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Christoph Madritsch,* Elisabeth Gadermaier,* Uwe W. Roder,† Christian Lupinek,* Rudolf Valenta,* and Sabine Flicker*

The timothy grass pollen allergen Phl p 1 belongs to the group 1 of highly cross-reactive grass pollen allergens with a molecular mass of ~25–30 kDa. Group 1 allergens are recognized by >95% of grass pollen allergic patients. We investigated the IgE recognition of Phl p 1 using allergen-specific IgE-derived single-chain variable Ab fragments (IgE-ScFvs) isolated from a combinatorial library constructed from PBMCs of a grass–pollen–allergic patient. IgE-ScFvs reacted with recombinant Phl p 1 and natural group 1 grass pollen allergens. Using synthetic Phl p 1–derived peptides, the binding sites of two ScFvs were mapped to the N terminus of the allergen. In surface plasmon resonance experiments they showed comparable high-affinity binding to Phl p 1 as a complete human IgE-derived Ab recognizing the allergens’ C terminus. In a set of surface plasmon resonance experiments simultaneous allergen recognition of all three binders was demonstrated. Even in the presence of the three binders, allergic patients’ polyclonal IgE reacted with Phl p 1, indicating high-density IgE recognition of the Phl p 1 allergen. Our results show that multiple IgE Abs can bind with high density to Phl p 1, which may explain the high allergic activity and sensitizing capacity of this allergen. The Journal of Immunology, 2015, 194: 2069–2078.

The number of patients suffering from allergic symptoms such as hay fever, asthma, allergic skin diseases, food allergy, or severe anaphylactic reactions has increased to nearly a third of the population in industrialized countries (1). A major hallmark of allergic diseases is the formation of specific IgE Abs against per se harmless environmental Ags called allergens. Owing to heavy and prolonged release of pollen, grasses represent one of the most potent and frequent allergens worldwide (2, 3). Grass pollen contains several allergens with high allergic activity and remarkably high stability to pH and temperature variations. Among those, group 1 allergens are the most prevalent and important (4, 5). In fact, one of the first pollen allergens ever purified was the group 1 allergen from rye grass (6). This allergen source is also suspected to be responsible for the initial hay fever epidemic in northern Europe against per se harmless environmental Ags called allergens. Owing to heavy and prolonged release of pollen, grasses represent one of the most potent and frequent allergens worldwide (2, 3). Grass pollen contains several allergens with high allergic activity and remarkably high stability to pH and temperature variations. Among those, group 1 allergens are the most prevalent and important (4, 5). In fact, one of the first pollen allergens ever purified was the group 1 allergen from rye grass (6). This allergen source is also suspected to be responsible for the initial hay fever epidemic in northern Europe.

The timothy grass pollen allergen Phl p 1 belongs to the group 1 of highly cross-reactive grass pollen allergens with a molecular mass of ~25–30 kDa. Group 1 allergens are recognized by >95% of grass pollen allergic patients. We investigated the IgE recognition of Phl p 1 using allergen-specific IgE-derived single-chain variable Ab fragments (IgE-ScFvs) isolated from a combinatorial library constructed from PBMCs of a grass–pollen–allergic patient. IgE-ScFvs reacted with recombinant Phl p 1 and natural group 1 grass pollen allergens. Using synthetic Phl p 1–derived peptides, the binding sites of two ScFvs were mapped to the N terminus of the allergen. In surface plasmon resonance experiments they showed comparable high-affinity binding to Phl p 1 as a complete human IgE-derived Ab recognizing the allergens’ C terminus. In a set of surface plasmon resonance experiments simultaneous allergen recognition of all three binders was demonstrated. Even in the presence of the three binders, allergic patients’ polyclonal IgE reacted with Phl p 1, indicating high-density IgE recognition of the Phl p 1 allergen. Our results show that multiple IgE Abs can bind with high density to Phl p 1, which may explain the high allergic activity and sensitizing capacity of this allergen. The Journal of Immunology, 2015, 194: 2069–2078.

They are recognized by >90% of grass pollen–allergic patients and occur as highly cross-reactive allergens in pollen of most, if not all, grass species, including temperate and tropical grasses (8, 9). Phl p 1 from timothy grass pollen is one of the best characterized group 1 allergens (10). It has been shown to contain the majority of group 1–specific IgE and T cell epitopes, and its three-dimensional structure has been solved by x-ray crystallography (11–14). Several clinical studies have demonstrated the high allergenic activity and clinical relevance of Phl p 1 (5, 15), and it can be used as a diagnostic marker for the identification of grass pollen–allergic patients (16) due to its strong cross-reactivity with group 1 allergens from related grass species as demonstrated by Kahn and Marsh (17). A key role as a possible initiator of grass pollen allergy has recently been attributed to Phl p 1 based on the longitudinal analysis of IgE reactivity toward a panel of different grass pollen allergens in birth cohorts (18). Interestingly, Phl p 1 was the grass pollen allergen with the highest prevalence of IgE recognition in early life whereas sensitization to other allergens occurred later in life (18).

In this study we investigated whether the high allergenic activity of Phl p 1 can be attributed to its recognition of IgE Abs from allergen patients. IgE-derived single-chain variable fragments (ScFvs) specific for Phl p 1 were isolated by combinatorial cloning from a grass pollen–allergic patient. Binding sites for the IgE-derived ScFvs were mapped and visualized on the three-dimensional structure of the allergen. The simultaneous high-affinity binding of the IgE-derived ScFvs was shown in surface plasmon resonance (SPR) experiments. Our study indicates that Phl p 1 bears a high number of distinct IgE epitopes providing a possible explanation for its high sensitizing potential and allergic activity.

Materials and Methods

Allergens, Abs, sera from allergic patients, and synthetic peptides

Recombinant allergens were purchased from Biomay (Vienna, Austria). The Phl p 1–specific IgE Fab clone 25 was isolated from a combinatorial...
library in a previous study (19) (Table I). The V regions of this clone were also grafted onto a complete human monoclonal IgG1 Ab designated P1 IgG1 and used to engineer ScFv. 25 H chain variable (VH) and L chain variable (VL) (14, 20). Serum samples from grass pollen–allergic patients were analyzed with approval of the Ethics Committee of the Medical University of Vienna in a retrospective and anonymous manner. The diagnosis of grass pollen allergy was based on a patient’s history of seasonal symptoms, skin prick testing, and allergen-specific IgE measurements as described (5). Grass pollen–allergic patients’ sera were also tested for IgE reactivity toward the four major recombinant grass pollen allergens, that is, Phl p 1, Phl p 2, Phl p 5, and Phl p 6, by ELISA. A comprehensive IgE reactivity profile toward >100 purified allergen molecules was established using ImmunoCAP ISAC assays (Thermo Fisher Scientific, Uppsala, Sweden) (21) (Supplemental Table I). Phl p 1–specific IgE Ab levels in patients’ sera were determined by ImmunoCAP g205 (Thermo Fisher Scientific). Phl p 1–derived peptides were synthesized and purified as described (22).

Isolation of grass pollen–specific ScFvs from an IgE library constructed from PBMCs of a grass pollen–allergic patient

After informed consent was obtained from a grass pollen–allergic patient with almost exclusive IgE reactivity to the four major grass pollen allergen groups, a heparinized 100-ml blood sample was collected. PBMCs were isolated by Ficoll gradient centrifugation (23) and RNA was prepared (24) and translated into cDNA (SuperScript first-strand synthesis system for RT-PCR; Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions using a primer specific for the C region of Ig (5′-GCTAC- TAGTGT TTTTGTGTCGAGG CACGTCCTGGTGC′-3′) (19). DNA coding for Vκ regions was PCR amplified (GoTaq green master mix; Promega, Madison, WI) using primers specific for the six Vκ families from the preselected IgE-derived cDNA transcripts (25). cDNA for the amplification of DNA coding for Vλ regions was obtained by using oligo(dT) primers for reverse transcription in a first step followed by PCR using primers specific for the six κ L chain families (25). PCR products were purified on 1% agarose gel, eluted (Wizard SV gel and PCR clean-up system; Promega), and DNAs coding for Vκ and Vλ families were pooled and introduced into an assembly PCR reaction (GeneAmp PCR core reagents; Life Technologies, Carlsbad, CA) with linker DNA coding for a 15-aa linker ([Gly3Ser]3) and specific primers overlapping between Vκ and Vλ regions and the linker DNA (20). The assembly PCR reaction was run for seven cycles (mousse ScFv module; Amersham Biosciences, Little Chalfont, U.K.) and the assembled sequences were further elongated by adding an equimolar mixture of Ig H chain V region (IGHV) and Ig κ chain J region (IGKJ) extension primers comprising restriction sites for SfiI (IGHV) and NotI (IGKJ) (25). PCR products were purified on a 1% agarose gel, eluted with a gel elution kit (Promega). Assembled DNA coding for ScFvs was digested with the restriction enzymes SfiI and NotI (New England Biolabs, Ipswich, MA) purified with a reaction cleanup kit (MinElute reaction cleanup kit; Qiagen), and transformations using a primer specific for the Phl p 1 region were done with increasing stringency in each round. Following rounds of panning were done with increasing stringency in each round.

**Table I. Summary of human IgE-derived Phl p 1 binders isolated from combinatorial libraries**

<table>
<thead>
<tr>
<th>Clone</th>
<th>Name</th>
<th>N-terminal aa</th>
<th>Original Description</th>
<th>Binding Region</th>
<th>Original Description</th>
<th>Accession No.</th>
<th>Length (aa)</th>
<th>No. Mutations</th>
<th>C-terminal aa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clone F8</td>
<td>IGHV 3–7 IGHD 3–10 IGHJ 3 11 8 IGKV 1–39 or IGKV</td>
<td>N-terminal aa</td>
<td>Original Description</td>
<td>N-terminal aa</td>
<td>0</td>
<td>26</td>
<td>KP315340</td>
<td>IGKJ 4 9 0 146–240</td>
<td></td>
</tr>
<tr>
<td>Clone A8</td>
<td>IGHV 3–9 IGHD 3–15 IGHJ 4 13 11 IGKV 3–25 IGLJ1 10 2</td>
<td>C-terminal aa</td>
<td>Original Description</td>
<td>C-terminal aa</td>
<td>8</td>
<td>14</td>
<td>KP315341</td>
<td>IGKJ 4 9 0 146–240</td>
<td></td>
</tr>
<tr>
<td>Clone 10</td>
<td>IGHV 3–9 IGHD 3–15 IGHJ 4 13 11 IGKV 3–25 IGLJ1 10 2</td>
<td>C-terminal aa</td>
<td>Original Description</td>
<td>C-terminal aa</td>
<td>8</td>
<td>14</td>
<td>KP315342</td>
<td>IGKJ 4 9 0 146–240</td>
<td></td>
</tr>
<tr>
<td>Clone 25</td>
<td>IGHV 3–9 IGHD 3–15 IGHJ 4 13 11 IGKV 3–25 IGLJ1 10 2</td>
<td>C-terminal aa</td>
<td>Original Description</td>
<td>C-terminal aa</td>
<td>8</td>
<td>14</td>
<td>KP315342</td>
<td>IGKJ 4 9 0 146–240</td>
<td></td>
</tr>
<tr>
<td>Clone 3</td>
<td>IGHV 3–7 IGHD 3–10 IGHJ 3 11 8 IGKV 1–39 or IGKV</td>
<td>N-terminal aa</td>
<td>Original Description</td>
<td>N-terminal aa</td>
<td>0</td>
<td>26</td>
<td>KP315340</td>
<td>IGKJ 4 9 0 146–240</td>
<td></td>
</tr>
<tr>
<td>Clone 6</td>
<td>IGHV 3–7 IGHD 3–10 IGHJ 3 11 8 IGKV 1–39 or IGKV</td>
<td>N-terminal aa</td>
<td>Original Description</td>
<td>N-terminal aa</td>
<td>0</td>
<td>26</td>
<td>KP315340</td>
<td>IGKJ 4 9 0 146–240</td>
<td></td>
</tr>
</tbody>
</table>

Determination of allergen and epitope specificity of isolated ScFvs by ELISA and dot blotting

ELISA plates (Maxisorp; Nunc) were coated with recombinant Ags (allergens, peptides, BSA, or human serum albumin [HSA], 5 μg/ml) in 100 mM NaHCO3 (pH 9.6). Plates were incubated for 1 h at 37°C, washed
twice with PBS containing 0.05% (v/v) Tween 20 (PBST), and saturated with PBST containing 5% (w/v) BSA. E. coli lysates containing ScFvs or purified ScFvs were diluted in PBST containing 1% (w/v) BSA and applied overnight at 4°C. Plates were washed with PBST and bound ScFvs were detected with either a mouse monoclonal anti–E tag (GE Healthcare, Little Chalfont, U.K.) or anti–His tag Ab (Dianova, Hamburg, Germany) diluted in PBST 1% (w/v) BSA. Bound mouse Abs were detected with an HRP-conjugated sheep anti-mouse Ab (GE Healthcare) and ABTS (1 mg/ml; Sigma-Aldrich, St. Louis, MO) as substrate. ODs were measured at 405 nm in an ELISA reader SpectraMax Plus (Molecular Devices, Sunnyvale, CA). Results represent means of duplicate determinations with variations of <5%.

Nitrocellulose strips (Whatman, Dassel, Germany) containing 1 µg of each recombinant allergen (Phl p 1, Phl p 2, Phl p 5, Phl p 6, Bet v 1, and the control protein BSA) were incubated with buffer A (40 mM Na2HPO4, 0.6 mM NaH2PO4, 0.5% [v/v] Tween 20, 0.5% [w/v] BSA, 0.05% [v/v] NaN3). E. coli lysates containing recombinant ScFvs specific for Phl p 1 were diluted 1:1 in buffer A and incubated overnight at room temperature. Blots were washed and subsequently incubated with a monoclonal anti–E tag Ab diluted 1:8000 and, after washing, with an HRP-conjugated sheep anti-mouse Ab diluted 1:2000 (GE Healthcare). Bound Abs were visualized with ECL substrate (GE Healthcare) by autoradiography.

Cross reactivity of IgE-derived ScFvs with nitrocellulose-blotted grass pollen extracts

Allergen extracts were prepared as described (27) and separated by SDS-PAGE under reducing conditions. Comparable amounts of pollen extracts were blotted onto nitrocellulose (28) and successful transfer was verified by Ponceau S staining of the membranes. Blots were blocked with buffer A and incubated with purified His-tagged ScFv fragments F8 and A8 overnight at room temperature. Bound ScFvs were detected with a monoclonal anti–His tag Ab (Dianova) and subsequently with alkaline phosphatase–conjugated rabbit anti-mouse Abs (BD Pharmingen, San Diego, CA).

Multiple sequence alignments of group 1 grass pollen allergen sequences

Group 1 grass pollen allergen amino acid sequences were obtained from the International Union of Immunological Societies allergen nomenclature subcommittee Web site (http://www.allergen.org) and aligned using the Clustal Omega sequence alignment Web tool (29).

Three-dimensional modeling of Phl p 1 and analysis of isolated gene sequences

The x-ray crystal structure of Phl p 1 (14) was acquired via the National Center for Biotechnology Information Molecular Modeling Database and visualized with the Cn3D 4.3 software (30). Phl p 1–derived peptides recognized by ScFvs were marked on the three-dimensional model using the sequence alignment viewer tool. V region DNA sequences were aligned with the most closely related V region alleles using the ImMunoGeneTics/V-QUEST Web tool (31, 32). Sequences were aligned according to the ImMunoGeneTics unique numbering system (33).

Determination of the affinities of Phl p 1 binders by SPR

Experiments were conducted on a Biacore 2000 instrument (GE Healthcare) at room temperature. A monoclonal anti–E tag Ab (GE Healthcare) was immobilized on a Sensor Chip CM5 to capture E-tagged ScFv F8 as described (20). Likewise, a monoclonal anti–His tag Ab (Abcam, Cambridge, U.K.) was immobilized using 10 mmol/l sodium acetate buffer (pH 3) as coupling buffer to capture His-tagged ScFv A8. In the reference cell an isotype-matched control Ab (mouse IgG1) was used. All Abs were immobilized in excess compared with subsequently tested ligands. Capturing levels of analyzed ScFvs were chosen to reach a calculated Rmax of 100 resonance units (RU). Multicycle kinetics were performed to determine binding affinities for Phl p 1. Allergen was diluted in HBS-EP (0.01 mol/l HEPES, 0.15 mol/l NaCl, 3 mmol/l EDTA, and 0.005% [v/v] surfactant P20 [pH 7.4]) to obtain 2-fold increasing concentrations (0.5–256 nmol/l) and injected at a flow rate of 30 µl/min for 5 min. Dissociation was measured by injecting HBS-EP buffer at 30 µl/min for 15 min. For control purposes, two runs were performed with the same allergen dilutions. Additionally, two cycles were run with HBS-EP alone to subtract background signals.

Regeneration of the sensor chip surface was achieved with 10 mmol/l glycine–HCl (pH 2) at 30 µl/min for 30 s. The BIAevaluation 3.2 (GE Healthcare) software package was used to fit binding curves and calculate kinetics and affinity constants using a 1:1 (Langmuir) binding model.

A single cycle kinetic approach was used to determine the binding affinity of P1 IgG1 (14). For this purpose, P1 IgG1 was immobilized on a Sensor Chip CMS to an immobilization level of 7000 RU using an amine coupling kit (GE Healthcare). An isotype-matched Ab (human IgG1) was immobilized in a reference cell to a level of 4000 RU as control. Three-fold increasing concentrations of Phl p 1 (5, 15, 45, 135, 255) were injected and ODs were measured at 405 nm in an ELISA reader SpectraMax Plus (Molecular Devices, Sunnyvale, CA). The x-ray crystal structure of Phl p 1 (14) was acquired via the National Center for Biotechnology Information Molecular Modeling Database and visualized with the Cn3D 4.3 software (30). The x-ray crystal structure of Phl p 1 (14) was acquired via the National Center for Biotechnology Information Molecular Modeling Database and visualized with the Cn3D 4.3 software (30).
405 nmol/l) diluted in HBS-EP were injected consecutively with a dissociation time of 1 min between each concentration and a final dissociation time of 20 min. The same procedure was performed with buffer alone. BIACore evaluation 3.2 (GE Healthcare) software was used to subtract the buffer cycle from the sample cycle to receive double referenced datasets. A 1:1 binding model was applied to fit measured data to calculate binding affinity (34).

Analysis of simultaneous allergen recognition using SPR

A Sensor Chip CM5 containing an immobilized anti-His tag mAb was prepared as described above. Phl p 1-specific ScFvs F8 or A8 were captured until ~250 RU were reached. Immediately thereafter, Phl p 1 was injected at a concentration of 500 nmol/l until the observed binding curve showed no more incline, indicating steady-state conditions. In the next step, a second E-tagged Phl p 1-specific ScFv (350 nmol/l) or complete P1 showed no more incline, indicating steady-state conditions. In the next experiment, E-tagged ScFvs having the same epitope specificity as the capturing His-tagged ScFvs were injected. Results are shown as binding curves recorded in real time with RU corresponding to binding interactions.

IgE-inhibition ELISA experiments

Aliquots of 50 ng recombinant Phl p 1 per well were coated and blocked as described above for ELISA experiments. Wells were then incubated with a 20-fold molar excess of purified ScFv F8, ScFv A8, P1 IgG1, or of an equimolar mixture of all three (F8, A8, P1 IgG1) reagents overnight at 4˚C. As positive control, polyclonal rabbit anti-Phl p 1 IgG diluted 1:100 in PBST 0.5% (w/v) BSA was used for IgE inhibition. As negative control, Bet v 1–specific rabbit IgG diluted 1:100 in PBST 0.5% (w/v) BSA was used. After overnight incubation, plates were washed with PBST and incubated with sera from Phl p 1–allergic patients diluted 1:5 in PBST 0.5% (w/v) BSA for 3 h at 37˚C. Bound IgE Abs were detected with alkaline phosphatase-conjugated anti-human IgE Abs (BD Pharmingen) using alkaline phosphatase substrate for development (Sigma-Aldrich). Optical densities corresponding to bound IgE were measured as described above for ELISA experiments. Results represent means of duplicates with error bars indicating SD.

To investigate possible effects of Phl p 1–specific patients’ IgE on the binding of ScFvs to Phl p 1, coincubation experiments were performed. Aliquots of 50 ng recombinant Phl p 1 per well were coated and blocked as described for the ELISA experiments. Wells were then incubated with a 20-fold molar excess of an equimolar mixture of purified ScFv F8 and ScFv A8 overnight at 4˚C. After overnight incubation, sera from Phl p 1–allergic patients diluted 1:5 in PBST 0.5% (w/v) BSA for 3 h at 37˚C. Bound IgE Abs were detected with alkaline phosphatase-conjugated anti-human IgE Abs (BD Pharmingen) using alkaline phosphatase substrate for development (Sigma-Aldrich). Optical densities corresponding to bound IgE were measured as described above for ELISA experiments. Results represent means of duplicates with error bars indicating SD.

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Table II. Characterization of recombinant Phl p 1, Phl p 1 fragment, and synthetic Phl p 1–derived peptides

<table>
<thead>
<tr>
<th>Name</th>
<th>Amino Acid Sequence</th>
<th>Position of Amino Acids</th>
<th>Number of Amino Acids</th>
<th>Molecular Mass (kDa)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phl p 1</td>
<td>1PKVFPGGPNITATYDGKWLAKSTVYKGKTGGGAG</td>
<td>1–240</td>
<td>240</td>
<td>28.5</td>
<td>Laffer et al. (10); Protein Data Bank: 1n10; UniProt: P43213</td>
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<tr>
<td>C-terminal</td>
<td>KVTHVEKGASNPLYVLKVKVNYGQGDDVAVAD</td>
<td>146–240</td>
<td>95</td>
<td>10.7</td>
<td>Flicker et al. (14)</td>
</tr>
<tr>
<td>Pep 1</td>
<td>HEVGSNSNPOLLKLIKVNYGQGDDVAVAD</td>
<td>151–177</td>
<td>28</td>
<td>2.9</td>
<td>Focke et al. (22)</td>
</tr>
<tr>
<td>Pep 2</td>
<td>EPVYYHTDNEEPAPYFHDFSLGHAFGAGRAC</td>
<td>87–117</td>
<td>32</td>
<td>3.5</td>
<td></td>
</tr>
<tr>
<td>Pep 3</td>
<td>1PKVFPGGPNITATYDGKWLAKSTVYKGKTGGGAG</td>
<td>1–30</td>
<td>32</td>
<td>3.5</td>
<td></td>
</tr>
<tr>
<td>Pep 4</td>
<td>GKVDKPPFSQMTGCNTPFIFKSGRC</td>
<td>43–70</td>
<td>28</td>
<td>2.9</td>
<td></td>
</tr>
<tr>
<td>Pep 5</td>
<td>CVRTTSTGKTEAEVIPEGKADTYESK</td>
<td>212–241</td>
<td>31</td>
<td>3.5</td>
<td></td>
</tr>
<tr>
<td>Pep 2 A</td>
<td>EPVYYHTDNEEPAPYFHDFSLGHAFGAGRAC</td>
<td>86–100</td>
<td>15</td>
<td>1.7</td>
<td>Gieras et al. (37)</td>
</tr>
<tr>
<td>Pep 2 B</td>
<td>APYFHDFSLGHAFGAMA</td>
<td>101–116</td>
<td>16</td>
<td>1.7</td>
<td></td>
</tr>
<tr>
<td>Pep 2 C</td>
<td>ITDDNEEPAPYFHDFSLGHAFGAMA</td>
<td>92–109</td>
<td>18</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Pep 2 D</td>
<td>DLGHAFGAMA</td>
<td>106–116</td>
<td>11</td>
<td>1.1</td>
<td></td>
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<tr>
<td>Pep 2 E</td>
<td>HAFGAMA</td>
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<td>0.7</td>
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</tr>
<tr>
<td>Pep 2 F</td>
<td>YHDFSLGHAFGAMA</td>
<td>103–114</td>
<td>12</td>
<td>1.3</td>
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</tr>
</tbody>
</table>

Added cysteine residues are underlined.

Results

Construction of a combinatorial library from a patient with exclusive grass pollen allergy: isolation of IgE-derived Phl p 1–specific single-chain fragments

We isolated three Phl p 1–specific ScFvs designated clones F8, A8, and E8 from a combinatorial library constructed from PBMCs of a grass pollen–allergic patient with an exclusive allergic sensitization to grass pollen allergens (Supplemental Table I). The patient suffered from allergic rhinoconjunctivitis during grass pollen season and had not undergone allergen-specific immunotherapy. This patient showed IgE reactivity to the grass pollen allergens Phl p 2, Phl p 4, Phl p 5, and Phl p 6 but the highest levels of IgE against the major grass pollen allergen Phl p 1 (Supplemental Table I).

The initial step of library construction was based on the generation of cDNA using a primer specific for the IgE C region, ensuring a preselection of IgE transcripts that then served as templates for the next PCR step with V H family–specific primers, a strategy that was already successfully implemented by several other investigators (14, 19, 36). An ScFv library comprising 5 × 10 6 independent clones was obtained. The analysis of randomly picked clones from the original library revealed the presence of highly diverse and nonredundant H and L chain variable sequence fragments. Sequences among the ScFv clones (Supplemental Fig. 1). Five rounds of panning were conducted against each of the four major grass pollen allergens (Phl p 1, Phl p 2, Phl p 5, and Phl p 6), which resulted in a strong enrichment of phages displaying Phl p 1–specific ScFv whereas no relevant enrichment of phages specific for the other three allergens was observed (Supplemental Fig. 2). Approximately 1000 enriched phage clones were picked and tested for reactivity to Phl p 1, 2, 5, and 6 by ELISA, which resulted in the isolation of two Phl p 1–specific ScFvs (i.e., F8 and A8). A third independent ScFv clone, E8, containing a V Δ region almost identical to clone A8 resulted from an additional ELISA screening (data not shown; Table I).

Allergen and epitope specificity of isolated ScFvs

ELISA experiments with ScFv F8 and A8 showed that they specifically reacted with Phl p 1 but not with other recombinant...
pollen allergens or control proteins (i.e., HSA, BSA) (Fig. 1A).

Binding of ScFv F8 and A8 to Phl p 1 was also confirmed in dot blot assays (Fig. 1B). A previously isolated Phl p 1–specific ScFv 25VH/25VL derived from clone 25 (Table I) (20) was used as positive control and also bound to Phl p 1. Buffer alone gave no signal (Fig. 1B).

Next we performed epitope mapping of ScFv F8 and A8 using a panel of Phl p 1–derived synthetic peptides that cover almost the complete sequence of Phl p 1 (Table II). ScFv F8 bound to the N-terminal peptide 2 (aa 87–117) whereas ScFv A8 reacted with the N-terminal peptide 3 (aa 1–30) (Fig. 1C). ScFv 25VH/25VL and the Phl p 1–specific IgG1 Ab utilizing the same V regions as ScFv

FIGURE 2. Cross-reactivity of IgE-derived ScFvs with natural group 1 allergens in different grass and corn species. Nitrocellulose-blotted pollen extracts (Ant o, sweet vernal grass; Ave s, oat; Cyn d, Bermuda grass; Lol p, rye grass; Phl p, timothy grass; Poa p, Kentucky bluegrass; Sec c, cultivated rye; Tri a, cultivated wheat; Zea m, maize) were exposed to His-tagged (A) ScFv F8 and (B) ScFv A8. Bound ScFvs were detected with a mouse monoclonal anti–His tag Ab followed by alkaline phosphatase–conjugated anti-mouse Ig Abs. Molecular masses are indicated on the right margin in kDa. (C) and (D) show multiple sequence alignments of the peptides Pep 2 C (epitope of ScFv F8) and Pep 3 (epitope of ScFv A8) with the corresponding group 1 sequences from different grasses according to sequence identity. Dashes represent identical amino acids and dots are gaps.

FIGURE 3. Position of Phl p 1–derived peptides on the three-dimensional surface representation of Phl p 1. Binding sites recognized by ScFv F8 (Pep 2, green, aa 87–117; Pep 2C, orange, aa 92–109), A8 (Pep 3, blue, aa 1–30), and of clone 25–derived IgG1 (C-terminal fragment, aa 146–240, yellow) on the Phl p 1 structure. The N and C termini are highlighted in red and magenta, respectively.
25VH/25VL did not react with any of the tested peptides (data not shown). Using six smaller peptides (i.e., Pep 2 A–F) representing portions of Pep 2 (Table II) the binding site of ScFv F8 could be mapped to Pep 2 C comprising aa 92–109 of Phl p 1 (Fig. 1D, Table II).

Cross-reactivity of IgE-derived ScFvs with natural group 1 allergens from different grass and corn species

When tested for reactivity with nitrocellulose-blotted pollen extracts from several grass and corn species, ScFv F8 showed distinct cross-reactivity with bands between 25 and 35 kDa in each of the tested grasses except Bermuda grass (Cyn d) (Fig. 2A). The alignment of the peptide epitope Pep 2 C recognized by ScFv F8 in timothy grass with the corresponding sequences in other grasses revealed a high sequence identity of the Pep 2 C–defined region in most of the grasses, with Bermuda grass showing a lower sequence identity (Fig. 2C). Two of the amino acid exchanges in the Bermuda grass sequence occurred also in other grasses without affecting the binding of ScFv F8, whereas the exchanges of aspartic acid to lysine and from proline to histidine most likely caused the loss of binding because they affected the charge of the peptide (Fig. 2C). The reactivity of ScFv A8 to the nitrocellulose-blotted extracts was less distinct. We found a clear signal between 25 and 35 kDa in timothy grass pollen extract (Phl p), a band below 25 kDa in Kentucky bluegrass (Poa p), whereas a more diffuse (Bermuda grass [Cyn d], ryegrass [Lo l], oat [Ave s], maize [Zea m], sweet vernal grass [Ant o]) and no (cultivated wheat [Tri a], cultivated rye [Sec c]) reactivity was observed for the other grasses (Fig. 2B). In accordance, we found that the Pep 3 region in Kentucky bluegrass showed the highest degree of sequence identity with timothy grass (Fig. 2D).

Visualization of the ScFv epitopes and of a known IgE epitope–containing fragment on the three-dimensional structure of Phl p 1

Next we highlighted the binding site for ScFv F8 and A8 on the three-dimensional structural model of Phl p 1 (Fig. 3) (Protein Data Bank: 1m10). Peptide 2 is indicated in green and orange. The orange part of Pep 2 represents Pep 2 C as the minimal binding site of ScFv F8. ScFv A8 reacted with Pep 3, which is colored in blue. Both binding sites are located at the N-terminal portion of Phl p 1 and do not overlap with the major IgE-reactive C-terminal fragment of Phl p 1 comprising aa 146–240, which is marked in yellow. This fragment contained also the binding sites for all monoclonal IgE Abs characterized so far by combinatorial cloning (Table I). Interestingly, Pep 2 (aa 87–117) and Pep 3 (aa 1–30) are located closely together on the Phl p 1 surface whereas Pep 4 (aa 43–70), whose sequence lies between the two peptides, is partly buried within the Phl p 1 structure. The localization of the epitopes on the three-dimensional structure of Phl p 1 thus shows that the IgE-reactive epitopes are located in three distinct areas of the allergen.

Sequence analysis of Phl p 1–specific IgE V regions

The V genes of the H chains of the two ScFvs F8 and A8 (Fig. 4A) belong to the IGHV3 subgroup and show a moderate number of amino acid exchanges as compared with the germline sequence that is in the range reported also for other Phl p 1–specific human IgE Abs (Table I). Analysis of the V L regions of ScFv A8 and F8 (Fig. 4B) revealed that they were in germline configuration. The L chain sequences of ScFv A8 and E8 were identical (data not shown). Table I provides a summary of characteristics of ScFv A8, F8, and E8 and of all other human monoclonal Phl p 1–specific IgE Abs isolated by combinatorial cloning to date. IGHV3 and IGHV1 were the exclusively used subgroups among the IgE VH.

Lengths of the CDRH3 of the Phl p 1 binders differed considerably, ranging from 6 up to 18 aa. Interestingly, the V regions (i.e., 5p1:3, p1–20) using IGHV1–18 had the shortest CDRH3 regions as compared with the binders belonging to IGHV3. With regard to L chain IGVK gene family usage, note that clone F8 uses IGVK 1–39, a gene that was also preferentially used by the Phl p 1–specific binders isolated from an unrelated patient (19). L chain CDR3 regions showed less variability in length, ranging only from 9 to 11 amino acids. Despite relatively low numbers of somatic mutations in the VH and VL regions of the Phl p 1 binders, the affinities for those Phl p 1 binders (F8, A8) that had been examined by SPR were all in the nanomolar to micromolar range (Table I).

Phl p 1–specific IgE-derived ScFvs F8 and A8 bind with high affinity to Phl p 1

Binding kinetics and affinity constants of ScFvs F8 and A8 for Phl p 1 were analyzed and compared with those of an IgE-derived complete Phl p 1–specific Ab (i.e., P1 IgG1) using SPR (Fig. 5). ScFvs captured on the sensor chip were subjected to increasing concentrations of Phl p 1 (0.5–256 nmol/l), and recorded data were fitted using a 1:1 binding model (Langmuir). Shown are five selected concentrations (16–256 nM) for clone F8 and A8 with a 15-min dissociation phase (Fig. 5A, 5B). Both clones showed fast onset of binding to Phl p 1 (F8, k a = 1.5 × 107/Ms; A8, k d = 4.5 × 107/Ms) with moderate dissociation at the beginning of the dissociation phase, especially in the case of clone F8.

**FIGURE 4.** Alignment of the Phl p 1–specific VH (A) and VL (B) amino acid sequences of clones F8 and A8 with the corresponding germline sequences. Framework (FR) and CDRs are indicated according to ImMunoGeneTics nomenclature; identical amino acids are displayed as dashes, mutations are in bold letters, and changes due to primer sequences are underlined.
ingly, dissociation slowed down and stabilized over time, leading to relatively low off rates \( (F8, k_\text{d} = 4.0 \times 10^{-4}/\text{s}; A8, k_\text{d} = 2.6 \times 10^{-4}/\text{s}). \) Calculated affinities for clone A8 were in the nanomolar range \( (K_D = 5.7 \times 10^{-9} \text{M}) \) whereas clone F8 showed a lower affinity \( (K_D = 2.6 \times 10^{-8} \text{M}). \) Note that the recorded curves did not strictly follow the fitted Langmuir model, which resulted in less accurate calculations. Therefore, several other mathematical

**FIGURE 5.** Affinity determination of Phl p 1–specific ScFvs and of a recombinant Phl p 1–specific IgE-derived Ab using SPR. ScFvs F8 (A) and A8 (B) were captured via anti–E tag Abs to the chip surface and Phl p 1 was injected in 2-fold increasing concentrations from 16 to 256 nM. Recorded binding curves (colored) were fitted with a 1:1 binding model (black) to calculate binding affinities. (C) A Phl p 1–specific IgE-derived Ab (Table I, clone 25) was immobilized directly on the chip and subjected to increasing concentrations of Phl p 1 (blue). Affinity was determined by applying a 1:1 single cycle kinetic model (black). RU \((y\text{-axes})\) indicate signal intensities; association and dissociation times are shown in seconds \((x\text{-axes})\). \(k_a\) and \(k_d\) rates and calculated \(K_D\) are shown.

**FIGURE 6.** Demonstration of simultaneous ScFv and Ab binding to Phl p 1 by SPR. (A–C) Phl p 1 captured by His-tagged ScFv A8 was subsequently probed with the IgE-derived Phl p 1–specific Ab (A), the E-tagged ScFv F8 (B), or E-tagged ScFv A8 (C). (D and E) Phl p 1 captured by His-tagged ScFv F8 was probed with the IgE-derived Phl p 1–specific Ab (D) or E-tagged ScFv F8 (E). (F) Experiment as in (A) but with additional exposure to E-tagged ScFv F8. (G) Experiment as in (B) but with additional exposure to IgE-derived Phl p 1–specific Ab. Arrow lengths correspond to injection time of molecules indicated. Graphs show RU \((y\text{-axes})\) displayed over time in seconds \((x\text{-axes})\).
models were tested to fit the data, including bivalent and multivalent interaction models. However, the 1:1 model was the most appropriate.

In the case of the monoclonal IgE-derived Phl p 1–specific IgG1 Ab, P1 IgG1, we used a single-cycle kinetic approach to determine $k_+ \text{ and } k_-$ (Fig. 5C). This method circumvents regeneration of the chip surface in cases where proper regeneration conditions cannot be established. P1 IgG1 was immobilized on the chip and sequentially exposed to increasing concentrations of Phl p 1 with dissociation intervals between each injection. Controls included a cycle with buffer alone and an unrelated Ab in a control cell. Results showed high affinity of P1 IgG1 for Phl p 1 with dissociation constants also in the nanomolar range ($k_+ = 1.9 \times 10^7$ M$^{-1}$s$^{-1}$; $k_-$ = $3.3 \times 10^{-4}$ s$^{-1}$; $K_D = 1.7 \times 10^{-10}$ M).

Simultaneous binding of two ScFvs and an Ab to Phl p 1 as demonstrated by SPR experiments

Having demonstrated high-affinity binding for three human IgE-derived Phl p 1–specific binders, we conducted SPR experiments to investigate whether they can bind simultaneously to Phl p 1. For this purpose we used ScFvs (F8 and A8) with different tags (E-tag and His-tag) to establish capturing conditions, which allowed us to study binding properties in real time. In a first set of experiments, anti–His tag Abs immobilized on the chip surface were used to capture His-tagged A8, which after exposure to Phl p 1 bound the complete P1 IgG1 (Fig. 6A) and the E-tagged ScFv F8 (Fig. 6B) but not E-tagged ScFv A8 (Fig. 6C). Likewise, captured His-tagged F8 was exposed to Phl p 1 and then reacted with P1 IgG1 (Fig. 6D) but not with E-tagged F8 (Fig. 6E). Moreover, it was possible to demonstrate simultaneous attachment of all three binders to Phl p 1 (Fig. 6F, 6G). Captured His-tagged ScFv A8 bound Phl p 1, and subsequently a 3000-fold higher concentration compared with $K_D$ of P1 IgG1 was applied to approach saturation of P1 IgG1 binding sites. Nevertheless, E-tagged ScFv F8 was able to bind Phl p 1 (Fig. 6F). Likewise, captured His-tagged A8 bound Phl p 1, E-tagged F8, and subsequently P1 IgG1 (Fig. 6G).

Experiments conducted in Fig. 6C and 6E exclude that dimerization or oligomerization of Phl p 1 was responsible for the simultaneous binding because no additional binding was observed when the same ScFv used for catching was tested for reactivity.

Evidence for binding of multiple independent IgE Abs to Phl p 1 demonstrated by competition experiments with polyclonal patients’ IgE

Having shown simultaneous reactivity of three IgE-derived Phl p 1 binders with Phl p 1, we investigated whether additional IgE Abs can attach to the allergen. For this purpose we used polyclonal serum IgE from allergic patients (Phl p 1–specific IgE levels were between 41.2 and 100 kUA/l) in inhibition experiments. None of the isolated ScFvs or the mixture caused a relevant inhibition of polyclonal IgE binding (Fig. 7) whereas polyclonal rabbit anti–Phl p 1 IgG strongly inhibited IgE binding to Phl p 1. No inhibition was observed when polyclonal rabbit Abs specific for an unrelated allergen (i.e., birch pollen allergen Bet v 1) were used (Fig. 7). To study whether the presence of high levels of Phl p 1–specific patients IgE might have an influence on the binding of ScFvs to the Phl p 1 allergen, coinubation experiments were performed, which showed that patients’ IgE had no profound effects on Phl p 1–specific ScFv binding (data not shown).

Discussion

The major timothy grass pollen allergen Phl p 1 belongs to a family of highly cross-reactive allergens present in most grass and corn species and represents one of the most frequently recognized allergens known to date (38). The analysis of the early development of grass pollen allergy in childhood indicates that group 1 allergens are the culprit allergens in the sensitization process, which may initiate subsequent sensitization against multiple other grass pollen allergens (18). Importantly, Phl p 1 is also highly potent in eliciting IgE-mediated allergic inflammation (5). Data from murine experimental model systems (37), molecular docking simulations (39) and indirect IgE inhibition data for human allergy (40) suggest that multiple high affinity IgE recognition is a key property of highly potent allergens.

Here we used human IgE-derived Phl p 1–specific ScFvs isolated from a combinatorial library constructed from PBMCs of a grass pollen allergic patient to study IgE recognition of Phl p 1. The isolated IgE-derived ScFvs belonged to the IGHV3 gene subgroup which together with the IGHV1 family seems to dominate in the so far known human allergen-specific IgE repertoire.
Interestingly, the corresponding L chains in two of the isolated clones (i.e., A8 and E8) were identical and showed virtually no mutations in comparison with the germline. In contrast to previously isolated Phl p 1–specific human IgE V regions, which were all directed against the major IgE epitope–containing C-terminal portion of Phl p 1 (14, 35), the IgE-derived ScFvs isolated by us are the first that recognized the N-terminal portion of the allergen. The presence of several IgE epitope–containing regions on Phl p 1 has been reported (42, 43), but so far simultaneous binding of several human IgE Abs to an allergen has not been demonstrated.

We therefore used the IgE-derived ScFvs F8 and A8 and the IgE-derived P1 IgG1 in a series of SPR (Biacore) experiments to determine whether indeed three IgE V regions from allergic patients can simultaneously bind to Phl p 1. This method enabled us to demonstrate the simultaneous binding of three monoclonal IgE–derived Abs specific for Phl p 1 in real time and to watch the buildup and stable formation of the resulting immune complexes. Our experiments show that there are at least three independently accessible IgE epitopes simultaneously available on the surface of Phl p 1. We were then interested to investigate whether even more IgE Abs can bind to Phl p 1 considering that it represents a rather small protein and that steric inhibition of three binders would eventually prevent the recognition of the allergen by additional IgE Abs. Interestingly, ELISA competition results showed that even an excess of the three binders did not cause relevant inhibition of patients’ IgE binding to Phl p 1. The fact that each of the three monoclonal binders showed very high affinity for Phl p 1 in the nanomolar or low micromolar range and that they were applied in huge excess to patients’ IgE makes it very unlikely that the lack of inhibition of patients’ IgE to Phl p 1 was caused by displacement of the binders by polyclonal IgE. We therefore think that our experiment indicates that in addition to the three binders even more IgE Abs can bind to Phl p 1.

Based on our results, we suggest that the large number and distinct spatial distribution of independent IgE epitopes that are recognized by high-affinity IgE Abs are reasons for the known high allergenic activity of Phl p 1 in terms of effector cell degranulation and induction of IgE-mediated immediate allergic inflammation. It is also quite tempting to speculate that the presence of multiple independent IgE epitopes on Phl p 1 contributes to the fact that it is the most potent sensitizing molecule in grass pollen that may be involved also in the proposed spreading of IgE sensitization toward other grass pollen allergens (18).

In summary, our study demonstrates simultaneous high-affinity IgE recognition of the major grass pollen allergen Phl p 1 as a possible explanation for its high allergenic activity and perhaps also for its important role in allergic sensitization to grass pollen. Furthermore, the isolated human IgE–derived ScFvs may be considered useful tools for diagnosis and eventually for allergen–specific targeted therapy approaches.

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