

Mapping of conformational IgE epitopes on Phl p 5a by using mimotopes from a phage display library

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Background: Phl p 5 represents a major allergen of timothy grass pollen (*Phleum pratense*). Detailed knowledge about the structures responsible for IgE binding would allow the design of a novel generation of allergy vaccines.

Objective: We aimed to characterize the IgE epitopes of Phl p 5a using phage display combined with a molecular modeling approach.

Methods: Phl p 5a-specific IgE from sera of patients with grass pollen allergy was used for screening of a random peptide phage library displaying constrained decamers.

Results: Fifteen phage clones that shared sequence motifs and could be grouped into families were selected by using Phl p 5a-specific IgE. Peptide alignment with the solvent-accessible amino acids of Phl p 5a revealed 3 sequence sections with frequent hits of identical or similar amino acids. On the surface of Phl p 5a, these sections assembled in compact patches, most likely representing conformational IgE epitopes, whereas no matching clusters were found on the back sides of the 2 Phl p 5a halves. In surface plasmon resonance experiments, the high-affinity interaction between IgE and Phl p 5 could be competed by phage-displayed peptides up to 24%, indicating that they represent true epitope mimics (ie, mimotopes). Allergen-specific immunogenicity of the mimotopes was proved in Biozzi mice. **Conclusion:** The selected mimotopes facilitated the localization of conformational IgE epitopes of Phl p 5. We suggest them to be suitable candidates for the development of an epitope-specific immunotherapy. (J Allergy Clin Immunol 2004;114:1294-300.)

Key words: Phl p 5, major grass pollen allergen, IgE, B-cell epitope, conformational epitope, phage display, mimotopes, molecular modeling

An estimated 50% of all patients with type I allergy are sensitized to grass pollen proteins. These allergens are

Abbreviations used

AR repeat: Arginine-rich repeat
cfu: Colony-forming units
WT: Wild type

potent elicitors of clinical symptoms, such as rhinitis, conjunctivitis, and asthma.¹ Phl p 5 represents one of the major pollen allergens of timothy grass (*Phleum pratense*) and accounts for IgE binding in up to 60% of patients with grass pollen allergy.² It exists naturally in 2 isoforms, Phl p 5a and Phl p 5b, which exhibit high sequence similarity but differ slightly in their molecular mass and biochemical behavior.^{3,4} Their function has largely remained unclear, even though ribonuclease activity has been proposed for Phl p 5b.⁵ Phl p 5 is highly homologous to group 5 allergens from other grass species, as well as to the group 6 allergen Phl p 6. The isoproteins are composed of 2 alanine-rich (AR) repeats, which form 4 α -helices each. Until now, no sequence similarities to any protein with known function were found, suggesting that group 5 and 6 allergens represent a distinct protein class of so-called 4-helix-bundle allergens.⁶

The IgE-binding capacity of Phl p 5b could be reduced by deleting parts of the molecule,⁷ suggesting the existence of continuous, as well as conformational, IgE epitopes. During recent years, phage display has become a useful tool to select mimotopes (ie, epitope mimics) from random peptide phage libraries comprising a huge repertoire of potential ligands.^{8,9} The mimotope sequences enable alignments with the antigen and the subsequent localization of structural B-cell epitopes.¹⁰⁻¹³ Furthermore, mimotopes can be used to design peptide vaccines for the direct induction of blocking antibodies, thereby excluding anaphylactogenic IgG specificities.^{14,15} The aim of our present work was to generate mimotopes of Phl p 5a, map them to its solvent-accessible surface, and examine their immunogenicity.

METHODS

Biopanning of phage display library and colony screening

Phl p 5a-specific IgE was purified from patient sera by means of affinity chromatography, as described previously,¹⁶ and the quality

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TABLE I. Characteristics of IgE-binding peptides selected from a constrained decameric random phage display library

Clone no.	Peptide sequence*	Frequency†	IgE reactivity‡	Relative inhibition (%)§	Control inhibition (%)§
Acidic elution					
F1	SRLGRSSAWV	9/21	a	14.7 ± 3.4	3.8 ± 2.5
F2	THWQLGERPD	1/21	b	4.4 ± 1.1	ND
F3	PSTPGERVRH	1/21	c	9.8 ± 10.8	ND
F4	RGGPDDLTAL	1/21	c	1.3 ± 2.8	ND
F5	PFWVRGTTW	2/21	c	17.5 ± 7.0	ND
F12	PSTPGSRQNM	1/21	c	ND	ND
F17	PSTPGDNPLV	1/21	c	7.9 ± 2.4	ND
F25	KFVVNGRWID	1/21	b	ND	ND
F26	KFLVNGRWID	1/21	b	10.0 ± 12.4	2.7 ± 1.8
F29	RLTENTEPLL	2/21	a	17.8 ± 7.8	2.2 ± 3.2
F50	FTWGGLRDKS	1/21	c	ND	ND
Competitive elution					
H4	ERAGAMERAN	22/25	b	7.2 ± 6.8	3.3 ± 1.4
H22	RSVSKEEPM	1/25	b	10.9 ± 2.6	2.6 ± 4.6
H32	KLKGFGAARV	1/25	a	23.8 ± 10.1	2.9 ± 3.4
H56	VQDLMKSSGV	1/25	b	5.5 ± 9.6	ND
Phage cocktail					
WT-phage	None		c	26.9 ± 7.6	4.3 ± 2.3
				1.0 ± 2.1	2.0 ± 1.3

ND, Not determined. The results are the means ± SD of 3 inhibition experiments.

*The flanking constraining cysteine residues are omitted here.

†From the most IgE-reactive phage clones, 21 clones from the acidic and 25 clones from the competitive elution method were isolated and single-strand DNA sequenced. The number of times each sequence was identified is given.

‡Reactivity of phage-displayed mimotopes to Phl p 5a-specific IgE in ELISA: a, OD of greater than 0.55; b, OD of between 0.4 and 0.55; and c, OD of less than 0.4.

§The percentage of inhibition of IgE binding specific for Phl p 5a or Bet v 1, respectively, was measured in BIACORE and calculated as follows:

$$\%Inhibition = R_{eq} (IgE + phage) \times 100 R_{eq} (IgE)$$

was checked in ELISA, SDS-PAGE, and immunoblotting experiments. For control, Bet v 1-specific IgE was used. The constrained random decapeptide library CL10, displaying a diversity of 1.04×10^9 different clones, was a kind gift of Dr L. Mazzucchelli (Bern, Switzerland).¹⁷ Biopanning was performed as described previously, coating Phl p 5a IgE on microtiter plates (Maxisorp; Nunc, Roskilde, Denmark).¹⁸ Bound phages were eluted either with glycine buffer (0.1 mol/L, pH 2.2) or competitively by using 10 µg/mL recombinant Phl p 5a in 10 mM Hepes/150 mM NaCl, pH 7.2. After 3 rounds of selection, 60 phage clones from each elution method were chosen, amplified, and tested for reactivity with Phl p 5a-specific IgE by means of capture ELISA, as described below. Phage clones with OD values of greater than the mean ± 3 SDs of the background control were chosen for further analyses.

Phage sequence analysis and peptide alignments

Single-strand phage DNA was extracted (QIAprep Spin M13 Kit; QIAGEN, Hilden, Germany) and subjected to sequencing (IBL, Vienna, Austria) by using the primer 5'-gtaccgtaacactgagtttcg-3' (MWG-Biotech, Ebersberg, Germany). Sequence comparisons with Phl p 5a (GI: 1171011) and Phl p 6 (GI: 2851456) were done by using the AlignX module of the Vector NTI Suite software (InforMax, Inc, Frederick, Md), which uses a modified Clustal W algorithm¹⁹ to create multiple sequence alignments. The solvent accessibility of Phl p 5a amino acid side chains was calculated by using the Swiss-PdbViewer program suite.

Molecular modeling of Phl p 5a

The 2 α-helix bundles of Phl p 5a were modeled separately by using X-ray diffraction data of Phl p 6²⁰ and Phl p 5b⁶ as templates for the N-terminal and C-terminal AR repeat, respectively (Brookhaven Protein Data Bank).²¹ The sequences were submitted to ProModII for

model building. The obtained models were examined for unfavorable van der Waals' overlaps, which were removed by means of minimal rotation around side-chain torsion angles. The quality of the models was evaluated with the WHAT_CHECK validation package,²² and loops in the problematic regions were modeled individually. The final models were energy minimized with the GROMOS 96 implementation in the Swiss-PdbViewer program suite.²³

Phage ELISAs

For capture ELISA, Phl p 5a-specific IgE, human myeloma IgE (PS-IgE; from K. and T. Ishizaka, La Jolla, Calif) and human monoclonal IgE (SUS11-IgE, purified from a hybridoma supernatant),²⁴ a gift from Professor B. Stadler (Bern, Switzerland), were coated (0.01 µg/mL). Monoclonal Phl p 5-specific Fab²⁵ was coated at 1:10 dilution. After blocking, plates were incubated with individual phage clones (1×10^8 colony-forming units [cfu]/well), wild type (WT)-phage, or buffer. For titration ELISA, plates were incubated with serial dilutions (1:10-1:106) of phage particles or with a phage pool composed of equal titer amounts of individual clones, starting at an equipotent titer of 3.85×10^{10} cfu/mL. Washing and developing was done, as described previously.¹¹

Surface plasmon resonance studies

Experiments were performed on a BIACORE X instrument (BIAcore, Uppsala, Sweden). CM5 sensor chips for standard amino-coupling procedure were used. Recombinant Phl p 5a or Bet v 1 were injected in 10 mM sodium acetate, pH 4.0, for coupling. All experiments were carried out at 20°C at a flow rate of 5 µL/min. Phage inhibition was performed with constant amounts of allergen-specific IgE incubated with equal amounts of phage clones (approximately 5×10^9 cfu of phage particles) or with a cocktail of all 12 investigated clones in a 1:1 ratio. Sensorgrams were fitted to a single-site binding model (1:1 Langmuir binding) with BIAevaluation 3.1 software. The degree of inhibition was calculated from the decrease

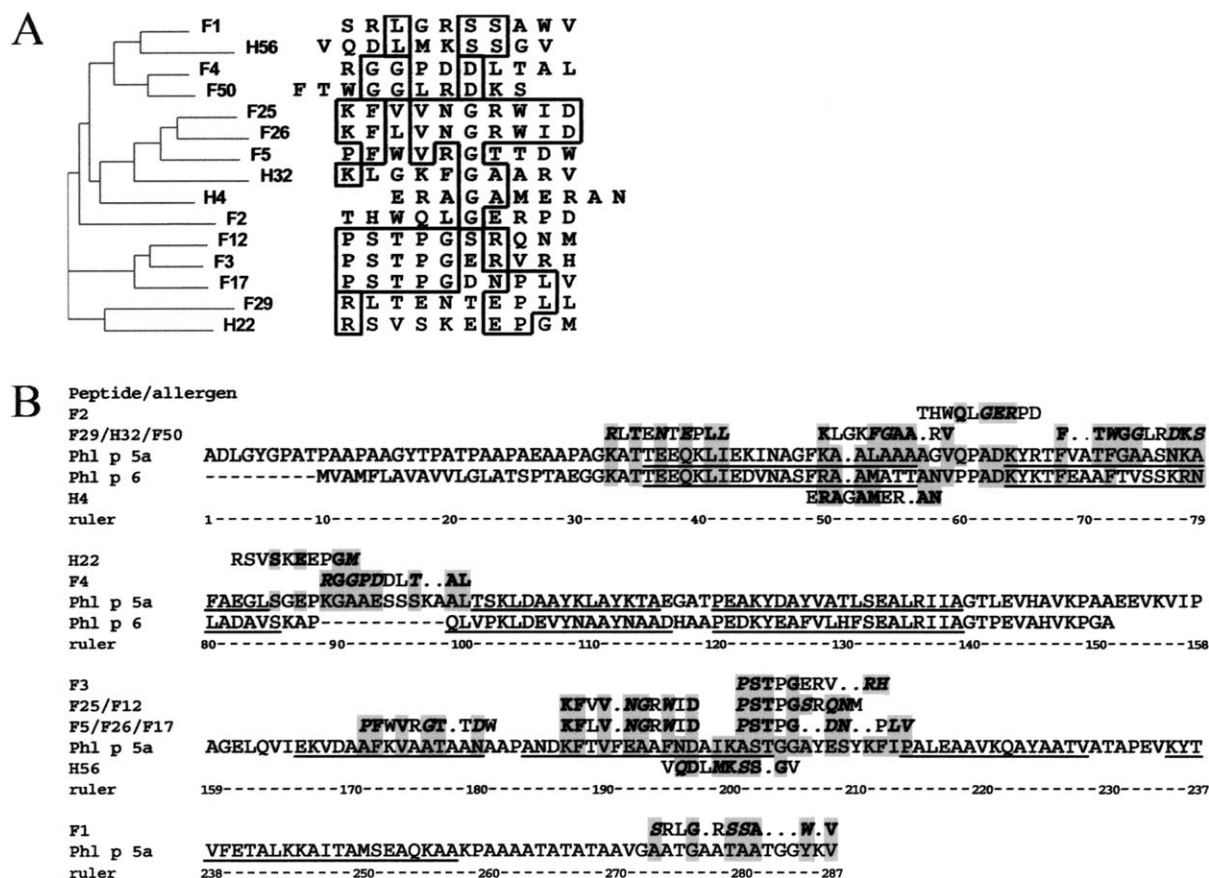


FIG 1. A, Phylogenetic tree indicating the degree of similarity between the selected peptides. Identical residues are boxed. **B**, Alignment of peptides with the amino acid sequences of Phl p 5a and Phl p 6. Identical residues are indicated in bold, and physicochemically related amino acids are indicated in bold-faced italics. Additionally, identical and related amino acids are boxed in gray. Predicted α -helices are underlined.

of the steady-state binding value R_{eq} compared with the R_{eq} value of the same IgE concentration without phage.

Mouse immunizations

CsCl-purified phage samples²⁶ of clones F1, F29, H32, and WT-phage were injected into 3-month-old Biozzi mice (Harlan-Winkelmann, Blackthorn, United Kingdom) intraperitoneally ($n = 5$ per clone). One hundred-microliter samples containing $10^{1.3}$ phage particles in 0.9% NaCl solution were injected at days 0, 14, and 28, and blood samples were obtained on days 0, 21, and 32. Experiments were performed according to European Community rules of animal care and with permission 66009/117-Pr/4/00 of the Austrian ministry of Science. Antibody titers to Phl p 5a or Bet v 1 were determined in ELISA, as described previously.²⁷ The statistical significance of the results was calculated by using the Student t test.

RESULTS

Sequence analysis and alignment of IgE-binding peptides

Forty-six phage clones reactive with Phl p 5a-specific but not Bet v 1-specific IgE (data not shown) were selected for sequencing and rendered 15 different peptide sequences (Table I). The sequences were grouped into families

according to their degree of similarity (Fig 1, A) and were aligned with the primary sequence of Phl p 5a, taking the solvent accessibility of its amino acids into consideration (Fig 1, B). Three regions, in which the peptides matched in a cumulative manner, could be identified as follows: (1) amino acid residues 33-100; (2) amino acid residues 171-214; and (3) amino acid residues 273-287 at the C-terminus. Alignments of peptides were also seen with Phl p 6 (Fig 1, B).

Mapping of IgE epitopes to the modeled structure of Phl p 5a

The peptide alignments were assigned to the Phl p 5a structure. The first peptide-matching region (amino acids 33-100) stretches from α -helix 1 and extends to the loop region between α -helices 3 and 4 (Fig 2, A). Through the up-down topology of this motif, amino acids 33-42 get into close vicinity with amino acids 82-100, forming a continuous surface patch (Fig 2, B). Similarly, amino acids 49-79 build a closed region on the molecular surface (Fig 2, B). The reverse side remained free from alignments (Fig 2, C). On the C-terminal AR repeat, the residues of the second alignment region (amino acids 171-214) again come close (Fig 2, D), also forming a compact area on the

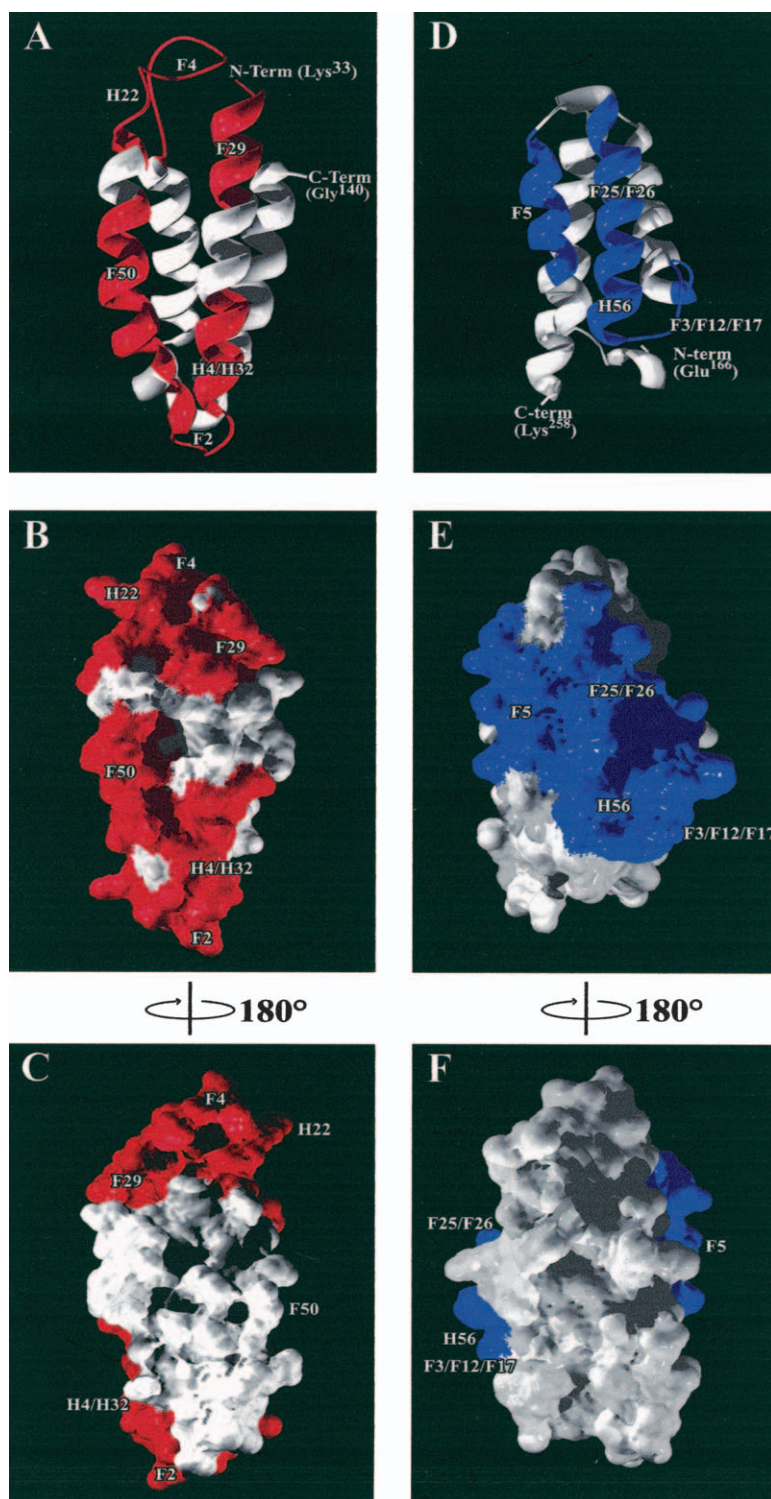


FIG 2. Molecular modeling and IgE epitope mapping on the 2 AR repeats of Phl p 5a. **A** and **B**, Ribbon diagrams visualizing the AR repeats. **C** and **D**, Molecular surface representations. Alignment regions with the peptides (designations correspond to clone numbers in Table I) are highlighted in red (AR repeat 1) and blue (AR repeat 2), respectively. **E** and **F**, One hundred eighty degree rotation of the 2 domains.

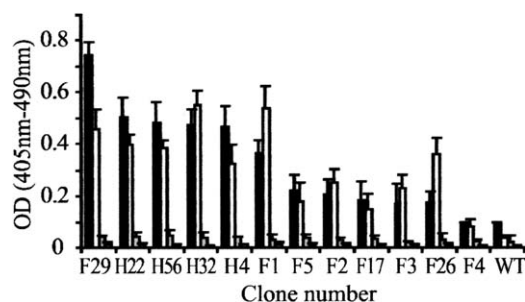


FIG 3. Phage-displayed peptides bind specifically to a monoclonal Phl p 5a-reactive Fab fragment (black columns) and to Phl p 5a IgE (white columns) but not to the isotype controls PS- and SUS11-IgE (gray columns). Twelve individual phage clones (1×10^8 cfu/mL) and WT-phage lacking an insert were added to the coated wells. The data represent the mean $\pm$ SD of 4 individual assays.

molecular surface (Fig 2, E). As before, no alignments with residues on the opposite side of the domain could be found (Fig 2, F). The presumptive IgE epitope at the C-

terminus (amino acids 273-287) could not be visualized because of a lack of structural data.

Specificity and mimicry of phage-displayed mimotopes

In capture ELISA phage clones bound exclusively to a Phl p 5a-specific monoclonal Fab antibody and to Phl p 5a-specific IgE but not to human PS- and SUS11-IgE antibodies (Fig 3). The specificity of 6 highly reactive phage clones was ascertained by titrating them against Phl p 5a-specific IgE (Fig 4, A and B) in comparison with control phage (WT-phage). A cocktail of phage clones rendered the mean value of single clone reactivities (Fig 4, C). The structural mimicry of the selected peptides with the IgE epitopes was tested in competition assays by using surface plasmon resonance (Table I and Fig 4). Individual phage clones were able to decrease the binding of specific IgE to Phl p 5a (Fig 4, D-F) but not to the control antigen Bet v 1 (Fig 4, G-I). A cocktail of clones led to the highest value of

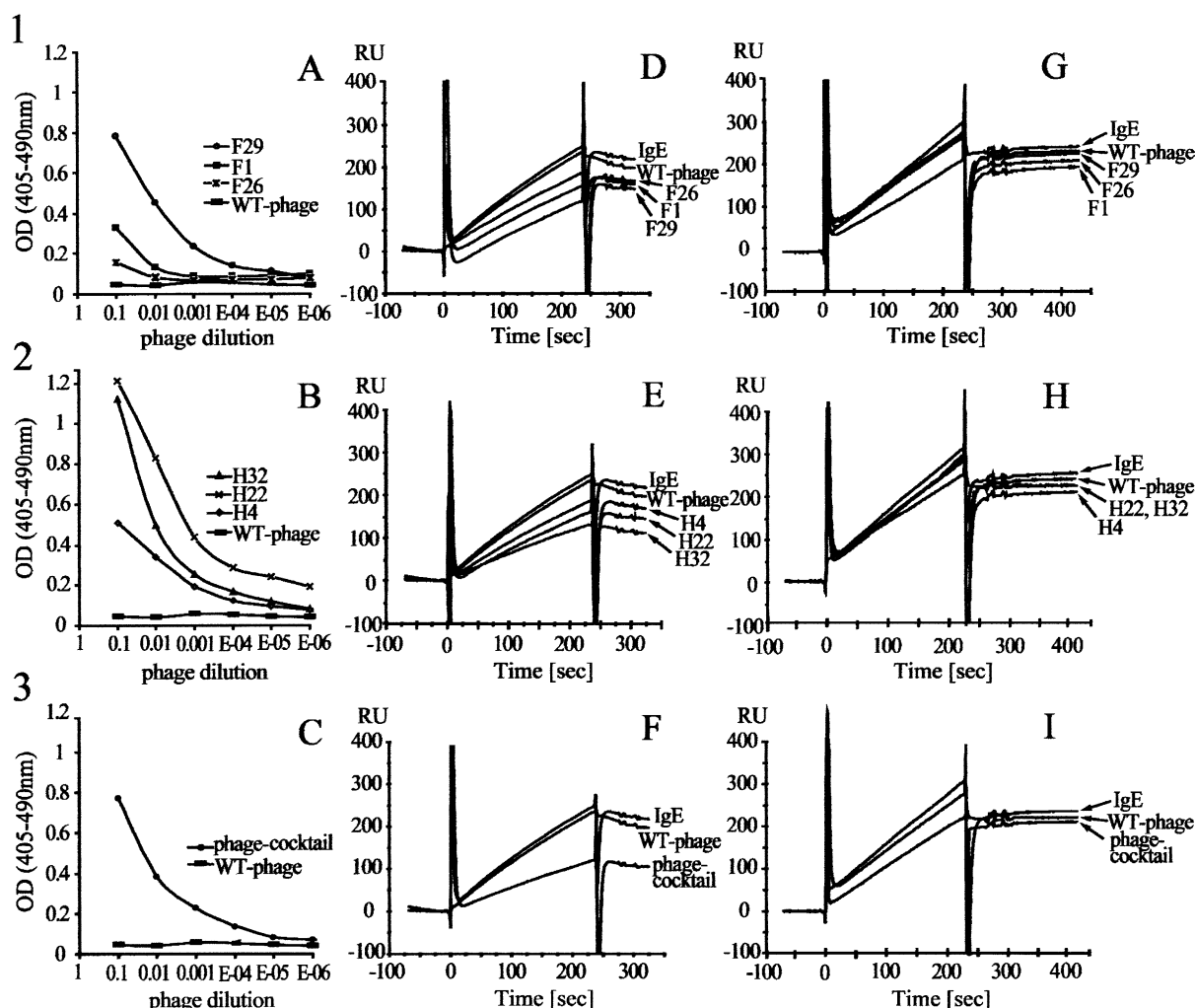


FIG 4. A-C, Phage clones of the acidic (1) and competitive (2) elution method and a phage cocktail (3) tested for IgE specificity in ELISA. D-F, BIAcore sensorgrams of Phl p 5-specific IgE binding to Phl p 5a. G-I, Bet v 1-specific IgE binding to Bet v 1 in the absence of phages (IgE), in the presence of WT-phage, and in the presence of phage clones. One typical sensorgram of 3 individual measurements is shown.

26.9% relative inhibition (Table I and Fig 4, F), whereas WT-phage did not interfere with IgE binding to Phl p 5a.

Immunogenicity of phage-displayed peptides

Phagotopes F1, F29, and H32, resulting in the highest inhibition, were injected into high-responder Biozzi mice.²⁸ All animals had strong and comparable responses against the phage proteins, indicating a successful immunization (data not shown). Sera were tested for allergen-specific immune response: mice immunized with the phage clones F1 and F29 produced antibodies against Phl p 5a, whereas injection of phage clone H32 and WT-phage did not induce Phl p 5-specific antibodies (Fig 5, A). None of the mouse sera recognized Bet v 1 (Fig 5, B).

DISCUSSION

In the present study we have used affinity-purified human Phl p 5a-specific IgE to select 15 different mimotopes. They reacted specifically with Phl p 5a-specific IgE, as well as with a human monoclonal anti-Phl p 5 Fab antibody,²⁵ excluding the undesired enrichment of peptides interacting with Fcε domains.

Comparison of the selected peptides with the primary sequence of Phl p 5a revealed 3 alignment hot spots. In the first Phl p 5a AR repeat, we localized 2 IgE epitopes. Amino acids 33-42 represent a continuous IgE epitope of the highly homologous grass pollen allergen Hol 15.²⁹ On the structural model of Phl p 5a, it became apparent that amino acids 33-42, together with amino acids 82-100, presumably compose a larger discontinuous epitope. The second alignment residues, amino acids 49-79, also form a compact surface patch. The existence of this IgE epitope is supported by dot-blot and mutation studies of Lol p 5.^{30,31} In the second domain of Phl p 5a, we localized a third IgE epitope. Deletions of Phl p 5b and Hol 15⁷ and amino acid substitutions of Lol p 5³¹ in this region reduced IgE binding. An additional IgE epitope could be located at the C-terminus, which has not yet been resolved structurally. The existence of this presumptive epitope is consistent with deletion analyses of Hol 15²⁹ and Lol p 5.^{30,31} No alignments of mimotopes were found along amino acids 101-170, containing the 26-amino-acid-long linker region between the 2 AR repeats. The absence of IgE epitopes agrees with data obtained with fragmented Phl p 5a.³ This region is prone to degradation,^{32,33} which could represent the reason for problems when resolving the structure of Phl p 5. Interestingly, the reverse sides of the AR repeats revealed no antigenic sites. It is tempting to speculate that interaction between the 2 domains could take place here, composing whole Phl p 5 in a back-to-back position. Taken together, IgE-captured peptides helped us to reveal conformational epitopes of Phl p 5a. Because group 5 allergens are highly conserved,^{29,34} our results might account for homologues in other grass species. Furthermore, there is evidence that Phl p 5 and Phl p 6 might share IgE epitopes.^{35,36} Phl p 5 mimotopes aligned with linear regions of Phl p 6, exactly matching the Phl p 6

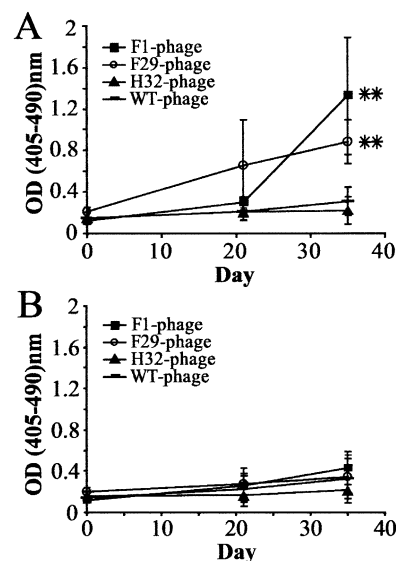


FIG 5. IgG reactivity of Biozzi mice immune sera elicited by phagotopes F1, F29, H32, and WT-phage toward Phl p 5a (A) and Bet v 1 (B). The x-axis shows the time schedule of blood taking. The y-axis shows mean IgG reactivities and SDs of mouse groups (n = 5), testing mice individually. ** $P < .05$ for F1- and F29-phage immune sera on day 35 compared with preimmune sera and WT-phage sera on day 35.

IgE binding domain (National Center for Biotechnology Information database).³⁶

The reported number of residues that are involved in B-cell epitopes³⁷ is in accordance with the size of the Phl p 5 epitopes identified in this work. This provides evidence that the mimotopes contain residues critically involved in antibody binding and, furthermore, structurally mimic IgE-reactive subregions of the whole antibody contact area. Moreover, it might explain why IgE binding could never be completely inhibited by individual or pooled phagotopes, as has been observed in other studies,^{38,39} and also why the Phl p 5-specific Fab fragment reacted with more than one mimotope.

Mimotopes can be applied as vaccines for the induction of a polyclonal yet epitope-specific immune response.⁴⁰ Biozzi mice are an antibody high-responder strain recommended for phage immunizations, when peptides are displayed on the low-copy phage protein pIII.³⁸ Two peptides indeed induced antibodies recognizing the original antigen, Phl p 5a. Thus they represent true mimotopes of Phl p 5a and can be used for epitope-specific immunotherapy.

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