Supplementary material

Multiple independent IgE epitopes on the highly allergenic grass pollen allergen Phl p 5

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SUPPLEMENTARY MATERIALS AND METHODS

Recombinant antibody fragments

Genes encoding Phl p 5-specific antibody fragments (Supplementary Table S1) have been selected by phage display technology [E1-E4] from combinatorial antibody libraries created from transcripts encoding allergic patients' IgE as found in peripheral blood B lineage cells. All these genes (Supplementary Table S1) except one (clone 5) were obtained after selection in a form that encode a single chain fragment variable (scFv) product. A gene, optimised for production in human cells with respect to codon usage, encoding clone 5 in scFv format was synthesized by GenART (Invitrogen, Carlsbad, CA). The genetic origin and mutational status of the genes encoding the heavy chain variable domains of the originally isolated antibody fragments were re-analyzed using the IMGT V-QUEST web interface [E5] The first 23 nucleotides of the sequences were eliminated from the analysis as they are encoded for by PCR primers used in the process of creating the libraries from which the binders have been selected.

Production of scFv-Fc in HEK293 cell line

In order to produce scFv fused to the Fc fragment of human IgE and IgG_2, the scFv-encoding gene sequence was digested with *Sfi*I and *Not*I and ligated into a vector (pFUSE-hIgG-Fc2; kindly provided by Dr. Franck Perez (Institut Curie, CNRS UMR144, Paris, France)) carrying the gene encoding the second and third constant domains of human IgG_2 [E6] or a related vector encoding the second, third and fourth constant domains of IgE [E7], respectively. A single clone carrying each sequence was identified and the correctness of the cloned gene was confirmed by gene sequencing (GATC Biotech, Konstanz, Germany). The plasmid was purified from Top 10 *Escherichia coli* cells (Invitrogen) using the Qiaprep Spin Miniprep Kit (Qiagen, Hilden, Germany) according to the manufacturer's description. The vector was transfected using Lipofectamin 2000 (Invitrogen) into 90% confluent HEK293 cells grown in MEM medium (Invitrogen) containing 10 % fetal calf serum (Invitrogen). ScFv-Fc fusion proteins (molecular weights approximately 110 kDa) were retrieved in the form of culture supernatants after 3 days of culture and used in that form. Clone 5 scFv-Fcε could for unknown reasons not be produced in this manner and this product is therefore excluded from all analysis. 4.2 scFv-Fc was poorly produced (<5µg/ml) and it could not reproducibly be implemented in several of the below described assays. It has therefore been excluded from such assays.

Production of the C-terminal domain of Phl p 5.0101 and mutated versions thereof

Genes encoding the C-terminal half (residues 181-312) of Phl p 5.0101 were synthesized (codon-optimised for protein production in *Escherichia coli*) and cloned into the pGEX-6P-1

vector (GE Healthcare, Chalfont St. Giles, United Kingdom) by GenART. Genes encoding mutated variants of this protein (each carrying one of the following mutations: K246A, K270A, K280A) were synthesized and cloned in the same way. These vectors were transformed into the *E. coli* T7 Express strain (New England Biolabs, Ipswich, MA). Production of the GST-fused proteins was induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) in a growing culture at OD_{600}≈0.5. After 3 hours of culture at 37°C the cells were harvested by centrifugation at 7 500 x g for 12 minutes and treated with lysozyme (Sigma-Aldrich, St. Louis, MO). Soluble proteins were purified using affinity chromatography with GSTrap FF columns (GE Healthcare) and dialyzed against PBS prior to use.

Immunoassay to determine reactivity to recombinant allergens

Recombinant allergens, either in purified form (recombinant allergens and recombinant C-terminal domain of Phl p 5.0101 and mutants thereof) or present in extracts (C-terminal domain of Phl p 5.0201 and mutants thereof) were coated onto 96 or 384-well microtitre plates (Corning, Corning, NY). After washing with 0.9% (w/v) NaCl and 0.05% (v/v) Tween 20, scFv displayed on phage, recombinant scFv-Fcγ2 or scFv-Fcε diluted in TPBSB buffer (0.1% (v/v) Tween 20 and 2% (w/v) BSA in PBS) were added and plates were incubated for 1 h at 37°C. After washing bound antibodies/antibody fragments were detected using horseradish peroxidase (HRP)-conjugated anti-M13 mouse monoclonal antibody (GE Healthcare, Chalfont St. Giles, United Kingdom), goat anti-human IgG (Invitrogen) or anti-human IgE (KPL, Guilford, UK) using 1-Step Ultra TMB-ELISA Substrate (Thermo Scientific, Rockford, Illinois, USA) as the chromogen or the chemiluminescence substrate SuperSignal ELISA Femto Maximum Substrate (Thermo Scientific). Absorbance and luminescence were read in a FLUO star OMEGA microtitreplate reader (BMG-Labtech, Ortenberg, Germany). In competition assays immobilized allergens were pre-incubated with scFv-Fc fusion proteins before being incubated with a scFv fused to an Fc of another isotype (IgE or IgG_2). Binding of the second antibody was finally assessed as described above.

Analysis of the inhibition of binding of scFv displayed on phage to immobilized allergen Phl p 5.0201 by soluble allergen derivatives (C-terminal domain or mutated versions thereof) was made by a similar methodology. In this case the phage solution was pre-incubated at 37°C for 1 h with different concentrations of soluble allergen or the C-terminal domains of Phl p 5.0201 prior to addition to a microtitre plate coated with.Phl p 5.0201. Detection of bound phages was performed as described above. ScFv p5b-7 (identical to p5-MA5) and scFv p5b-31 (identical to scFv 4.12 except for its use of a different light chain sequence) selected for binding to Phl p5.0201, both of which also cross-react to Phl p 5.0101) (data not shown) were used in this investigation.

Phl p 5 sandwich assay to assess epitope recognition

The relationship of epitopes detected by different binders was also assessed by a sandwich ELISA approach. Goat anti-human IgG (Invitrogen) was coated onto 96-well microtitre plates. After washing as above, recombinant scFv-Fcγ2 (0.5 µg/ml) diluted in TPBSB buffer were added and plates were incubated for 1 h at 37°C. Unbound scFv-Fcγ2 was washed away and the wells were incubated for 1 h at 37°C with recombinant Phl p 5.0101 (0.5 µg/ml) in TPBSB buffer. After another wash step, recombinant scFv-Fcε (25 kU/L as determined by an ImmunoCAP IgE assay (Phadia AB, Uppsala, Sweden)) diluted in TPBSB buffer was added and plates were incubated for 1 h at 37°C. Finally, after washing, bound scFv-Fcε was detected with goat anti-human IgE as described above using luminescent substrate.

Epitope mapping using peptide and allergen-specific anti-sera

Rabbit anti-sera had been obtained by immunization of 8 rabbits (Charles River Laboratories, Kislegg, Germany) with either purified recombinant Phl p 5.0101 or with seven different peptides spanning most of the N- and C-terminal domains of Phl p 5.0101 [E8]. ELISA plates (MaxiSorp, Nunc, Roskilde, Denmark) were coated with Phl p 5.0101 (100 ng/well in 0.05 M sodium bicarbonate (pH 9.6)) overnight. The plates were washed twice with PBST (PBS containing 0.05% v/v Tween 20) and blocked with PBST containing 1% w/v BSA at 37°C for 2 h. After blocking, the wells were pre-incubated for 5 h at 37°C with either one of seven different anti-peptide anti-sera diluted 1:100 in PBST/0.5% BSA, or with a Phl p 5-specific anti-serum diluted 1:100 in PBST/0.5% BSA, or, for control purposes, with buffer alone. After washing five times with PBST, the plates were incubated overnight at 4 °C in duplicates with different cell culture supernatants containing human Phl p 5-specific scFv-Fcγ2 fusion proteins in PBST/0.5% w/v BSA. After washing five times with PBST, the binding of scFv-Fcγ2 fusion protein was assessed using horseradish peroxidase-conjugated rabbit polyclonal anti-human IgG antibody (Dako, Glostrup Denmark) diluted 1:2000 in PBST/0.5% w/ BSA for 1 h at 37°C using ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) *a*s the chromogen. Absorbance at 405 nm (reference filter, 490 nm) was determined with an ELISA reader (Spectramax PLUS; Molecular Devices, Sunnyvale, CA). The results represent means of duplicate determinations with variations of less than 10%.

Blocking serum IgE allergen binding with recombinant scFv-Fcγ2

The inhibitory capacity of recombinant antibody fragments on allergic patients IgE binding was studied by ELISA inhibition studies [E9-E11]. ELISA plates (MaxiSorp) were coated with Phl p 5.0101 (20 ng/well in 0.05 M sodium bicarbonate (pH 9.6) overnight. The plates were washed twice with PBST and blocked with PBST containing 1% BSA at 37°C for 3 h. After

blocking, the wells were incubated overnight at 4°C with either 1) a mix of cell culture supernatants containing four human Phl p 5-specific scFv-Fcγ2 (clone 5, 4.3, 4.13 and P5-AB5), each binding an independent epitope (see Table 1 and Supplementary Figure S1) on either the N- or the C-terminal domain of Phl p 5. The different scFv-Fcγ2 were present each at 7 µg/ml (to guarantee 10 times excess) in the final mixture ensuring excess of competitors over allergen; 2) a cell culture supernatant containing the monoclonal Bet v 1-specific antibody Bip 1 [E12] diluted 1:1; 3) a Phl p 5-specific rabbit antiserum at a concentration of 10 µg Phl p 5-specific IgG/ml; 4) a Bet v 1-specific rabbit antiserum [E13] at a concentration of 10 µg Bet v 1-specific IgG/ml. Each of these samples had been diluted in PBST containing 0.5% BSA and competitors were always in excess to allergen. After incubation sera from grass pollen allergic patients (characterized by clinical history, positive skin prick test, and presence of grass pollen extract-specific IgE in serum) (final dilution: 1: 10) were added without prior removal of the blocking scFv-Fcγ2. After incubation (1 h, 37°C) bound IgE antibodies were detected with an alkaline phosphatase-conjugated mouse monoclonal anti-human IgE antibody (BD Pharmingen, NJ, USA) using 4 Nitrophenyl phosphate disodium salt hexahydrate (Sigma-Aldrich, St. Louis, Mo, USA) as the chromogen. Absorbance at 405 nm (reference filter, 550 nm) was determined with an ELISA reader (Spectramax PLUS). The absorbance values corresponding to IgE represent means of duplicate or triplicate determinations with variations of less than 10%.

To further investigate binding of patients' IgE antibodies to Phl p 5 already bound to several scFv-Fc, allergen/scFv-Fcγ2 complexes were preformed in solution and then exposed to sera of grass pollen allergic patients. In detail, 5 µg/ml goat anti-human IgG (Invitrogen) in 0.05 M sodium bicarbonate (pH 9.6) was coated onto ELISA plates (MaxiSorp). The plates were washed twice with PBST and blocked with PBST containing 1% BSA at 37°C for 3 h. In parallel, complexes consisting of 0.1 µg/ml Phl p 5 and 3.5 µg/ml clone 5 or of 0.1 µg/ml Phl p 5 and a mixture of four scFv-Fcγ2 (clone 5, 4.3, 4.13 and p5-AB5, each at 3.5 µg/ml) were preformed in tubes at room temperature. These complexes were loaded onto wells containing immobilized anti-human IgG and incubated over night at 4°C. After washing five times with PBST, the plates were incubated in triplicates with sera from grass pollen allergic patients diluted 1:10 in PBST/0.5% BSA for 1 h at 37°C. Bound IgE antibodies were detected with an alkaline phosphatase-conjugated mouse monoclonal anti-human IgE antibody (BD Pharmingen), as described above.

Affinity determination

The affinity of scFv-Phl p 5.0101 interaction was determined using a surface plasmon resonance based methodology on the MASS-1 platform (Sierra Sensors, Hamburg, Germany). Briefly, a polyclonal goat anti-human IgG antiserum (Invitrogen) was immobilized

onto a High Capacity Amine sensor (Sierra Sensors). ScFv-Fcγ2 (30-128 RU) was caught on the chip's B surface. Its A surface was run without immobilized scFv-Fcγ2 and used for background subtraction. PBS containing 0.005% Tween 20 was used as running buffer. Phl p 5.0101 (1.5-400 nM) was injected for 2 minutes over both surfaces with a flow rate of 25 µl/min. The dissociation phase was monitored for 5 minutes at the same flow rate. Flow channels were regenerated using 0.1 M HCl. Reaction rate kinetics and affinity constants were calculated using a 1:1 binding with mass transport limitations model using the AnalyserR2 version 0.1.5.0 software (Sierra Sensors).

Supplementary Table S1. Genes encoding and summary of properties of Phl p 5-specific IgE[a]

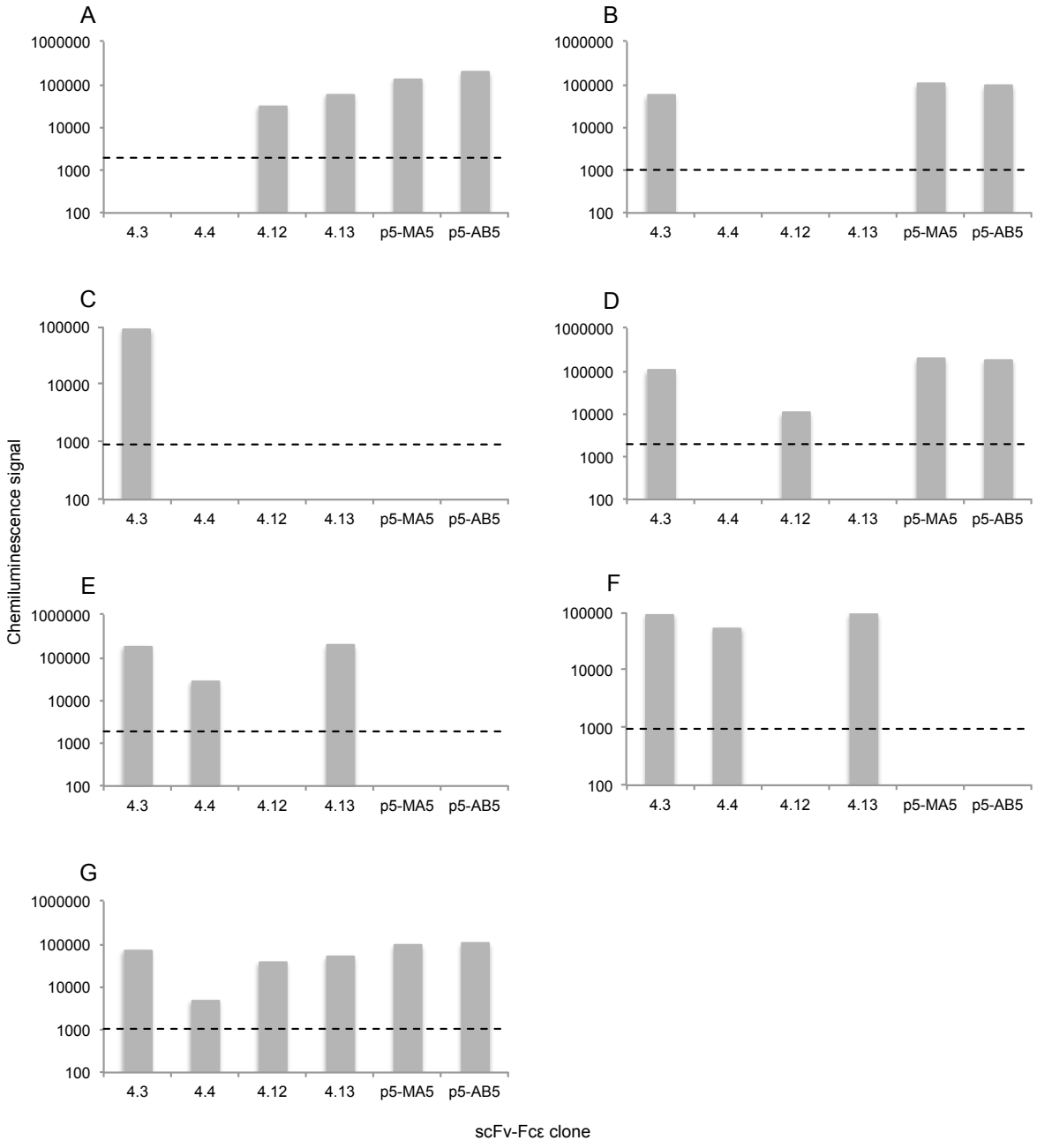
Clone	Selecting antigen	Heavy chain variable gene	Mutation frequency	CDRH3 length	Domain reactivity[b]	Isoallergen reactivity		GenBank accession number	Binding constants[c] to Phl p 5.0101 (M^{-1})			Reference
						01	02		k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_A (M^{-1})	
4.2	Phl p 5.0101	IGHV5-a	5.3	20	N	+	-	DQ065730	ND[d]	ND	ND	E1
4.3	Phl p 5.0101	IGHV3-11	6.8	15	N	+	+	DQ065731	3.2×10^{5}	9.4×10^{-4}	3.4×10^{8}	E1
clone 5	Phl p 5.0101	IGHV3-30 / 3-30-3	12.4	17	N	+	-	X95746 and X95747	1.2×10^{6}	2.3×10^{-4}	5.3×10^{9}	E4
4.4	Phl p 5.0101	IGHV3-23	5.3	21	C	+	-	DQ065732	4.0×10^{4}	2.8×10^{-2}	1.4×10^{6}	E1
4.13	Phl p 5.0101	IGHV1-18	5.3	23	C	+	-	DQ065735	4.9×10^{5}	1.3×10^{-1}	3.9×10^{6}	E1
4.12	Phl p 5.0101	IGHV1-69	6.4	20	C	+	+	DQ065734	2.3×10^{4}	1.9×10^{-3}	1.2×10^{7}	E1
p5-AB5	Phl p 5.0101	IGHV3-23	2.3	16	C	+	+	EF601887	2.5×10^{5}	2.7×10^{-4}	9.4×10^{8}	E3
p5-MA5	Phl p 5.0101	IGHV1-18	3.8	15	C	+	+	EF601894	6.9×10^{5}	2.4×10^{-2}	2.9×10^{7}	E3
p6-12	Phl p 6.0101	IGHV3-21	3.0	13	*	+	-	EF607121	ND	ND	ND	E2
p6-19	Phl p 6.0101	IGHV3-11	4.2	9	*	+	ND	EF607122	ND	ND	ND	E2

[a] For many of these clonotypes, several sequence variants are available. In such cases one clone was selected for this study.

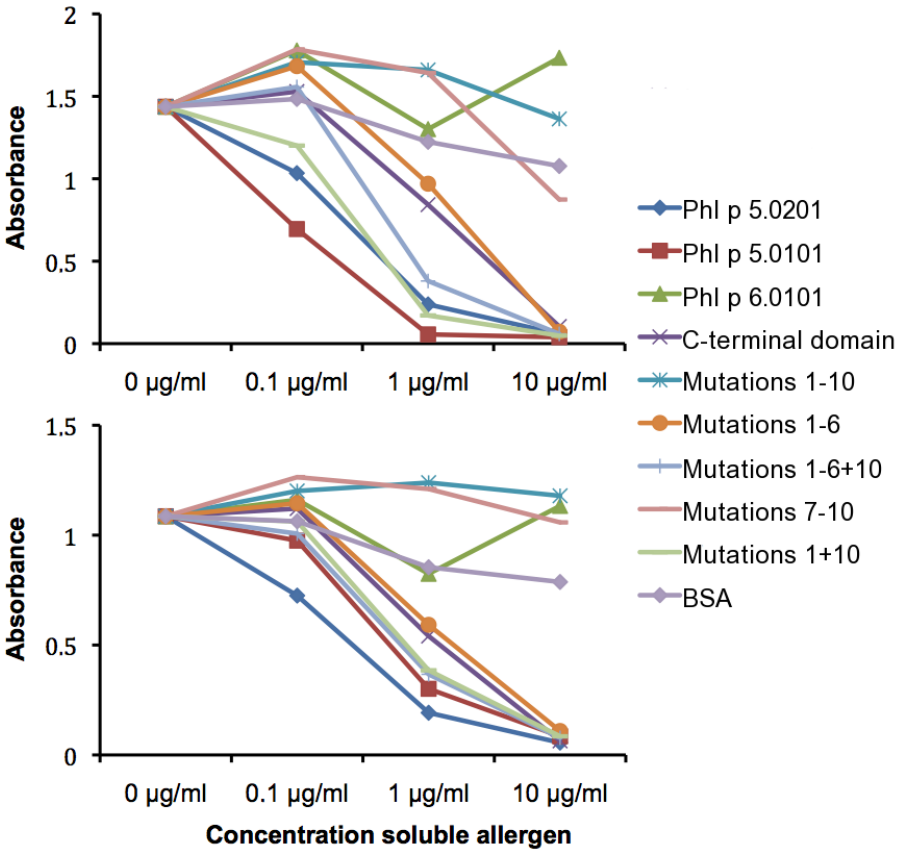
[b] N, C indicates binding the N-terminal or C-terminal domain of Phl p 5, respectively; *: not tested but homology would suggest that it recognizes the N-terminal domain of Phl p 5.

[c] Determined using the scFv-Fcγ2 construct.

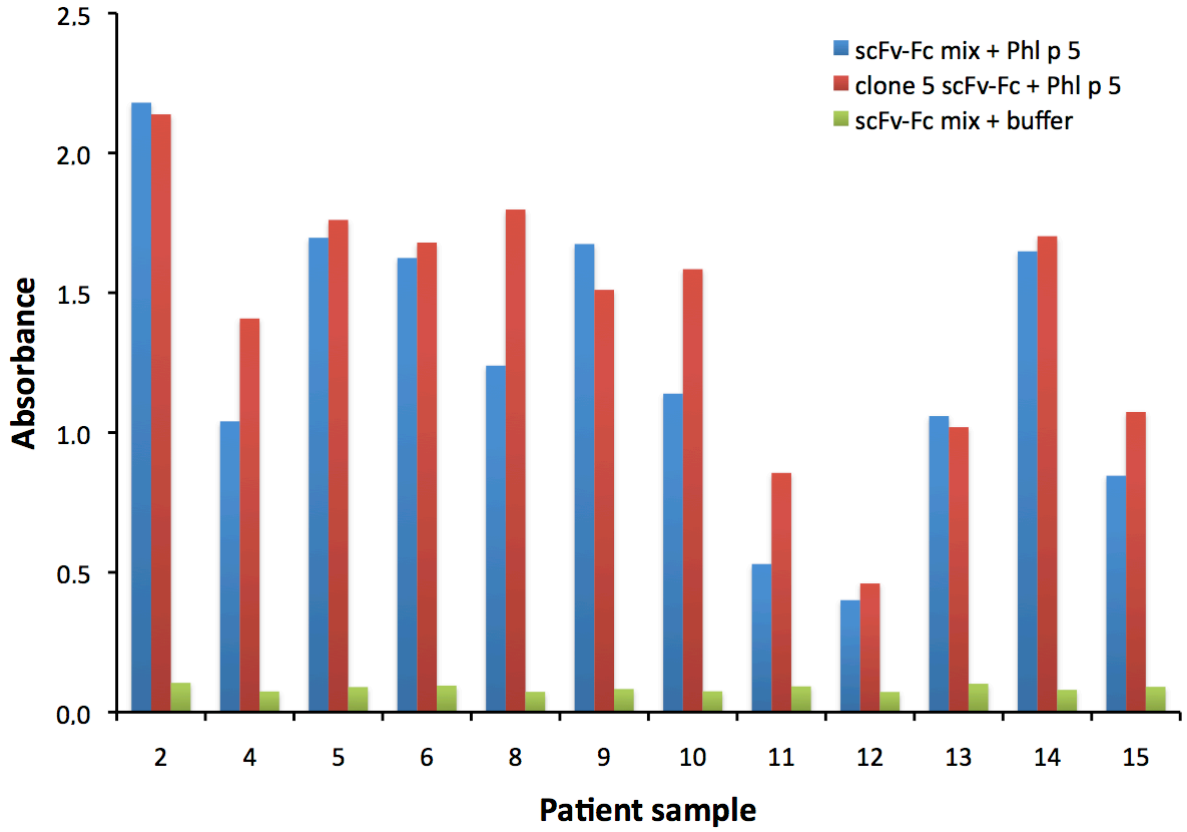
[d] not determined



Supplementary Figure S1. Sandwich immunoassay detecting the ability of a second antibody (scFv-Fcε) to bind allergen caught on an immobilized antibody (scFv-Fcγ2). Immobilized antibodies 4.3 (A), 4.4 (B), 4.12 (C), 4.13 (D), p5-AB5 (E), p5-MA5 (F), and clone 5 (G) were investigated. Dashed lines denote a signal intensity corresponding to 1% of the maximum signal obtained in each assay. Background signal is deducted and signals less than twice the background are not shown. The results represent means of duplicate determinations with coefficient of variation less than 12%.



Supplementary Figure S2. Blocking of the binding of scFv P5b-7 (identical to p5-MA5) (top) and P5b-31 (a sequence variant of 4.12 that uses a light chain different from that found in 4.12) (bottom) (both selected by phage display for binding to Phl p 5.0201 (data not shown)), displayed on phage, to immobilized Phl p 5.0201 by pre-incubation of the displayed antibody fragment with recombinant allergens or recombinant C-terminal domain of Phl p 5.0201 or mutated variants thereof (1: K149A; 2: K160A; 3: K166A; 4: K190A; 5: K193A; 6: K204A; 7: K214A; 8: K238A; 9: K248A; 10: K283A). The results represent means of duplicate determinations with variations of less than 8%.



Supplementary Figure S3. Binding of serum IgE from 12 grass pollen allergic donors to complexes of Phl p 5.0101 and one (clone 5; red) or four (clone 5, 4.3, 4.13, and p5-AB5; blue) scFv-Fcγ2 fusion proteins that had been caught on immobilized anti-human IgG.

Supplementary Figure S4. Sequence similarities (light grey shading) and identities (dark grey shading) between the N-terminal sequence of Phl p 5.0101, and the N-terminal parts of the N- and C-terminal domains of Phl p 5.0101.

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