	5.0	9.5	6.5	0
1	4.5	5.0	6.5	0
0.2	2.5	4.0	4.5	0
Timothy grass	10.0	10.5	12.0	0
Birch	0	3.0	7.0	0
Histamine	7.0	8.0	4.5	5
NaCl	0	0	0	0

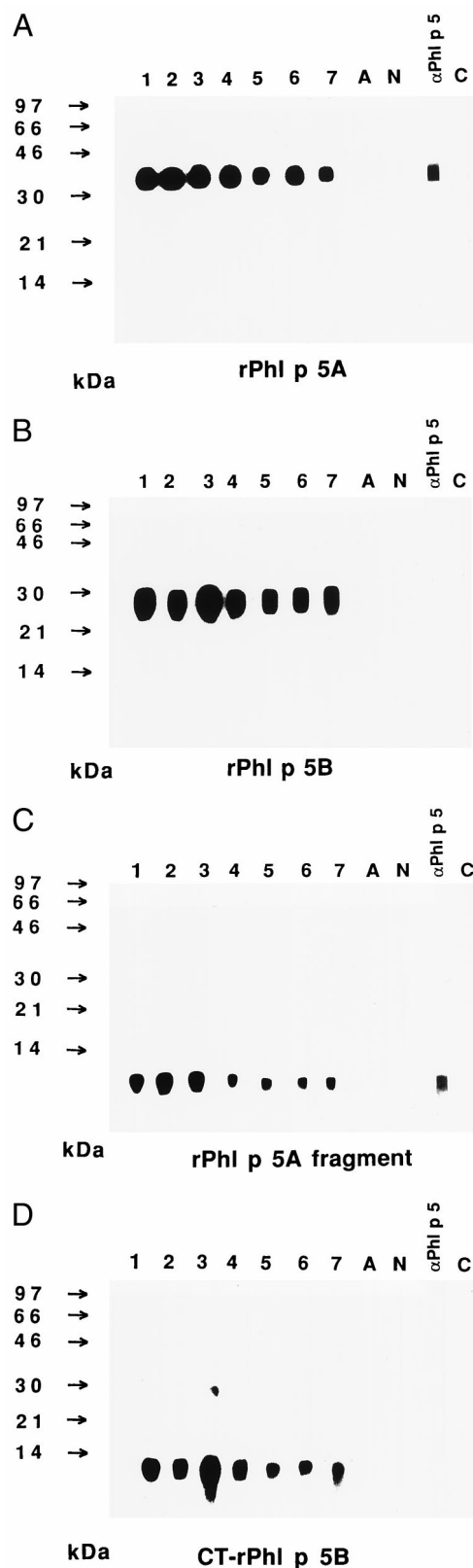


FIGURE 2. Serum IgE reactivity and binding of the recombinant human IgE Fab with isoforms and recombinant fragments of the major timothy grass pollen allergen, Phl p 5. Nitrocellulose-blotted complete rPhl p 5A (A) and rPhl p 5B (B) as well as of a N-terminal rPhl p 5A (C) and a C-terminal rPhl p 5B fragment (D) were exposed to serum IgE from 7 grass pollen-allergic patients (lanes 1–7), from an allergic individual without grass pollen allergy (lane A), from a nonatopic individual (lane N), the bacterial extracts with (lane α Phl p 5) or without (lane C) recombinant human IgE Fab. Bound IgE Abs were detected with ^{125} I-labeled anti-

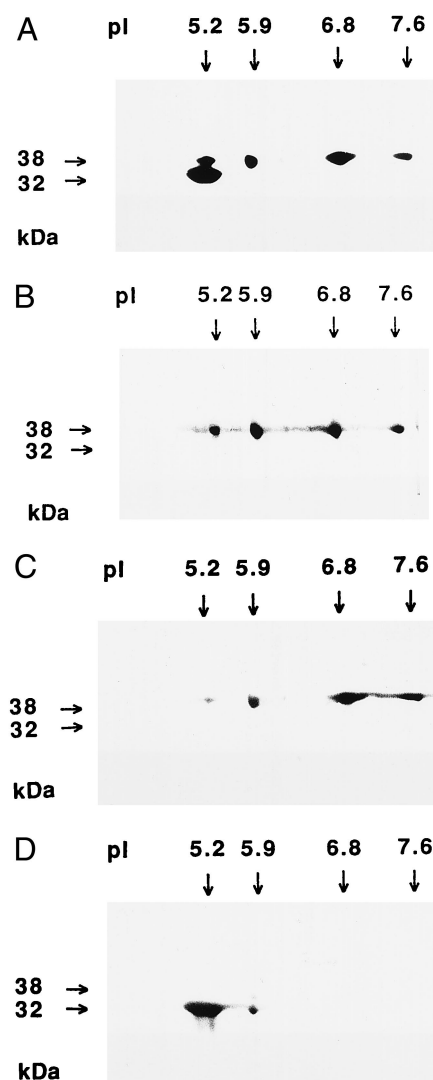


FIGURE 3. The recombinant human IgE Fab reacts specifically with natural Phl p 5A but not Phl p 5B isoforms in two-dimensionally separated timothy grass pollen extract. Two-dimensionally separated timothy grass pollen extract was blotted onto nitrocellulose and probed with serum IgE from a Phl p 5-allergic patient (A) with the recombinant human IgE Fab (clone 5A) (B) and with mouse mAbs with specificity for rPhl p 5A (C) or rPhl p 5B (D). Molecular masses and isoelectric points are indicated in the left margin and at the top, respectively.

allergen fragments (Fig. 2, C and D, lanes 1–7). The recombinant human IgE Fab reacted with complete rPhl p 5A and the N-terminal recombinant rPhl p 5A fragment, but did not bind to rPhl p 5B or the C-terminal rPhl p 5B fragment (Fig. 2, A–D, lane α Phl p 5). Bacterial extracts without the recombinant human IgE Fab did not react with blotted rPhl p 5 isoforms nor with their fragments (Fig. 2, A–D, lane C). Sera from a birch pollen-allergic patient without grass pollen allergy (lane A) and a nonatopic individual (lane N) contained no IgE Abs to any of the blotted proteins (Fig. 2, A–D). Our finding, that the recombinant human IgE Fab reacts specifically with an epitope on the N-terminal fragment

human IgE Abs and the human IgE Fab with an AP-conjugated goat anti-human Fab antiserum. Molecular masses are displayed in kilodaltons in the left margin.

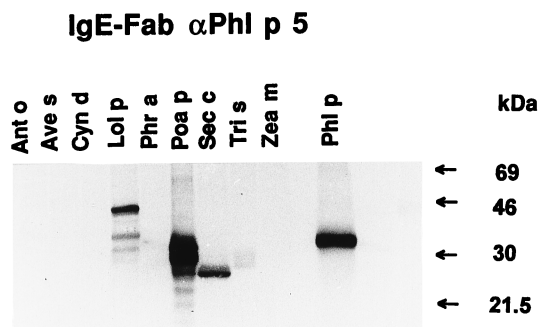


FIGURE 4. The recombinant human IgE-Fab cross-reacts with natural group 5 allergens from different grass and corn species. Nitrocellulose-blotted grass pollen extracts (Ant o: sweet vernal grass; Ave s: oat; Cyn d: Bermuda grass; Lol p: rye grass; Phr c: common reed; Poa p: Kentucky Blue grass; Sec c: rye; Tri s: wheat; Zea m: maize; Phl p: Timothy grass) were exposed to the rFab. Bound rIgE Fabs were detected with an AP-coupled goat anti-human Fab antiserum. Molecular masses are indicated in kilodaltons in the right margin.

of rPhl p 5A, is confirmed by experiments performed with two-dimensional separated natural timothy grass pollen extract (Fig. 3, A–D). Serum IgE from a grass pollen-allergic patient reacted with natural Phl p 5A (38 kDa) and Phl p 5B (32 kDa) pI variants (Fig. 3A). The recombinant human IgE Fab (Fig. 3B) as well as a Phl p 5A-specific mouse moAb BG6 (Fig. 3C) reacted exclusively with the natural Phl p 5A pI variants at 38 kDa. The pI variants at 32 kDa were identified as being Phl p 5B-derived by a Phl p 5B-specific mouse moAb Bo9 (Fig. 3D).

The recombinant human IgE Fab cross-reacts with group 5 allergens from various grass and corn species

To investigate whether the recombinant human IgE Fab cross-reacts with natural group 5 allergens from different grass and corn species, nitrocellulose-blotted natural pollen extracts from several grass and corn species were tested (Fig. 4). The IgE Fab reacted strongly with group 5 allergens in timothy grass, Kentucky Blue-grass, rye grass, and rye, and exhibited weaker reactivity to 30-kDa moieties in common reed and wheat (Fig. 4). No reactivity was observed with pollen extracts from monocots reportedly lacking or containing low levels of group 5 allergens (sweet vernal grass, oat, Bermuda grass, and maize) (32). Thus the recombinant human IgE Fab detected a cross-reactive epitope in group 5 allergens from various grass and corn species.

CD analysis of purified rPhl p 5A, the rPhl p 5A fragment, and rPhl p 6

The far UV CD spectrum of rPhl p 5A taken at 20°C (Fig. 5A) is characterized by two broad minima at 207 and 221 nm. Similarly, rPhl p 5A fragment shows two broad minima at 208 and 222 nm and a maximum at 191 nm (Fig. 5B). The far UV CD spectra of rPhl p 5A, the rPhl p 5A fragment, and rPhl p 6 recorded at 20°C, indicate a common α helical protein fold (Fig. 5D). This finding is in agreement with the PHD secondary structure prediction performed on the multiple sequence alignment of group 5 allergens and Phl p 6 (data not shown).

Thermal denaturation of rPhl p 5A was monitored by CD spectroscopy and showed a biphasic unfolding curve (data not shown), whereas the unfolding transition of the rPhl p 5A fragment was monophasic and highly cooperative with a melting point of ~62°C

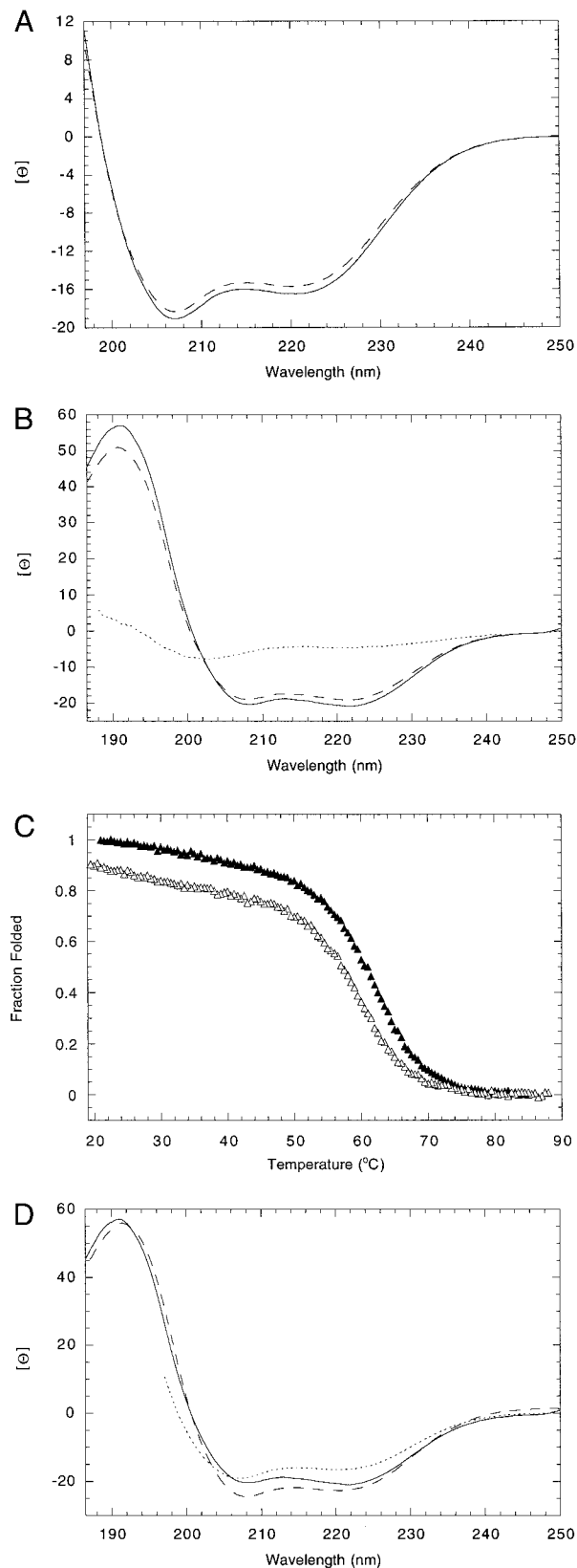


FIGURE 5. Far-ultraviolet CD analysis. A, Purified rPhl p 5A at 20°C (continuous line) and at 20°C after cooling from 90°C (dashed line). B, Purified rPhl p 5A fragment at 20°C (continuous line), at 90°C (dotted line) and at 20°C after cooling from 90°C (dashed line). Spectra are expressed as mean residue ellipticity ([Θ]). C, Thermal denaturation (▲) and cooling (△) of rPhl p 5A fragment expressed as fraction of folded protein. D, Comparison of rPhl p 5A (dashed line), rPhl p 5A fragment (continuous line), and rPhl p 6 (dotted line) at 20°C.

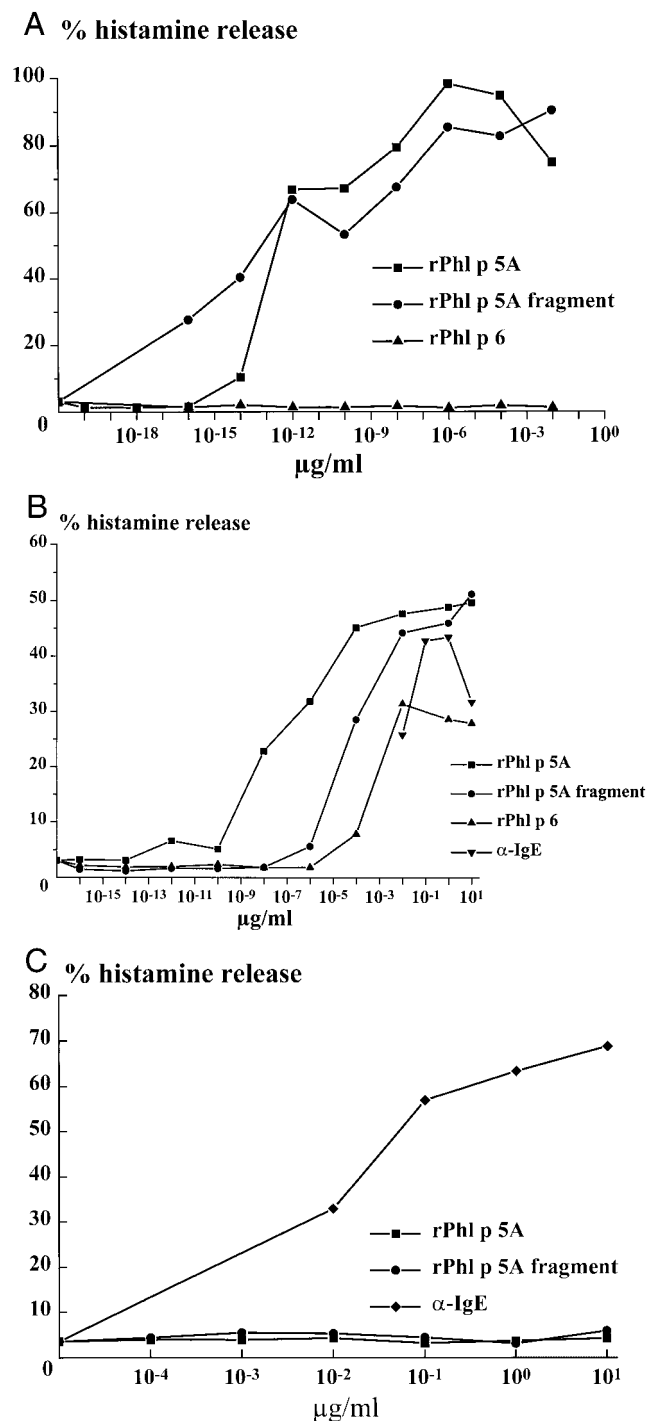


FIGURE 6. Induction of histamine release with complete rPhl p 5A, the rPhl p 5A fragment and rPhl p 6. Granulocytes from two grass pollen-allergic patients (A and B) and a nonatopic individual (C) were incubated with various concentrations of the allergens or a monoclonal anti-human IgE Ab (x-axis). Histamine release is expressed as percentage of total histamine on the y-axis.

(Fig. 5C). The characteristics of the CD spectra recorded for the thermal denaturation and cooling of the rPhl p 5A fragment and rPhl p 6 were very similar (data not shown). Cooling of rPhl p 5A and the rPhl p 5A fragment, which had been subjected to thermal denaturation, yielded an almost complete refolding of the molecules (Fig. 5, A–C).

The IgE Fab-defined rPhl p 5A fragment represents a frequently recognized allergen domain containing several IgE epitopes

When we analyzed sera from 58 grass pollen-allergic individuals and from three nonatopic persons for the presence of rPhl p 5A and rPhl p 5A fragment-specific IgE, we found that 44 sera (76%) displayed IgE reactivity with rPhl p 5A and the rPhl p 5A fragment (data not shown). The IgE binding capacity of complete rPhl p 5A and the rPhl p 5A fragment was analyzed in detail for 26 Phl p 5A-reactive sera in a quantitative RAST-based assay using two different serum dilutions (1:4 and 1:8). Using the 1:8 serum dilutions, we found that the rPhl p 5A fragment bound between 7.8 and 51.1% (mean: 24.0%) of the Phl p 5A-specific IgE (Table I). Similar results were obtained for the 1:4 serum dilutions, indicating that 5–42.1% (mean: 20.6%) of Phl p 5A-specific IgE was directed against the fragment (data not shown).

The percentage of rPhl p 5A-specific Abs bound by the rPhl p 5A domain corresponded to its size but suggested that it contains binding sites for IgE Abs with other specificities in addition to the Fab-defined epitope. This assumption was supported by competition experiments. The IgE Fab and a complete bivalent IgG1 Ab generated by expressing the variable region of the Fab fused to a human IgG1 constant region were used in >1000-fold excess to serum IgE (1.5 μg/ml). Preincubation of the rPhl p 5A fragment with the IgE Fab and the complete recombinant bivalent IgG1 derived from the Fab inhibited IgE binding of eight sera to a low degree (Fab: 3.7–20.6% inhibition; mean, 11.1%; IgG1: 0.4–36.8%; mean: 22.9%) (Table II). By contrast we found that polyclonal anti-Phl p 5A IgG Abs profoundly (73.9–88.7%; mean, 83.2%) inhibited IgE binding of the same eight sera to the rPhl p 5 fragment (Table II).

Complete rPhl p 5A and the recombinant Phl p 5A fragment strongly induce histamine release from basophils of sensitized patients

Exposure of basophils from four grass pollen-allergic patients to purified rPhl p 5A and the rPhl p 5A fragment resulted in maximal histamine release already at very low allergen concentrations (10⁻⁴ μg/ml). Therefore, both proteins were further diluted and exposed to basophils of a patient containing IgE Abs against complete rPhl p 5A and the rPhl p 5A fragment, but not to rPhl p 6 (Fig. 6A). In this patient, the rPhl p 5A fragment and rPhl p 5A started to induce histamine release at concentrations between 10⁻¹⁶ and 10⁻¹⁴ μg/ml and between 10⁻¹⁴ and 10⁻¹² μg/ml, respectively (Fig. 6A). The fact that the rPhl p 5A fragment more potently induced histamine than complete rPhl p 5A was of note because quantitative IgE binding tests showed that in this patient only 19.5% of Phl p 5A-specific IgE were directed against the Phl p 5A fragment. rPhl p 6, a structurally related allergen, did not induce histamine release even at 10⁻² μg/ml (Fig. 6A).

Next we used complete rPhl p 5A, the rPhl p 5A fragment, and rPhl p 6 to compare the IgE binding capacity of allergens with similar structural fold but different IgE binding capacity with their ability to induce histamine release. Parallel RAST measurements and basophil histamine release experiments were performed in the same patient (Fig. 6B). We found that complete rPhl p 5A bound ~5-fold more IgE Abs than the rPhl p 5A fragment and was ~1000-fold more active in inducing basophil histamine release (Fig. 6B; data not shown). rPhl p 5 fragment bound 2.7-fold more IgE than rPhl p 6 and appeared 100-fold more active in the basophil histamine release assay (Fig. 6B; data not shown). Although complete rPhl p 5A as well as the rPhl p 5A fragment induced a comparable maximal histamine

release of ~53%, a maximal histamine release of no more than 32% was achieved by rPhl p 6 (Fig. 6B). Anti-human IgE Abs were less active than rPhl p 6 but induced a higher maximal histamine release in this patient (Fig. 6B). Neither complete rPhl p 5A nor the rPhl p 5A fragment induced histamine release from basophils of a nonatopic person up to a concentration of 10 $\mu\text{g/ml}$, whereas the monoclonal anti-human IgE Ab yielded a dose-dependent histamine release (Fig. 6C).

rPhl p 5A and the rPhl p 5A fragment induce immediate type skin reactions in grass pollen-allergic patients

To further investigate the *in vivo* allergenic activity of rPhl p 5A and the rPhl p 5A fragment, skin prick tests were performed (Table III). Three grass pollen-allergic patients and one nonallergic individual were skin prick tested with five concentrations of purified rPhl p 5A fragment and with the complete rPhl p 5A allergen. In all grass pollen-allergic patients, but not in the nonallergic individual, all concentrations of the rPhl p 5A fragment, as well as of the complete allergen, induced immediate type skin reactions (Table III). The recombinant Phl p 5A fragment elicited skin reactions that were similar (A) or sometimes even stronger (B, C) than those elicited by complete rPhl p 5A over the whole range of concentrations in all three grass pollen-allergic patients. Timothy grass and birch pollen extract elicited immediate type skin reactions in the sensitized patients but not in the nonatopic individual. Histamine elicited a wheal reaction in all three atopic and in the nonallergic person.

Discussion

We report the characterization of a highly allergenic domain of the major timothy grass pollen allergen, Phl p 5A, which contains the binding site for a human monoclonal IgE Ab. The corresponding recombinant human IgE Fab was isolated from a combinatorial library constructed from lymphocytes of a grass pollen-allergic patient (26). The rPhl p 5A fragment represents an alanine-rich protein domain that occurs as a highly conserved segment in group 5 allergens from various grass and corn species. The fact that certain group 5 isoallergens (e.g., rPhl p 5B (40) and Lol p 5A) (50) as well as Phl p 6, a structurally related low m.w. timothy grass pollen allergen (51, 25), lacked several amino acids present in the Phl p 5A domain may explain the ability of the IgE Fab to discriminate Phl p 5A from Phl p 5B, Lol p 5A, and Phl p 6. However, the IgE Fab cross-reacted with group 5 allergens from grass and corn species reportedly containing group 5 allergens, indicating that the defined epitope-containing domain represents a highly cross-reactive structure.

When we analyzed the rPhl p 5A fragment by CD, we found that the allergen domain consisted exclusively of α helical secondary structure, in agreement with the secondary structure prediction and preliminary nuclear magnetic resonance analysis of the isolated protein module. In fact, nuclear magnetic resonance analysis of the rPhl p 5A domain indicates that it consists of four α helical bundles that are connected by short loops on top (A. Pastore and R. Valenta, unpublished data). Although the secondary structure of the rPhl p 5A domain differs from that of several other allergens analyzed to date (reviewed in 5, 6), the protein module as well as the complete rPhl p 5A allergen showed a remarkable tendency to refold after thermal denaturation. The ability to refold after denaturation was also reported for several other important allergens from plants and fish (12,

13, 15, 22–25). Therefore, stability and refolding capacity may be relevant features of potent allergens as they may facilitate the survival of these proteins in the environment and in the target organs of atopy and thus allow them to elicit and maintain allergic immune responses. This assumption would be also supported by the finding that food allergens exhibit high resistance against low pH and digestive enzymes (52).

The rPhl p 5A fragment represents an important allergenic protein domain because it was recognized by IgE of almost 80% of grass pollen-allergic patients. Several experiments suggest that the rPhl p 5A domain contains several IgE epitopes and thus exhibits high allergenic activity. Quantitative IgE binding experiments showed that the rPhl p 5A fragment bound between 7.8 and 51.1% (mean: 24%) of Phl p 5A-specific IgE Abs. In addition to the IgE Fab-defined epitope, several other IgE epitopes must be present on the rPhl p 5A fragment because neither the IgE Fab nor a complete recombinant bivalent IgG1 Ab, obtained by genetic engineering of the Fab-derived variable regions to IgG1 constant domains, inhibited significantly IgE anti-rPhl p 5A fragment reactivity. Both competitors were used in excess to IgE but achieved only moderate inhibition (Fab: 11.1%; IgG1: 22.9%) of IgE binding to the fragment, whereas polyclonal IgG inhibited IgE binding almost completely (Table II). The presence of several other cross-reactive IgE epitopes on the domain also explains why the deletion of the corresponding domain from the Phl p 5B isoform, which was not recognized by the Fab, strongly reduced the IgE binding capacity and allergenic activity of Phl p 5B (53).

Although the rPhl p 5A fragment comprised only approximately one-third of the complete rPhl p 5A allergen we found that the recombinant allergen domain exhibited high allergenic activity when tested for its capacity to elicit histamine release from basophils as well as immediate type skin reactions. The rPhl p 5A fragment accounted only for ~20% of the complete allergens IgE binding capacity but elicited comparable immediate skin reactions in all three grass pollen-allergic patients. We chose the basophil histamine release assay to evaluate the allergenic activity of the rPhl p 5A fragment in more detail for two reasons 1) serum-derived IgG Abs or factors are less likely to influence the release of histamine from isolated granulocytes than skin reactions; and 2) for the *in vitro* histamine release tests, allergens can be diluted to extremely low concentrations in protein-containing buffers avoiding loss of allergen due to adsorption to the tubes.

The rPhl p 5A fragment induced histamine release already at very low concentrations (10^{-12} $\mu\text{g/ml}$). Interestingly, we found that the fragment induced in one donor (Fig. 6A) histamine release at a lower concentration than the complete allergen. The latter finding is in agreement with a previous study reporting that proteolytic cleavage of Phl p 5 in nasal secretions increased the allergenic activity of the molecule (40).

As yet we cannot provide a definitive explanation for the high allergenic potency of the rPhl p 5A allergen domain, but the possibility that aggregation of the fragment might have been responsible for the strong biological activity seems unlikely because neither CD measurements nor native PAGE and gel filtration provided evidence for the formation of aggregates. Alternatively we suggest that a favorable arrangement/geometry of IgE epitopes on the rPhl p 5A domain may be responsible for the efficient cell activation. In this context it has been reported that cross-linking of only a small proportion of effector cell-bound IgE can yield maximal responses (54). Furthermore, it has been shown that upon cross-linking the high affinity IgE receptor becomes associated with rafts, representing membrane

microdomains (55–57). Thus it may be speculated that optimal structural arrangement of IgE epitopes contributes to highly efficient cross-linking of FcεRI and influences membrane clustering and signal transduction.

In conclusion, we suggest the rPhl p 5A domain together with its corresponding IgE Fab as paradigmatic tools for the analysis of structural requirements for highly efficient effector cell activation. This example may perhaps also teach us general strategies to inactivate the allergenic activity of major allergens and retain their immunogenicity for specific immunotherapy.

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