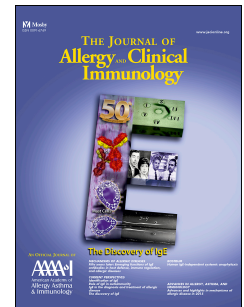


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Flexible IgE epitope containing domains of Phl p 5 cause high allergenic activity

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Summary

This is the first study determining the three-dimensional structure of Phl p 5a which reveals a novel mechanism for high allergenic activity based on flexibly connected IgE-reactive domains which cross-link effector cell-bound IgE more efficiently than isolated rigid globular proteins. These findings may also form a basis for specific immunotherapy strategies.

Key words: Grass pollen, allergy, allergen, protein structure, NMR spectroscopy

Abbreviations used:

IgE: Immunoglobulin E
FcεRI: high affinity IgE receptor
kD: kilodalton
SAXS: small-angle X-ray scattering
NMR: nuclear magnetic resonance
NOE: nuclear Overhauser effect
RDC: residual dipolar couplings
NOESY: nuclear Overhauser effect spectroscopy
Å: Angstrom
RMSD: root-mean-square deviation

54 ns: nanosecond
55 PRE: paramagnetic relaxation enhancement
56 Rg: radius of gyration
57 Dmax: maximum particle size
58 CTD: C-terminal domain
59 NTD: N-terminal domain
60 P1-7: peptide 1-7

61 **To the Editor:**

62 Group 5 allergens are cross-reactive allergens found in most grass species. When
63 released from grass pollen especially after rainfalls in the form of respirable
64 submicronic particles they cause severe asthma attacks in sensitized patients. Group
65 5 allergens are abundant 30 kD monomeric proteins which are localized in the
66 amyloplasts of pollen grains and are supposed to play a role in pollen germination.
67 One of the most striking features of group 5 allergens is that they potently trigger
68 mast cell and basophil degranulation even at extremely low concentrations.^{1,2}
69 Attempts to obtain the full three-dimensional structure of group 5 allergens by X-ray
70 crystallography were so far not successful and only partial structures or models were
71 obtained.³⁻⁵ We succeeded to solve the three-dimensional structure of Phl p 5a by
72 solution NMR spectroscopy. It indicates that Phl p 5a and the members of the family
73 of highly cross-reactive group 5 grass pollen allergens (for sequence alignment see
74 Fig E2) display a modular assembly (i.e., consist of domains which are similar to
75 each other) consisting of two flexibly-connected domains of similar size which contain
76 identical secondary structural elements, i.e., four parallel alpha helices (see Fig 1,
77 and Fig E3 Table E1, Methods and Results section in this article's Online Repository
78 at www.jacionline.org). The structure of Phl p 5a (30–260) consists of two domains
79 that are flexibly connected by a central linker region. Inspection of the NOESY-type
80 spectra did not indicate any inter-domain contacts. Both domains display a 4-helix-

bundle-like fold showing the same up-down-up-down topology (see Fig E3, A). Each helix bundle is stabilized by a hydrophobic core. The helices in domain 1 are found to be oriented almost in parallel, and the additional insert (ranging from residue 85 – 95) forms a flexible and unstructured loop. In domain 2, helix $\alpha 5$ is bent towards its hydrophobic core. This forces helix $\alpha 6$ and helix $\alpha 7$ apart from each other, leading to an elongated loop connecting these two helices, in contrast to the tight loops observed for the other helix-helix connections.

The modular assembly of two flexibly-connected domains is a novel structural feature among allergens which usually assume a compact structure containing several, often spatially-oriented binding sites for patients' IgE antibodies. Earlier epitope mapping data indicated that group 5 allergens contain several IgE binding sites in their N- as well as C-terminal portion.^{2,6} Furthermore, it has been shown that a recombinant fragment of Phl p 5a which resembles the N-terminal domain reacted strongly with allergic patients IgE and was extremely potent in inducing cross-linking of basophil bound IgE antibodies.² In order to investigate if both Phl p 5a domains demonstrate IgE reactivity, we expressed two Phl p 5 fragments, NTD and CTD, representing the isolated N-terminal and C-terminal allergen domain, respectively (see Fig E3, E4, in this article's Online Repository at www.jacionline.org). Purity, correct mass and fold comparable to Phl p 5a were demonstrated for NTD and CTD by SDS-PAGE, mass spectrometry and circular dichroism (see Fig E1, Methods and Results section in this article's Online Repository at www.jacionline.org). Direct IgE binding assays as well as antibody probes raised against peptides from each domain in IgE inhibition experiments showed that each of the isolated domains is important for the binding of allergic patients' IgE antibodies (see Fig E4, E5, Table E2, Methods and Results section in this article's Online Repository at www.jacionline.org). Equivalent IgE

binding capacity of the Phl p 5a and the equimolar mix of the isolated domains NTD and CTD was demonstrated for sera from patients tested in basophil activation experiments (Fig 2A, Table E4, Fig E8, Methods and Results section in this article's Online Repository at www.jacionline.org). Sequence and structural comparison of the two Phl p 5a domains showed that they contain similar sequences as well as structural elements pointing to the possibility that they not only contain independent IgE epitopes but possibly also harbor repetitive IgE epitopes (see Fig E3, in this article's Online Repository at www.jacionline.org) which was confirmed by antibody binding and IgE inhibition assays (see Fig E4, E5, Table E2, Methods and Results section in this article's Online Repository at www.jacionline.org). In fact, the presence of repetitive antibody binding sites has so far only been observed recently for high molecular weight glutenins, a family of wheat food allergens containing identical repetitive sequence elements. Importantly, we show that the Phl p 5a allergen in which the two domains are connected by the flexible linker is more potent in cross-linking IgE on basophils and in inducing basophil degranulation as compared to an equimolar mix of the isolated domains (see Fig 2A, Fig E8 and Methods and Results section in this article's Online Repository at www.jacionline.org). The results obtained in the basophil experiments may need confirmation by *in vivo* testing in patients but this goes beyond the scope of this study.

To illustrate the dynamic properties of group 5 proteins, Fig 2B provides a schematic diagram comparing a rigid molecule (left) with two flexibly-connected domains (right). Due to the flexibility of the two IgE epitope-containing domains, the Phl p 5a allergen should be more potent than other allergens in initiating the cross-linking of effector cell bound IgE antibodies. Upon binding of one domain, the other domain is still mobile and able to sample a much larger volume than for instance an 8-helix-bundle,

or a rigid dimer of a four-helix-bundle, making it more efficient in binding to a second IgE antibody. The elongated conformation of the two folded domains, including the flexible linker, is able to expand up to 120 Å (see Fig E6, Methods and Results section in this article's Online Repository at www.jacionline.org) which allows for efficient interaction to other IgE molecules on the cell surface. Another important structural feature for respiratory allergens that could be identified for Phl p 5a is the observation of recurring sequential and structural elements. Group 5 proteins can thus be considered as highly repetitive molecules that possess similar sequences not only on both domains but also within the same four-helix-bundle.

According to our results, different possibilities for effector cell degranulation by Phl p 5a can therefore be envisaged (see Fig 2C). First, Phl p 5a may crosslink IgE antibodies by IgE epitopes present on one or both of the domains which would correspond to the classical mode of cross-linking known so far (mode 1). However, there are at least two additional novel possibilities of cross-linking. Cross-linking may be achieved by different (mode 2) or repetitive IgE epitopes (mode 3) on each of the domains. This process is facilitated by the flexible domains and their high rotational and translational freedom to recruit additional IgE antibodies into the emerging complexes. In agreement with this assumption, we know that the Phl p 5a allergen indeed accounts for a high percentage of grass pollen-specific IgE antibodies.¹ Furthermore, we know from experimental models with artificial allergen constructs that the intensity of effector cell degranulation increases with the number of engaged IgE molecules.⁷

More importantly, the structural analysis of Phl p 5a not only provides an explanation why Phl p 5a is such a potent allergen, it also explains why it has been difficult to produce recombinant Phl p 5a variants with reduced allergenic activity to be safely

administered as vaccines into patients. It is known that allergen-specific immunotherapy especially with grass pollen allergens can induce severe side effects due to the allergenic activity of allergens in the vaccines. Therefore, it is a long-sought goal to engineer recombinant and synthetic vaccines with reduced allergenic activity. Attempts to construct hypoallergenic Phl p 5a variants have so far proven difficult, because it becomes obvious from the structural analysis of the protein that larger fragments comprising each of the domains or point mutations will not be sufficient for reducing the allergenic activity. In fact, each of the isolated domains showed strong IgE reactivity. According to the structural data, one possibility for constructing a safe and effective vaccine for Phl p 5a is to incorporate several non-allergenic small peptide elements from both domains in a carrier-bound vaccine.^{8,9}

In summary, our study reveals a novel mechanism responsible for the high allergenic activity of an allergen and provides the structural basis for the engineering of safe candidate vaccines to treat patients sensitized to group 5 allergens.

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200 **Accession codes.** Protein Data Bank: Atomic coordinates of Phl p 5a (30-260) have
201 been deposited under accession code 2M64. BioMagResBank: Chemical shift data
202 has been deposited with entry number 19107.

203

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213

214 **AUTHORS CONTRIBUTIONS**

215 C. G., E. S., C.M., M.F-T., Y.D. and S.K. produced proteins, M.F-T. and E. S.
216 performed cloning, C.M., N.N., S.F. and M.F-T. performed immunological
217 experiments, C.G., E. S., and S.K. performed NMR assignment and C. G. and N.T.
218 performed structure calculation. T.M. performed SAXS measurements and modeling.
219 C. G., N. T., S.F., R.V., and K. Z. designed experiments and wrote the manuscript. All
220 authors analyzed data, discussed the results and provided input for the manuscript.

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COMPETING FINANCIAL INTERESTS

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FIGURES AND TABLES

FIG 1. Structure of Phl p 5a and domain mobility. (A) Overlay of the 10 lowest energy models of both structured regions (left: domain 1 in blue, right: domain 2 in orange) showing the unstructured domain-linking loop and counter-domain in transparent form. Labeling of the helices is performed in a sequential manner starting at the N-terminal domain. Backbone dynamics of Phl p 5a are shown as ^{15}N longitudinal relaxation rates (B), transverse relaxation rates (C), overall correlation times (D) and solvent PRE (paramagnetic relaxation enhancement) rates (E), experimental values of structured domains in color, flexible termini and linker in grey, calculated values in black, outliers may arise from high solvent exposure and increased hydrogen exchange with water). Values are plotted as a function of residue number and trimmed mean values of structured regions are indicated by horizontal bars. (F) Position of peptides within the Phl p 5a structure. The regions of the peptides are labeled in different colors using a linear gradient ranging from red, displaying the highest average inhibition rate of IgE binding, to blue, with the lowest inhibition. Gray regions were not part of the study. The flexible N-terminus containing P1 was modeled into the structured part of the molecule.

FIG 2. (A) Basophil degranulation experiments. RBL cells expressing the human Fc ϵ RI were sensitized with sera from ten grass pollen allergic patients (#2, 18-26) and then incubated with different concentrations of Phl p 5 or with an equimolar mix of NTD+CTD (0.016nM, 0.0053nM, 0.0018nM, from left to right). The release of β -hexosaminidase is shown as percentage of the total β -hexosaminidase contents of the cells. The horizontal black bars denote the mean values. Asterisks indicate p-values of 0.0022 (**), < 0.0001 (****) and 0.0007 (***), respectively. (B) Facilitated cross-linking of IgE by Phl p 5a through presence on different domains and repetitive epitopes. In Phl p 5a the two domains (domain 1 blue, domain 2 orange) are connected by an unstructured linker region (right) and are able to sample a much larger volume than a rigid single domain molecule (left). The presence of repetitive IgE epitopes is indicated by the fillings (blank and spotted). (C) The unstructured linking region between the domains of Phl p 5a facilitates binding to a second IgE antibody on the surface of mast cells and effector cells and thus may induce more rapid initial cross-linking of Fc ϵ RI and degranulation (IgE antibody in gray). Additional IgE molecules with specificity for either a different epitope on the same domain or for a repetitive epitope on the other domain can be engaged leading to enhanced cross-linking and degranulation.

FIG 1

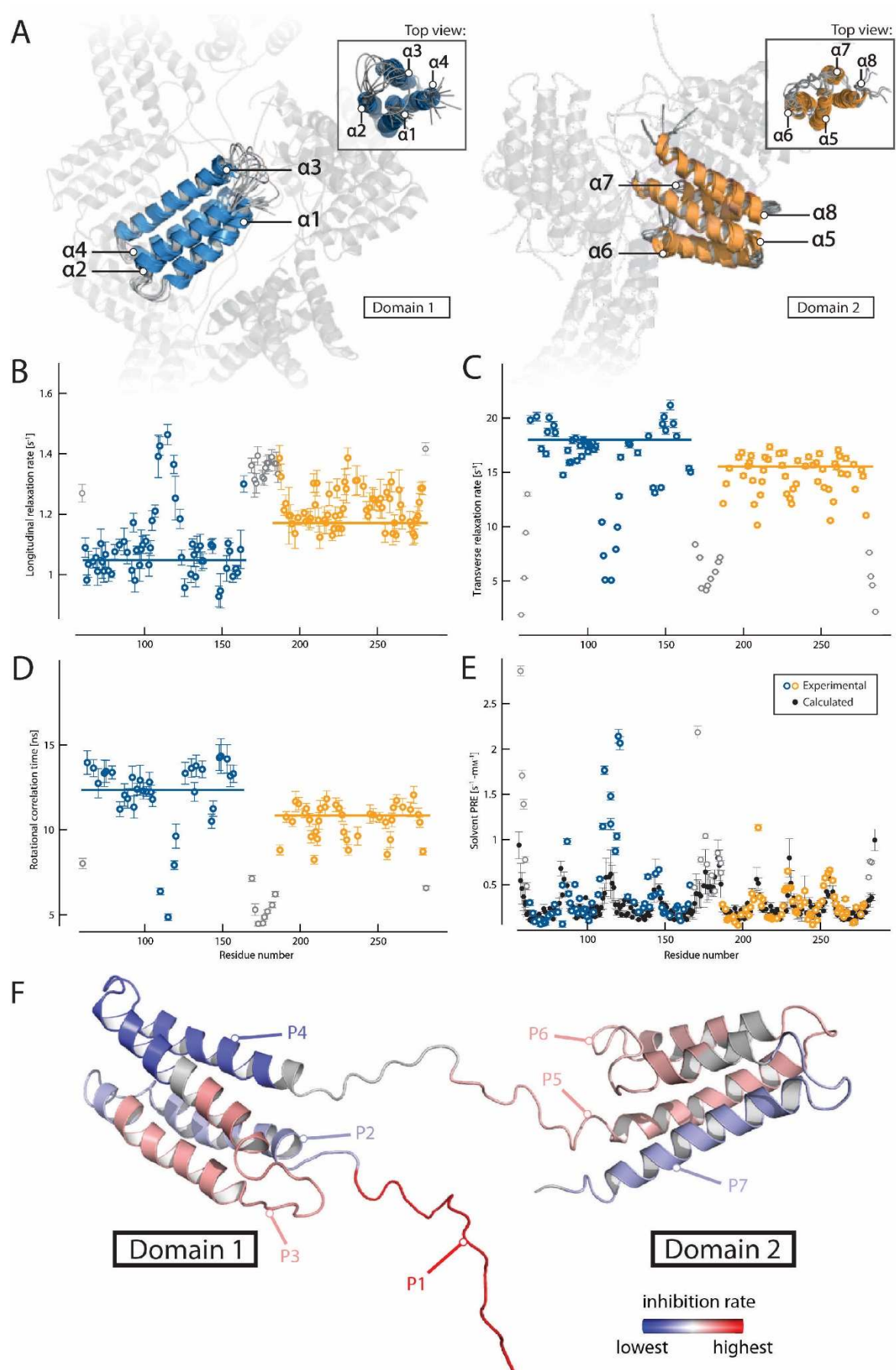
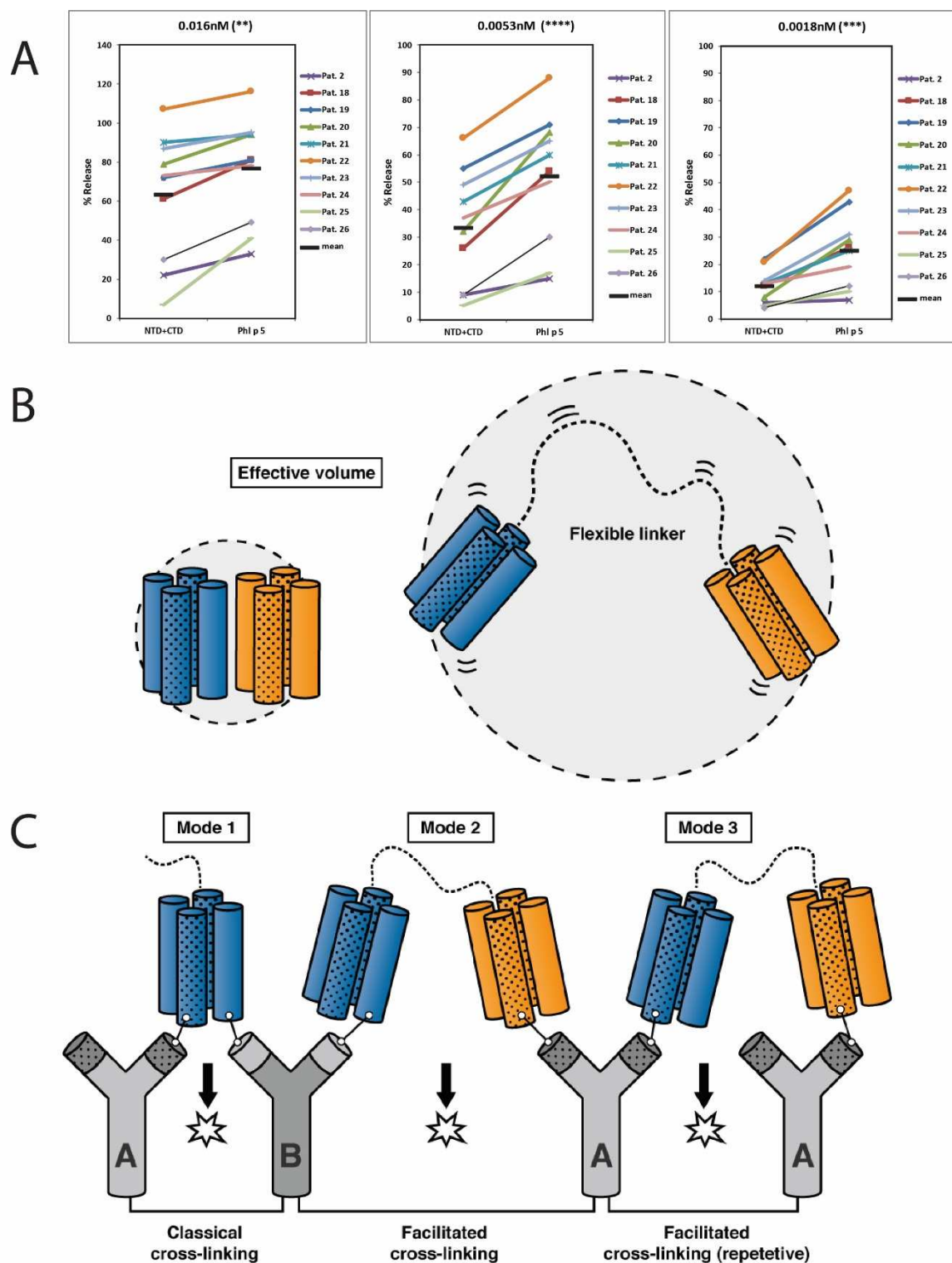


FIG 2



Online Repository

Flexible IgE epitope containing domains of Phl p 5 cause high allergenic activity

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SUPPLEMENTAL METHODS

Expression, purification and physicochemical characterization of Phl p 5a and of Phl p 5a fragments

E. coli BL21 (DE3), harboring either the expression vector pET17b including the gene of the full-length Phl p 5a or truncated protein (res. number 30-260), was cultured in minimal medium containing both (U-¹³C)-glucose and ¹⁵NH₄Cl to produce uniformly enriched, double-labeled ¹³C-, ¹⁵N-protein or LB medium for full-length protein for SAXS analysis. No tags have been present in these constructs. The cultures were grown at 37°C to an OD of 0.6 and protein expression was induced over night by addition of a final concentration of 0.5mM IPTG. The recombinant protein was obtained from the soluble fraction after (NH₄)₂SO₄ precipitation (50% w/v) and dialysis against 50 mM KPi buffer at pH 5.0. The solution was purified via anion exchange (monoQ) followed by size exclusion chromatography (HiLoad 16/600, Superdex 75) and yielded 13.3 mg/L of the pure protein.

DNA encoding two N-terminal (NTD, tNTD) fragments with N-terminal hexahistidine tags and one C-terminal fragment (CTD) of Phl p 5a containing a C-terminal hexahistidine tag were synthesized (GenScript, Piscataway, NJ, USA), cloned into expression vector pET17b, and expressed in *E. coli* BL21 (DE3). Protein expression was induced by addition of isopropyl β-d-thiogalactopyranoside (IPTG, Invitrogen, San Diego, CA) to a final concentration of 0.5 mM at OD of 0.6. Bacteria were grown in shaking flasks over night at 37°C and harvested by centrifugation after induction for 14 hours. *E. coli* were then homogenized and the soluble proteins in the cleared lysates were purified via Ni-NTA agarose under native conditions (Qiagen, Hilden, Germany). Proteins were dialyzed against 10 mM Tris buffer and protein

concentrations were determined by measuring protein-specific ODs at 280 nm^{E1} and by using a Bradford Protein Assay Kit (Biorad, Munich, Germany). Determinations of protein concentrations were performed for all of the proteins in parallel and shortly before the physicochemical analysis and immunological experiments.

Purified rPhl p 5 and NTD and CTD (1 µg of Phl p 5 and equimolar amounts of NTD or CTD and a mix of NTD and CTD) were separated by 14% SDS-PAGE with (i.e., reducing conditions) or without (i.e., non-reducing conditions) of 2-mercaptoethanol in the sample buffer and visualized by Coomassie-staining.

IgE-binding capacity of purified rPhl p 5, NTD and CTD and presence of His Tag on NTD and CTD were tested by Western blotting. Nitrocellulose strips containing blotted proteins were incubated with sera from 2 grass pollen allergic and one non-allergic individuals (in 1:10 dilution) or monoclonal mouse anti-His tag Ab (Dianova, Hamburg, Germany, 1:2000). Bound IgE antibodies were detected with ¹²⁵I-labeled anti-human IgE antibodies (RAST RIA, Demeditec Diagnostics, Germany, 1:10) and with alkaline phosphatase-coupled rabbit anti-mouse Abs (BD Pharmingen, San Diego, CA, USA). Bound ¹²⁵I-labeled antibodies were visualized by autoradiography^{E2}.

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-ToF) mass spectra of rPhl p 5a, rNTD and rCTD were acquired in a linear mode on a Microflex mass spectrometer (Bruker, Billerica, MA) as described^{E3} with sinapic acid (dissolved in 60% acetonitrile, 0.1% trifluoroacetic acid) as a matrix.

Circular dichroism (CD) measurements were done using a JASCO (Tokyo, Japan) J-810 spectropolarimeter as described^{E3}.

Sera from grass pollen allergic patients

Sera were obtained from patients (n=46) with a grass pollen allergy as diagnosed by case history indicative of seasonal grass pollen allergy, a positive skin prick test reaction to grass pollen extract and by the presence of IgE antibodies specific from grass pollen allergens as described.^{E4} Anonymized serum samples from these patients were used for the serologic experiments (IgE-binding and competition experiments) with approval of the ethics committee of the Medical University of Vienna. Sera from which sufficient volumes were available and which showed equal IgE reactivity to the mix of NTD and CTD as well as to complete Phl p 5 were used for basophil experiments.

Reactivity of Phl p 5a peptide-specific antibodies with Phl p 5a fragments and inhibition of allergic patients IgE binding by ELISA

Rabbit antibodies raised against seven peptides (anti-P1-anti-P7)^{E4}, four from the N-terminal (anti-P1-anti-P4) and three from the C-terminal Phl p 5a domain (anti-P5-anti-P7), were used to test for cross-reactivity with the two Phl p 5a domains. For this purpose ELISA plates (Nunc Maxi-Sorp, Roskilde, Denmark) coated with the N-terminal domain (NTD), a truncated form of the N-terminal domain lacking the first flexible peptide P1 (tNTD), the C-terminal domain (CTD) and the complete Phl p 5a (Biomay AG, Vienna, Austria), were incubated with peptide-specific rabbit anti-sera (diluted 1:20.000). Bound rabbit antibodies were detected with horseradish peroxidase-conjugated donkey anti-rabbit antibodies (GE Healthcare, Little Chalfont, UK, diluted 1:2000) followed by ABTS detection.

To study the presence of cross-reactive IgE epitopes on the N-terminal and C-terminal domains, sera from 17 allergic patients (diluted 1:10) were pre-incubated with NTD and CTD (0.5 µg of fragment per 100 µl, 1 h at room temperature) and incubated with ELISA plate bound recombinant purified Phl p 5a-fragments (1 µg/mL). For control purposes, sera were not pre-adsorbed or pre-adsorbed with an immunologically unrelated allergen from birch, Bet v 1 (Biomay AG). Remaining IgE reactivity to the two domains was detected with mouse-anti-human IgE antibodies conjugated to alkaline phosphatase (BD Pharmingen, San Diego, CA). IgE competition ELISAs with peptide-specific rabbit antibodies to detect major IgE-reactive areas were done with sera from 29 grass pollen allergic patients as described.^{E4}

To test if the IgE binding capacity to Phl p 5 is equivalent to that of the sum of the isolated domains NTD and CTD for sera used in basophil experiments IgE ELISA and IgE ELISA inhibition experiments were performed. Sera from grass pollen allergic patients (n= 10, diluted 1:10) were pre-incubated either with Phl p 5 or with an equimolar mixture of NTD and CTD (75nM, 1 h 30 min at room temperature) and then incubated with ELISA plate-bound recombinant purified Phl p 5 (15nM). For control purposes, sera were pre-adsorbed with buffer. Remaining IgE reactivity to Phl p 5a was detected with mouse-anti-human IgE antibodies conjugated to alkaline phosphatase (BD Pharmingen).

To test IgE binding capacity of the Phl p 5 derived peptides plates were coated with 1 µg of Phl p 5 or peptides, incubated with 1:10 diluted sera and bound IgE was detected as described above.

Rat basophil leukaemia cell (RBL) mediator-release assay

Rat basophil leukaemia cells (RS-ATL8)^{E5} transfected with the human high affinity IgE receptor FcεRI (alpha, beta and gamma), were loaded with IgE antibodies from grass pollen allergic patients. Sera were diluted 1:10 in cell culture media. Cells were washed 3 times with Tyrode's buffer (Sigma–Aldrich) and then exposed to different concentrations (0.05 nM, 0.016nM, 0.0053nM, 0.0018nM and 0.0006nM) of either complete rPhl p 5a or an equimolar mixture of the isolated N-terminal and C-terminal domains. To increase and stabilize the secretion of β-hexosaminidase, 50% D₂O was added to the Tyrode's buffer used for the dilution of Phl p 5 or NTD and CTD.^{E5} Total β-hexosaminidase content was determined after lysis of the cells with 1% Triton-X100. β-hexosaminidase release is shown as percentage of total mediator content determined by detergent lysis. For control purposes, IgE-loaded cells were incubated with Tyrode's buffer only. Furthermore, cells were incubated with allergens or Tyrod's buffer only. The release of β-hexosaminidase was measured as described.^{E6}

NMR spectroscopy

NMR samples of 0.3 mM concentration of Phl p 5a were prepared in 50 mM potassium phosphate buffer, 20 mM NaCl at pH 6.2 and contained either 10% or 100% D₂O. Experiments were performed on Bruker 500, 600, 700, 800 and 900 MHz and Varian 600 MHz NMR spectrometers at 24.8 °C. Assignment of the resonances was gained through acquisition of HSQC, HNCACB, CBCA(CO)NH, HNCO, HN(CA)CO, CC-(CO)NH, HCCH-(CO)NH, HBHA(CO)NH and CT-CHSQC spectra. Proton NOEs were obtained from 3D ¹⁵N-edited NOESY and 4D ¹⁵N/¹³C-edited and

¹³C/¹³C-edited spectra. For RDC analysis in 15 mg/ml pf1 phage modified versions of CT-HNCO experiments were used in standard buffer and an addition of 50 mM NaCl.

Structure calculation

Dihedral angles were determined by Talos+^{E7} based on backbone chemical shifts and 294 statistically significant angles were used as structural restraints with at least 20° margin. No generic hydrogen bonds were used for structure calculation. The peak intensities of NOESY (nuclear Overhauser effect spectroscopy) cross-peaks were translated into a continuous distribution of proton-proton distances. Structures of Phl p 5a were then calculated using the simulated annealing method of XPLOR-NIH.^{E8} The calculations employed 2301 inter-residual proton-proton restraints, 147 φ and 147 ψ dihedral restraints and 85 NH RDC (residual dipolar couplings) for the first domain and 77 for the second domain. The introduction of a smooth statistical torsion potential in the final annealing steps improved the orientation of side chains by reducing the number of NOE (nuclear Overhauser effect) violations.^{E9} All molecule illustrations have been created using PyMOL.^{E10}

Small-angle X-ray (SAXS) scattering

SAXS data from solutions of Phl p 5a were collected on an in-house SAXS instrument (SAXSess mc2, Anton Paar, Graz, Austria) equipped with a Kratky camera, a sealed X-ray tube source, and a one-dimensional reverse-biased silicon diode array detector (Mythen 1K, Dectris, Baden, Switzerland). The scattering patterns were measured with a 30 minute exposure time (3 frames, each 10 minutes)

for several solute concentrations in the range from 1 to 10 mg/ml. To exclude radiation damage, the individual frames of the 10 minute exposure were compared, and no changes were detected. Using the sample-detector distance of 2.7 m, a range of momentum transfer of $0.01 < s < 0.6 \text{ \AA}^{-1}$ was covered ($s = 4\pi \sin(\theta)/\lambda$, where 2θ is the scattering angle and $\lambda = 1.5 \text{ \AA}$ is the X-ray wavelength).

All SAXS data were analyzed using the package ATSAS (version 2.5).^{E11} The data were processed with SAXSQuant (version 3.9) and extrapolated to infinite dilution using the program PRIMUS.^{E12} The forward scattering, $I(0)$, and the radius of gyration, R_g , were evaluated using the Guinier approximation^{E13}, assuming that at very small angles ($s < 1.3/R_g$), the intensity is represented as $I(s) = I(0) \exp((-s^2 R_g^2)/3)$. The values of $I(0)$ and R_g , as well as the maximum dimension, $D_{\max}$, and the inter-atomic distance distribution functions, $(P(r))$, were computed using the program GNOM.^{E14} The scattering from the high resolution models was computed using the program CRY SOL.^{E15} The masses of the solutes were evaluated by comparison of the forward scattering intensity with that from a bovine serum albumin reference solution (Sigma Aldrich, molecular weight 66 kDa).

SUPPLEMENTAL RESULTS

Physicochemical characterization of recombinant proteins

The Coomassie-stained SDS-PAGE shows that recombinant purified rPhl p 5a and rNTD and rCTD migrate under reducing as well as under non-reducing conditions as monomeric proteins (Fig E1A, reduced, and not reduced). The MALDI analysis of the purified proteins confirmed the correct mass (rPhl p 5a: calculated: 28585 Da,

measured: 28565 Da; rNTD: calculated 17382 Da, measured: 17368 Da; rCTD: calculated: 14911 Da, measured: 14896 Da) (Fig E1B).

The CD spectrum of rPhl p 5a is characterized by 2 broad minima at 207 and 221 nm, typical for a folded α helical protein (Fig E1, C). Comparable spectra were recorded for rNTD and rCTD (Fig E1, D). Comparable IgE binding was found by IgE-immunoblotting (Fig E1, E, left) and rNTD and rCTD but not rPhlp 5a reacted with an anti-His tag antibody (Fig E1, E, right).

Homologues of Phl p 5a and related allergens

Phl p 5a is a representative member of a family of highly allergenic proteins found in grasses and in corn (see Fig E2). Phl p 5a and the related members of the family of group 5 allergens show high sequence similarity and accordingly are highly cross-reactive allergens.^{E16} The only variation in their sequence is found at the terminal regions and within an insert that is missing in some homologues (ranging from approximately residue 119 to 130) (see Fig E2). The protein was reported to be expressed selectively in pollen tissue in the amyloplasts but its biological function is not known.^{E16} The N-termini (the first 30-70 amino acids) contain several PxxP and other poly-proline motifs, presumably SH2 and SH3 interaction sites, whereas the about 35–60-residue long threonine-rich C-termini are likely to be phosphorylation recognition sites as suggested by computational analysis.^{E17} The high conservation in the central region of the protein indicates that it might be important for its function. An isoform of Phl p 5, Phl p 5b (71% sequence similarity with Phl p 5a) was reported to show RNase activity^{E18} but neither its partial protein structure^{E19} nor the Phl p 5a full-length structure solved by us display any known RNase-like fold.

Sequence similarity can also be found between the shorter group 6 pollen allergens and the N-terminal region of group 5 allergens (see Fig E2). It has been speculated that groups 5 and 6 allergens have evolved from a common ancestor gene^{E20} but despite about 70% sequence similarity there is only weak immunological cross-reactivity between the two.^{E21}

Group 5 grass pollen allergens consist of two highly flexibly-connected domains

We present the structure of Phl p 5a, which was determined by NMR spectroscopy using experimental nuclear Overhauser effect (NOE), residual dipolar couplings (RDC) and chemical shift data. Full-length Phl p 5a contains roughly 30 intrinsically unstructured residues at each terminus based on their random-coil backbone NMR chemical shifts. Resonances were assigned and identified to correspond to residues 1–35 at the N-terminus and 255–287 at the C-terminal region. A shorter truncated construct lacking the flexible termini was created in order to improve the spectral quality. Truncation of the protein did not alter the position of the residual NMR resonances and therefore does not influence the structure of the remaining 231-residue protein (see Fig E7). The structure of Phl p 5a (30–260) was then solved using 2301 inter-residue NOEs and 162 RDCs (see Table E1). It consists of two domains that are flexibly connected by a central linker region. Inspection of the NOESY-type spectra did not indicate any inter-domain contacts. Both domains display a 4-helix-bundle-like fold showing the same up-down-up-down topology (see Fig E3, A). Each helix bundle is stabilized by a hydrophobic core. The helices in domain 1 are found to be oriented almost in parallel, and the additional insert

(ranging from residue 85 – 95) forms a flexible and unstructured loop. In domain 2, helix $\alpha 5$ is bent towards its hydrophobic core. This forces helix $\alpha 6$ and helix $\alpha 7$ apart from each other, leading to an elongated loop connecting these two helices, in contrast to the tight loops observed for the other helix-helix connections. The fold of domain 1 is similar to the structure of Phl p 6^{E22}, a representative member of group 6 allergens. The RMSD between domain 1 of Phl p 5a and Phl p 6 is 2.9 Å, whereas the additional insert in domain 1 of Phl p 5a is not present in Phl p 6. The structure of domain 2 shows highest similarity to the crystal structure of the homologous C-terminal region of Phl p 5b with an RMSD of 2.3 Å. The partial Phl p 5b crystal structure appeared as a homo-dimer with a cysteine-bridge positioned at the stretched loop between helices $\alpha 6$ and $\alpha 7$.^{E19} This stabilization might be the reason for the helical turn located in this loop, which is absent in the NMR solution structure of Phl p 5a. However, it is unlikely that the dimer formation of the crystal structure resembles the native protein, since all other known group 5 homologues do not possess any cysteine residues. The structure of an immunodominant rPhl p 5 fragment (aa 56-165) was found to consist of a four-helix bundle held together by hydrophobic interactions between the aromatic rings and aliphatic side chains but this work did not provide definitive information about the complete Phl p 5a structure.^{E23}

To confirm our findings of independent domains of Phl p 5a we conducted ¹⁵N relaxation measurements. The structured residues of the larger domain 1 (residues 35–140) display an average longitudinal relaxation rate of $1.04 \pm 0.05 \text{ s}^{-1}$ for domain 1 and $1.18 \pm 0.03 \text{ s}^{-1}$ for domain 2 (residues 163–255). This is significantly different indicating that there is a large amount of relative flexibility between the two domains (see Fig 1, B). The average transverse relaxation rate of domain 1 is also quite different ($17.9 \pm 1.4 \text{ s}^{-1}$) than that of domain 2 ($15.9 \pm 0.7 \text{ s}^{-1}$) (see Fig 1, C). The

relative mobility between the two domains is also highlighted by their average rotational correlation times as determined from their relaxation data^{E24} (see Fig 1, D). Correlation times were determined to be 12.5 ± 2.0 ns for the first domain and 11.1 ± 0.5 ns for the second domain, whereas the expected value for a globular molecule of 230 amino acids is about 16.2 ns.^{E25} The shorter observed correlations times than expected reflect relative mobility between the two domains. On the other hand, the correlation time for individual proteins of the size of each domain should be between 7.5 and 8.2 ns. This longer measured correlation time compared to the expected value for individual domain suggests some restricted mobility in the presence of the neighboring domain.

Since any well-defined or even transient domain orientation might influence the binding to IgE, we carried out solvent PRE (addition of a paramagnetic, inert gadolinium complex to determine the accessibility on the protein's surface^{E26}) and SAXS measurements to obtain further insights into the dynamic behavior of the two domains. As can be seen in Figure 1, E, the measured solvent PRE values agree very well with the back-calculated values for the 10 lowest energy NMR structures with random inter-domain-orientation. Chemical shifts, relaxation data, and solvent PRE values also highlight the flexibility of the loop (between residues 143 and 160) connecting domain 1 and domain 2. Using SAXS, we characterized the overall conformation of full-length Phl p 5a and further corroborated the NMR results (Fig E6). The average dimensions represented by the radius of gyration (R_g) and maximum size (D_{max}) of Phl p 5a are 35.4 ± 0.5 and 117 Å, respectively. These parameters and the pair-wise distance distribution functions ($P(r)$) (see Fig E6, A) are characteristic for a modular protein in which the two structured domains are flexibly connected. Starting from the NMR structures of the two folded domains of Phl p 5a,

we used the ensemble optimization method (EOM)^{E27} and SAXS data to generate an ensemble of structures (see Fig E6, B). It should be noted here that SAXS data represent a snapshot of all conformations present in solution, and that an ensemble of structures – rather than a single structure – is appropriate to characterize dynamic proteins such as Phl p 5a. The resulting ensemble of structures is in good agreement with the SAXS data (discrepancy χ less than 0.2). The Rg/Dmax distribution of the selected ensemble covers the Rg/Dmax ranges of 25–45/70–140 Å, corresponding to a large portion of completely extended domain configurations. Given the Rgs/Dmax of the individual domains and a theoretical Dmax of a 'closed' protein in the order of approximately 90 Å, more than 80% of the particles are located in the subset of extended structures. Equivalently, full-length Phl p 5a is highly flexible and is in equilibrium with a minor population (< 20%) of closed conformations (see Fig E6, C).

The Phl p 5a domains contain repetitive sequences and structural elements

Both domains of Phl p 5a show a similar, all-alpha helical fold and the sequence alignment between the two domains shows a similarity of about 45%. The structural comparison of both domains yields an RMSD value of 2.6 Å for all residues and 1.9 Å for residues in secondary structure (see Fig E3, A).

Interestingly, further sequential alignment within each domain reveals a similarity of approximately 50% between helix $\alpha 1$ – $\alpha 2$ and $\alpha 3$ – $\alpha 4$ in the first domain and between helix $\alpha 5$ – $\alpha 6$ and $\alpha 7$ – $\alpha 8$ in the second domain. Moreover, two similar helical segments can be found twice within each domain and four times within the full-length protein (residues in $\alpha 2$: 61–78, $\alpha 4$: 120–137, $\alpha 6$: 184–201, $\alpha 8$: 232–249 and $\alpha 1$: 41–59, $\alpha 3$: 100–128, $\alpha 5$: 164–182, $\alpha 7$: 183–201, see Fig E3, C). These similar sections in the

helices $\alpha 2$, $\alpha 4$, $\alpha 6$, and $\alpha 8$ overlap with an average RMSD of 1.3 Å for the backbone heavy atoms. A comparable similarity can be found in the regions of helices $\alpha 1$, $\alpha 3$, $\alpha 5$, and $\alpha 7$ (see Fig E3, C). Other preserved elements are proline residues that can be found serially in all but one loop. As a result of sequential and structural analogy, similar charge distributions can be observed in the electrostatic surface map of each domain, displaying the conserved locations of charged residues (see Fig E3, B). One precondition for IgE binding is the presence of (at least partial) secondary structured elements. The recurring patterns in sequence, structure, and consequently surface charge profile in Phl p 5a (see Fig E3, B-C) provide multiple potential targets for IgE binding. To search for the presence of repetitive epitopes in the two domains, we expressed the isolated domains as recombinant proteins for cross-reactivity experiments and used antibody probes specific for peptides (P1-P7) located in the domains (Fig E1, E4).

Presence of cross-reactive IgE epitopes in the two Phl p 5a domains

In a first set of experiments, antibody probes raised against seven peptides, four from the N-terminal and three from the C-terminal Phl p 5a domain, were tested for reactivity with the complete Phl p 5a molecule, the N-terminal domain (NTD), a truncated form of the N-terminal domain lacking the first flexible peptide P1 (tNTD), and the C-terminal domain (CTD) (see Fig E4, A). Each of the seven antibody probes (anti-P1 – anti-P7) reacted with the complete Phl p 5a allergen, anti-P2 – anti-P4 recognized NTD and tNTD, anti-P1 selectively bound to NTD, and anti-P5 – anti-P7 reacted with CTD reflecting the location of the peptides on the individual proteins (see Fig E4, B). Interestingly, anti-P2 and anti-P4 cross-reacted with the C-terminal

domain demonstrating the presence of epitopes which are duplicated in the two domains of Phl p 5a (see Fig E4, B). In fact, peptides P2 and P4 contained the sequences and structures which were identified in the sequence and structural alignment in figure E3, C, to be similar in helices $\alpha 6$ and $\alpha 8$.

Next, we searched for the presence of cross-reactive IgE epitopes on the N-terminal and C-terminal domain. For this purpose sera from allergic patients were pre-incubated with NTD and CTD to deplete the sera for IgE antibodies specific for each of the two domains. For control purposes, sera were both pre-adsorbed or not pre-adsorbed with an immunologically unrelated allergen from birch, Bet v 1. Pre-adsorbed sera were then tested for remaining IgE reactivity to the two domains (Table E2). We found that the pre-adsorption of the sera with the domains strongly reduced IgE binding to the very same domain and, interestingly, also sera were found in which CTD inhibited IgE binding to NTD and vice versa (Table E2, patient 1 and patient 7). Pre-incubation of sera with the unrelated control allergen, Bet v 1, did not cause any relevant inhibition to the Phl p 5a domains.

Visualization of the major IgE-reactive areas in the two domains of the Phl p 5a structure

Binding sites for IgE antibodies of allergic patients on the Phl p 5a allergen belong to the conformational type, whereas Phl p 5a peptides without structural fold lack IgE reactivity^{E4} (Table E3). However, allergen-derived peptides can be used to establish antibody probes which can be used to block regions defined by the peptides on the folded allergen to see if this affects patients IgE antibody binding. The degree of blocking of IgE binding caused by the anti-peptide antibodies then provides information whether the peptides are either part of or close to the conformational IgE

epitopes and thus allows an indirect definition of the IgE epitopes. Figure E5 shows the degree of inhibition of allergic patients IgE binding to Phl p 5a obtained with seven anti-peptide anti-sera. According to these blocking experiments, antibodies specific for peptides P1, P2, and P3 as well as P5, P6, and P7 define major IgE-reactive portions of Phl p 5a, whereas a lower degree of inhibition of IgE binding was observed with the anti-P4 antiserum. Figure 1, F shows the localization of the portions of Phl p 5a which are involved in binding allergic patients IgE antibodies. The IgE epitopes are present on the N-terminal as well as on the C-terminal domain which is in good agreement with the finding that each of the allergic patients tested in Table E2 showed IgE reactivity to NTD and CTD. On the individual domains, IgE epitopes seemed to be present on certain areas appearing on one side of the domain whereas the opposite areas were less reactive with IgE (see Fig 1, F). Such a clustering of IgE epitopes has also been found for other allergens and may be important for the development of allergy vaccines capable of targeting protective antibody responses against selected portions of the allergen.^{E22,E28,E29} Interestingly, anti-peptide P1 antibodies showed the strongest inhibition although P1 was found to be unstructured in the full length protein.

Flexibly-connected Phl p 5 domains are significantly more allergenic than a mix of the isolated domains

To test if the flexible connection between the two Phl p 5a domains contributes to the ability of the allergen to cross-link effector cell bound IgE antibodies and thus to its allergenic activity, we performed basophil activation experiments. Basophils which had been loaded with IgE antibodies from Phl p 5 allergic patients who showed

comparable IgE reactivity to rPhl p 5a and to the mix of rNTD and rCTD (Table E4) were exposed to the complete Phl p 5a allergen in which both domains are connected or to an equimolar mixture of the isolated N- and C-terminal domains. We found that Phl p 5a induced significantly stronger β -hexosaminidase release in each of the three four tested concentrations (0.016 nM, 0.0053 nm, 0.0018 nm and 0.0006 nm) compared to the equimolar mixture of the isolated domains ($p \leq 0.0022$). The mean releases for the concentrations 0.016 nM, 0.0053 nm, 0.0018 nm were 13% to 19% higher for Phl p 5a compared to the fragment mix (see Fig 2, A, Fig E8). Figure E8 shows in dose response curves that rPhl p 5a has greater allergenic activity than the mix of rNTD and rCTD for each of the tested sera. To confirm that the significant reduced release of the isolated fragments is not due to variable binding of IgE to Phl p 5 and the fragments, we demonstrated that the IgE binding capacity of the Phl p 5 is equivalent to that of the sum of the isolated domains NTD and CTD (Table E4).

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SUPPLEMENTAL FIGURES AND TABLES

FIG E1. Characterization of rPhl p 5a, rNTD and rCTD (A) Comparison of purified rPhl p 5a, rNTD and rCTD in SDS PAGE in the presence (left: reduced) or absence (right: non-reduced) of 2-mercaptoethanol. M, molecular mass markers (kDa). (B) Mass spectrometric analysis of rPhl p 5, rNTD and rCTD. The mass/charge (m/z) ratios are shown on the x-axes, and the signal intensities (a.u.) are displayed in the y-axes. (C) Circular dichroism analysis of rPhl p 5a. (D) Circular dichroism analysis of rNTD and rCTD. The mean molar ellipticities (y-axes) are shown at different wavelengths (x-axes). (E) Immunoblot of rPhl p 5a (P), rNTD (N), rCTD (C) probed with IgE from grass pollen allergic patients (P1, P2) and from a non-atopic person (Co) or with an anti-His tag antibody, B: buffer control.

FIG E2. Comparison of group 5 allergens from different grass species. Labeling of the individual proteins is based on the botanical denotation of the genus and their classical numerical annotation to the pollen family (Phl p = *Phleum pratensis*, Poa p = *Poa pratensis*, Lol p = *Lolium perenne*, Hor v = *Hordeum vulgare*, Tri t = *Triticum turgidum*, Sec C = *Secale cereale*, Dac G = *Dactylis glomerata*, Bra d = *Brachypodium distachyon*, Fes p = *Festuca pratensis*, Hol l = *Holcus lanatus*, Tri a = *Triticum aestivum*). The group 6 grass pollen allergen Phl p 6 is included as a representative member of group 6 allergens and shows high similarity to domain 1 of group 5 proteins. Dashes represent gaps.

FIG E3. Structural and sequential comparison of the two domains. (A) Structural presentation of the alignment of domain 1 (blue) and domain 2 (orange). (B) The electrostatic surface (negative charges in red, positive charges in blue) shows large areas with similar charge distribution. (C) A high degree of conservation of similar segments is found between the helices. Helices $\alpha 2$ and $\alpha 4$ from the first domain shown high similarity not only between each other but also to helices $\alpha 6$ and $\alpha 8$ in the second domain. Similar segments can also be found for $\alpha 1$ and $\alpha 3$ from the first domain and $\alpha 5$ and $\alpha 7$ from the second domain. (D) The

presence of the homologue helices is indicated on a scheme of both domains for helices $\alpha 2$ - $\alpha 8$ (spotted) and $\alpha 1$ - $\alpha 7$ (blank) and highlights the repetitive presence of homologue peptide sequences.

FIG E4. (A) Amino acid sequence of Phl p 5a. Recombinant fragments (blue: NTD, tNTD; orange: CTD) and synthetic peptides are marked and positions of the α -helices is indicated. (B) Histogram showing the reactivity (optical density values corresponding to bound antibodies) of anti-peptide antibodies with Phl p 5a (gray), NTD (light blue), tNTD (blue) and CTD (orange). The cross-reactions of anti-P2 and anti-P4 with the antibodies are marked with an asterisk.

FIG E5. Percentages inhibition of allergic patients (n=29) IgE binding to Phl p 5 by anti-peptide (anti-P1 – anti-P7) or anti-Phl p 5 antibodies (x-axis). Horizontal bars denote median inhibitions for each of the antibodies.

FIG E6. Overview of the full-length Phl p 5a SAXS analysis including the flexible termini. (A) SAXS data showing the experimental radial density distribution of full-length Phl p 5a. (B) Ensemble of 10 final structures generated by EOM modeling when superimposed at domain 1 (blue) and showing domain 2 in orange. (C) Distribution of frequencies of the models with given R_g (left) and D_{max} (right) in the initial pool of structures with randomized inter-domain linkers and in the selected ensembles.

FIG E7. Overlay of $^1H^{15}N$ HSQC spectra. The spectrum in blue displays the resonances of the full-length protein while the spectrum in red shows the spectrum of the truncated construct. No structural changes appeared in the shorter construct because altered peak positions are solely found at the truncated regions.

FIG E8. Basophil degranulation experiments. RBL cells expressing the human Fc ϵ RI were sensitized with sera from ten grass pollen allergic patients (#2, #18-26) and then incubated with increasing concentrations of rPhl p 5a or with an equimolar mix of rNTD+rCTD (0.016nM, 0.0053nM, 0.0018nM, 0.0006nM) or buffer (x-axes). The release of β -hexosaminidase is shown as percentage of the total β -hexosaminidase contents of the cells +/-SD on y-axes.

TABLE E1. NMR and refinement statistics for protein structures.

TABLE E2. Table of allergic patients IgE reactivity to NTD and CTD of Phl p 5a. Shown are the optical density values corresponding to allergic patients' (#1-17) IgE antibodies bound to NTD and CTD without inhibitor (no) or after pre-incubation of sera with NTD, CTD, or the unrelated birch pollen allergen, Bet v 1 (inhibitor). Percentages of inhibition of IgE binding are displayed in brackets. Sera, in which CTD inhibited IgE binding to NTD and vice versa, are displayed in bold.

TABLE E3. Table showing allergic patients IgE reactivity to rPhl p 5a and Phl p 5 derived peptides. Optical density values corresponding to allergic patients' (#1-26) IgE binding are displayed.

TABLE E4. Inhibition of IgE binding to solid phase-bound Phl p 5 by pre-incubation of sera with Phl p 5 or with the equimolar mixture of NTD and CTD. Shown are the optical density values corresponding to allergic patients' (#2, 18-26) IgE antibodies bound to Phl p 5 without inhibitor (no) or after pre-incubation of sera with Phl p 5 or with a mix NTD+CTD (inhibitor). Percentages of inhibition of IgE binding are displayed (% Inhibition).

Table E1. NMR and refinement statistics for protein structures

Distance constraints	2302	1139	1163
Total number of NOE restraints	2302	1139	1163
Intra-residue	-	-	-
Inter-residue	2302	1139	1163
Sequential ($ i - j = 1$)	362	215	147
Medium-range ($ i - j < 4$)	822	358	464
Long-range ($ i - j > 5$)	1118	566	552
Hydrogen bonds	-	-	-
Dihedral angle restraints	294	161	133
ϕ	146	80	66
ψ	148	81	67
Residual dipolar Couplings	162	85	77
Q_{free} (%) [*]	3.38 +/- 0.98		
Structure statistics**			
Violations (mean and s.d.)			
Distance constraints (Å)	0.049 ± 0.002		
Dihedral angle constraints (°)	0.736 ± 0.101		
Max. dihedral angle violation (°)	6.71		
Max. distance constraint violation (Å)	0.68		
Deviations from idealized geometry			
Bond lengths (Å)	0.003 ± 0.000		
Bond angles (°)	0.571 ± 0.005		
Impropers (°)	0.570 ± 0.012		
Average pairwise r.m.s. deviation** (Å)			
Heavy		1.14	1.06

^{*} RDC quality factor was calculated for all RDCs and among 20 refined structures

^{**}Structural statistics and pairwise r.m.s. deviation were calculated among 10 refined structures.

Unstructured termini and the flexible insert in domain 1 (residues 109-119) have been neglected.

TABLE E2. Table of allergic patients IgE reactivity to NTD and CTD of Phl p 5a.

inhibitor	Binding to NTD				Binding to CTD			
	no	NTD	CTD	Bet v 1	no	NTD	CTD	Bet v 1
%inhibition	OD	OD (%)	OD (%)	OD (%)	OD	OD (%)	OD (%)	OD (%)
Patient1	0.612	0.107 (83)	0.339 (44)	0.628 (0)	0.631	0.358 (43)	0.184 (71)	0.632 (0)
Patient2	0.144	0.074 (48)	0.140 (2)	0.143 (1)	0.230	0.227 (1)	0.084 (63)	0.224 (2)
Patient3	3.006	0.723 (76)	2.806 (7)	2.962 (1)	1.726	1.673 (3)	0.526 (69)	1.719 (0)
Patient4	0.809	0.196 (76)	0.811 (0)	0.786 (3)	1.088	1.077 (1)	0.197 (82)	1.100 (0)
Patient5	0.215	0.061 (71)	0.211 (1)	0.217 (0)	0.497	0.478 (4)	0.186 (62)	0.515 (0)
Patient6	0.318	0.086 (73)	0.313 (2)	0.301 (5)	0.662	0.648 (2)	0.167 (75)	0.670 (0)
Patient7	1.237	0.119 (90)	0.838 (32)	1.160 (6)	1.558	1.373 (12)	0.525 (67)	1.600 (0)
Patient8	0.704	0.167 (76)	0.663 (6)	0.641 (9)	0.688	0.688 (0)	0.121 (82)	0.686 (0)
Patient9	0.245	0.112 (54)	0.230 (6)	0.221 (10)	0.555	0.497 (10)	0.137 (75)	0.506 (9)
Patient10	0.318	0.067 (79)	0.284 (10)	0.294 (7)	0.412	0.416 (0)	0.140 (64)	0.441 (0)
Patient11	0.341	0.055 (84)	0.302 (11)	0.283 (17)	0.390	0.411 (0)	0.058 (85)	0.427 (0)
Patient12	0.118	0.050 (58)	0.113 (4)	0.113 (5)	0.176	0.177 (0)	0.071 (60)	0.182 (0)
Patient13	0.374	0.106 (72)	0.363 (3)	0.364 (2)	0.346	0.364 (0)	0.082 (76)	0.375 (0)
Patient14	0.096	0.047 (50)	0.099 (0)	0.098 (0)	0.126	0.128 (0)	0.058 (54)	0.143 (0)
Patient15	1.470	0.263 (82)	1.454 (1)	1.448 (1)	2.051	1.96 (4)	0.496 (76)	2.078 (0)
Patient 16	0.131	0.050 (61)	0.125 (4)	0.131 (0)	0.328	0.328 (0)	0.109 (67)	0.338 (0)
Patient 17	0.248	0.055 (78)	0.244 (1)	0.251 (0)	0.278	0.270 (3)	0.069 (75)	0.288 (0)

TABLE E3. Allergic patients (1-26) IgE reactivity to Phl p 5a (r) and Phl p 5 derived peptides (P1-P7). NHS: Normal healthy serum.

Patient	r	P1	P2	P3	P4	P5	P6	P7
1	0.457	0.035	0.016	0.020	0.016	0.049	0.030	0.029
2	0.488	0.034	0.023	0.016	0.027	0.046	0.031	0.003
3	2.522	0.136	0.019	0.013	0.030	0.024	0.029	0.015
4	1.426	0.044	0.022	0.080	0.030	0.024	0.024	0.023
5	0.234	0.009	0.011	0.015	0.020	0.025	0.015	0.019
6	1.421	0.032	0.031	0.038	0.024	0.061	0.033	0.024
7	2.446	0.027	0.021	0.022	0.015	0.026	0.033	0.029
8	1.284	0.083	0.022	0.016	0.015	0.038	0.058	0.082
11	1.370	0.027	0.018	0.026	0.042	0.023	0.048	0.029
12	0.465	0.026	0.020	0.024	0.014	0.040	0.015	0.014
13	0.570	0.019	0.013	0.022	0.011	0.015	0.015	0.012
14	0.341	0.026	0.027	0.051	0.018	0.049	0.066	0.036
15	2.394	0.054	0.023	0.023	0.055	0.015	0.028	0.019
16	0.393	0.024	0.015	0.015	0.027	0.014	0.023	0.016
17	0.460	0.010	0.038	0.013	0.012	0.019	0.017	0.015
18	2.599	0.064	0.000	0.001	0.000	0.003	0.000	0.000
19	3.141	0.016	0.003	0.021	0.003	0.000	0.022	0.000
20	2.498	0.095	0.001	0.011	0.006	0.006	0.038	0.005
21	2.868	0.030	0.019	0.018	0.016	0.003	0.013	0.017
22	2.918	0.105	0.000	0.001	0.000	0.000	0.001	0.000
23	3.189	0.033	0.014	0.037	0.024	0.027	0.026	0.023
24	2.609	0.036	0.018	0.019	0.016	0.019	0.042	0.097
25	0.648	0.046	0.017	0.017	0.011	0.024	0.047	0.021
26	1.440	0.026	0.017	0.029	0.021	0.056	0.042	0.032
NHS	0.019	0.005	0.006	0.000	0.001	0.000	0.004	0.000
mean	1.59089	0.043	0.017	0.023	0.019	0.025	0.029	0.023

TABLE E4. Inhibition of IgE binding to solid phase-bound Phl p 5 by pre-incubation of sera with Phl p 5 or with the equimolar mixture of NTD and CTD. Shown are the optical density values corresponding to allergic patients' (#2, 18-26) IgE antibodies bound to Phl p 5 without inhibitor (no) or after pre-incubation of sera with Phl p 5 or with a mix NTD+CTD (inhibitor). Percentages of inhibition of IgE binding are displayed (% Inhibition).

Inhibitor	IgE binding to Phl p 5				
	no	Phl p 5	NTD+CTD	Phl p 5	NTD+CTD
	OD	OD	OD	% Inhibition	% Inhibition
Pat 2	0.268	0.095	0.099	65	63
Pat 18	1.168	0.288	0.354	75	70
Pat 19	1.120	0.268	0.238	76	79
Pat 20	0.785	0.191	0.180	76	77
Pat 21	0.971	0.139	0.164	86	83
Pat 22	1.306	0.378	0.370	71	72
Pat 23	1.665	0.187	0.261	89	84
Pat 24	0.820	0.170	0.163	79	80
Pat 25	0.335	0.070	0.068	79	80
Pat 26	0.564	0.066	0.089	88	84

Fig E1

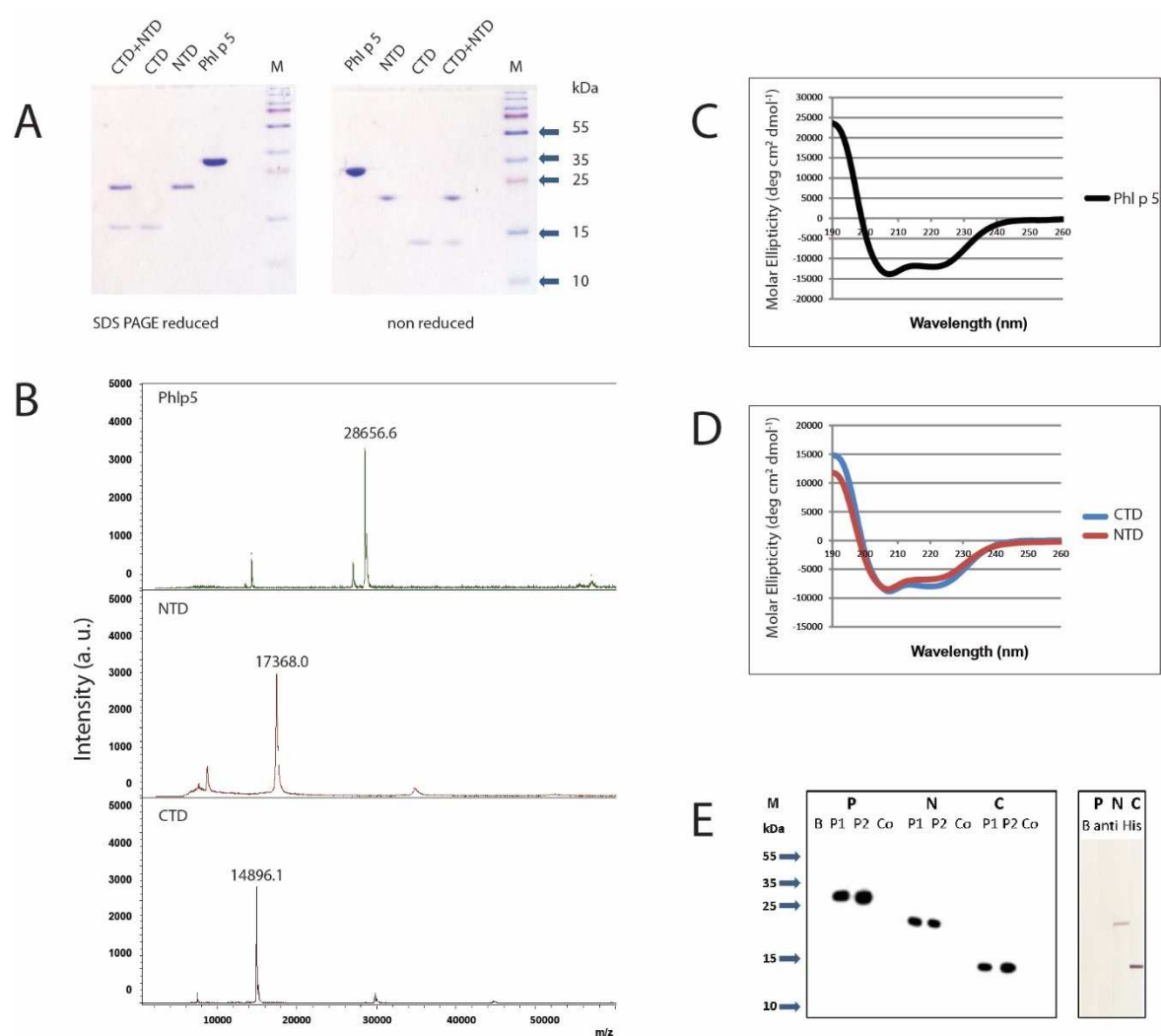
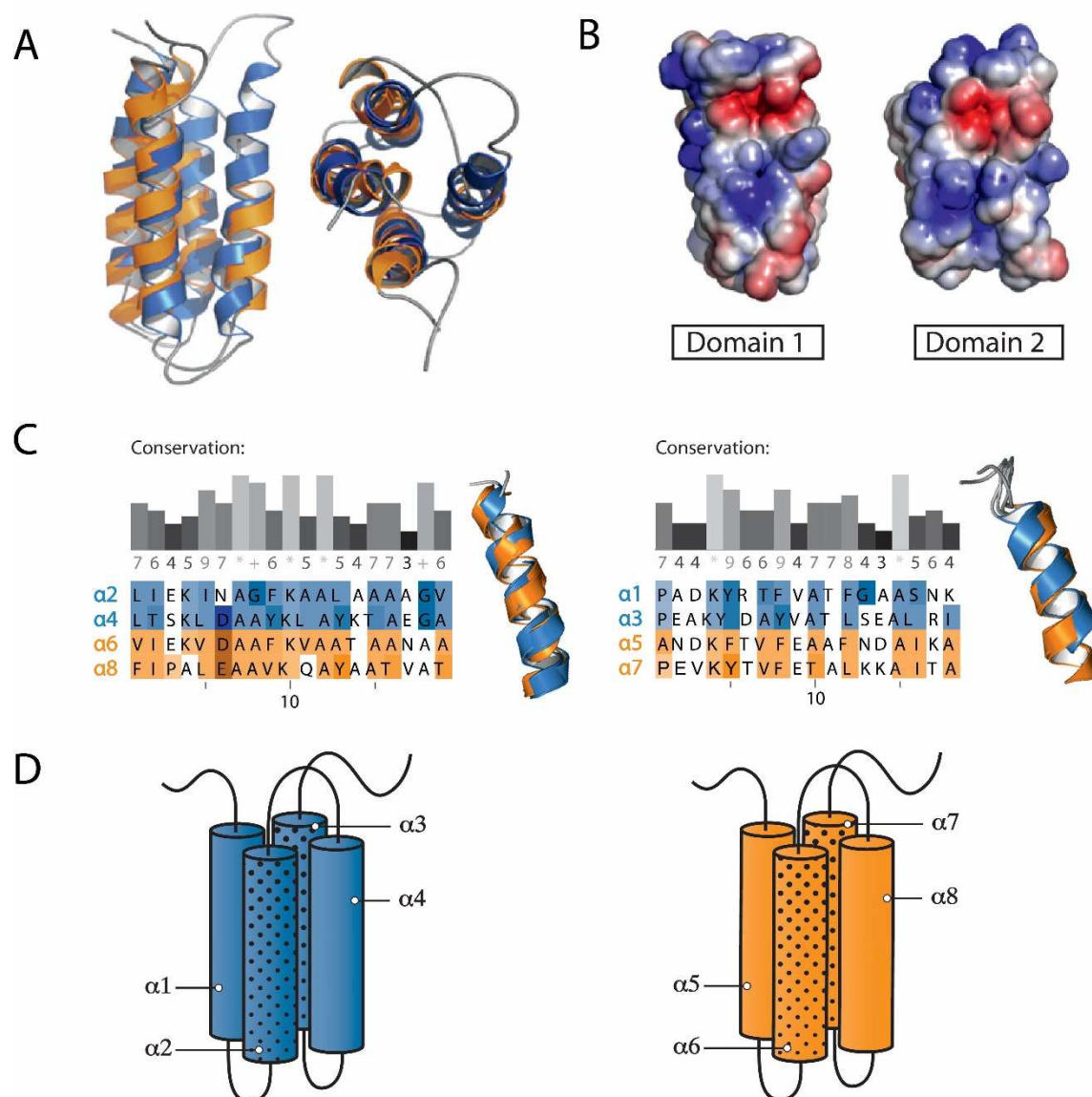


Fig E2

Phil_p5a/1-287	1	-----ADLGYG--PATPAAPAAGYTPATPAAPAA--EAPAGKATTEEQKLEIKINAGFKAALAAAAAGVQPADKVFRTFVATFGAASNKAFABEG	83
Phil_p5b/1-295	1	SVKRSNGSAEVHRGAVPRRGGPRGGRSYYAADAGYAPATPA-----AAGAEAGKATTEEQKLEIDINVGFKAAVAAAAAVPAADKFKTFEAAFTSSSKAA-----	95
Poa_p5/1-307	1	-----MAVQKYTTVALFL-VALVVGPAAASYAADLSYGA--PATFAAPAAGYTFAPAGA-----APKATTDEQKMIKINVGFKAAVAAAAAGVPAANFYKTFVATFGAASNKAFABEA	103
Loi_p9/1-339	1	-----MAVQKHTTVALFLAVALVAGPAASYAADAGYAPATPATPAAPATAATPATPATPATPAAVPSGKATTEEQKLEIKINAGFKAALAAAAAVVFPADKFKTFVETFGTATNKAFVEG	113
Hor_v5/1-296	1	-----MAVQQYTTVALFLAIALVAGPAVSYAT--YAP-----AAPAATYAPAAAGQPKATTPEOKLMECINDGFKAAVAAAAAVFPADKFKTFEATFAAASNKAFABEV	95
Tri_15/1-243	1	-----MAVQQYTTVALFLAVALVAGPAVSYGT--YAP-----AAP-----GTQPKATTPEOKLMENINNGFKAAVAAAAAVPAADKFKTFEATFSAAASNKAFABEV	42
Sec_c5/1-292	1	-----MAVQQYTTVALFLAVALVAGPAVSYGT--YAP-----AAP-----GTQPKATTPEOKLMENINNGFKAAVAAAAAVPAADKFKTFEATFSAAASNKAFABEV	87
Dac_g5/1-265	1	-----MAVQQYTTVALFLAVALVAGPAVSYGT--YAP-----AAP-----GTQPKATTPEOKLMENINNGFKAAVAAAAAVPAADKFKTFEATFSAAASNKAFABEV	69
Bra_d5/1-273	1	-----MEYRMALFLAVALVAGPAFAFA--YAPVTPA-----TPAAGYPKASTDEQKMIEDINAGFKTAVAAAANAPPADKFKTFEAAFKKASTGY-----	83
Fes_p5/1-298	1	-----MAVQKYTTVALFLAVALVAGPAASYAADAGYTPAAAAATPATPA-----ATPAAGGKATTDEQKLLDENVAGFKAAVAAAAANAPPADKFKTFEAAFTSSSKGFLAT	101
Hol_15/1-264	1	-----MAVQKYTTVALFLAVALVAGPAASYAADAGYTPAAAP-----AAAGAGGKATTDEQKLLDENVAGFKTAVAAAANAPPADKFKTFEAAFTSSSKASIAAA	68
Tri_a5/1-231	1	-----MAVQKYTTVALFLAVALVAGPAASYAADAGYTPAAAP-----AAAGAGGKATTDEQKLLDENVAGFKTAVAAAANAPPADKFKTFEAAFTSSSKASIAAA	49
Phil_p6/1-111	1	-----MAVQKYTTVALFLAVALVAGPAASYAADAGYTPAAAP-----AAAGAGGKATTDEQKLLDENVAGFKTAVAAAANAPPADKFKTFEAAFTSSSKASIAAA	53
Phil_p5a/1-287	34	LSGEPKGAES--SKAALTSKLDAAVKLAYKTAEGATPEAKYDAYVATLSEALRIIAGTLEVHAVKPAEEVK-----VIPAGELQVIEKVDAAFKVAATAANAAPANDKFTVFEEA	194
Phil_p5b/1-295	96	-----TAKAPGLVPKLDAAYSVAYKAAVGAATPEAKYDFVASTLEALRVIAGALEVHAVKPVTEEPGM-----AKIPAGELQVIDKIDAAFKVAATAANAAPANDKFTVFEEA	198
Poa_p5/1-307	104	LSTEPKGAADV--SKAALTSKLDAAVKLAYKSAEGATPEAKYDDYVATLSEALRIIAGTLEVHAVKPAEEVK-----ATPAGELQVIDKIDAAFKVAATAANAAPANDKFTVFEEA	214
Loi_p9/1-339	114	LASG-----YAD--QSKNQLTSKLDAAKLAKEAAGATPEAKYDAYVATLSEALRVIAGTLEVHAVKPAEEVKV--GAIIPAAEVQLIDKVDAAFKVAATAANAAPANDKFTVFEEA	222
Hor_v5/1-296	96	LKGAATQIAGQS--SMAKLSLSLELSYKLAIDKAGATPETKYDAYVATLSEALRVIAGTLEVHAVKPAEEVK-----GVFAGELKVIDKIDAAFKVAATAANAAPANDKFTVFEEA	208
Tri_15/1-243	43	LKGAATQIYAAQS--SMAKLSLSLELSYKLAIDKAGATPETKYDAYVATLSEALRVIAGTLEVHAVKPAEEVK-----GVFAGELKVIDKIDAAFKVAATAANAAPANDKFTVFEEA	155
Tri_15/1-243	88	LTAAASGQIPAAQSDSMARLSTLSLSYKLAIDKAGATPETKYDAYVATLSEALRVIAGTLEVHAVKPAEEVK-----GVFAGELKVIDKIDAAFKVAATAANAAPANDKFTVFEEA	200
Dac_g5/1-265	70	-----ATKVPFLFVAKLDAAVAVAYKTAAGATPEAKYDAFVAALTEALRVIAGALEVHAVKPAEEVPA--AKIPAGELQVIDKIDAAFKVAATAANAAPANDKFTVFEEA	172
Bra_d5/1-273	84	-----KAGVSAKLDATYQAAVKAADGATPEAKYDVFVATLSEALRVVAGAVEAHAVKPAEEELAAGAGGKMPADELKVIXKIDAAFKVAATAANAAPANDKFTVFEEA	186
Fes_p5/1-298	102	-----ATKAPGLVPKLDATYDIAVKAAGATPEAKYDAFVATLSEALRVIAGALEVHAVKPAEEVPA--AKIPAGELQVIDKIDAAFKVAATAANAAPANDKFTVFEEA	204
Hol_15/1-264	69	-----ATKAPGLIPOLNAATNTAYAAAAGATPEAKYDAFVATLSEALRVIAGALEVHAVKPAEEVGA--AKIPAGELQVIDKIDAAFKVAATAANAAPANDKFTVFEEA	171
Tri_a5/1-231	50	FKGS-----NASTFTAKIGMAQKLAIDKAGATPEAKYDSFIAILSEALRIIAGTLEIHGVKPAEEVK-----GPIPAAEVQVNDKIDTAFAATAANAAPANDKFTVFEEA	153
Phil_p6/1-111	54	-----VSKAPQLVPKLDDEVYNAAYNAADHAAPEDKYEAFVLHSEALRIIAGTPEVHAVKPGA-----	111
Phil_p5a/1-287	195	FNDIAIKASTGGAYESYKFIIPALEAAVKQAYAAVATAPFEVKYTVFETALKKAITAMSEAKKAAKPAATAATATAAAGVAA-----TGAAATAAGGYKV	287
Phil_p5b/1-295	199	FNKAIKESTGGAYDYTYKFIIPALEAAVKQAYAAVATAPAVKYTVFETALKKAITAMSEAKKAAKPAATAATATAAAGVAA-----TGAAATAAGGYKV	295
Poa_p5/1-307	215	FNDIAIKASTGGAYQSYKFIIPALEAAVKQSYAAVATAPAVKYTVFETALKKAITAMSEAKKAAKPAATAATATAAAGVAA-----TGAAATAAGGYKV	307
Loi_p9/1-339	223	FNNAIKVLGLAAYDSYKFIPTLVAAVKQAYAAKQATAPFEVKYTVFETALKKAVTAMSEAKKAAKPAATAATATAAAGVAA-----TGAAATAAGGYKV	339
Hor_v5/1-296	209	FNKAIKETGGAYESYKFIIPALEAAVKQAYAAVATAPEVKYTVFETALKKAINAMTQTEKPAKPAATAATAT--ATV-----AAGAAATAGGYKV	296
Tri_15/1-243	156	FNKAIKETGGAYESYKFIIPALEAAVKQAYAAVATAPEVKYTVFETALKKAINAMTQTEKPAKPAATAATAT--ATV-----AAGAAATAGGYKV	243
Sec_c5/1-292	201	FNKAIKETGGAYAYTYFVPALESAAVKQAYAAVATASAPVKYTVFETALKKAINAMTQTEKPAKPAATAATATAAAGVAA-----TGAAATAAGGYKV	292
Dac_g5/1-265	173	FNKAIKETGGAYESYKFIPTLEAAVKQAYAAVATAPEVKYTVFETALKKAITAMSEAKKAAKPAATAATATAAAGVAA-----TGAAATAAGGYKV	265
Bra_d5/1-273	187	FNKAIKETGGAYESYKFIIPALEAAVKQAYAAVATAPEVKYTVFETALKKAITAMSEAKKAAKPAATAATATAAAGVAA-----TGAAATAAGGYKV	273
Fes_p5/1-298	205	FNKAIKETGGAYETKFIIPALEAAVKQAYAAVATAPEVKYTVFETALKKAITAMSEAKKAAKPAATAATATAAAGVAA-----TGAAATAAGGYKV	298
Hol_15/1-264	172	FNKAIKETGGAYEAYKFIIPLETAVKQAYAAVATAPEVKYTVFETALKKAITAMSEAKKAAKPAATAATATAAAGVAA-----TGAAATAAGGYKV	264
Tri_a5/1-231	154	FDKAIKETGGAYAGYKFIIPALESAVKQAYAAVATAPEVKYTVFETALKKAITAMSEAKKAAKPAATAATATAAAGVAA-----TGAAATAAGGYKV	231
Phil_p6/1-111			

FIG E3



FIGE4

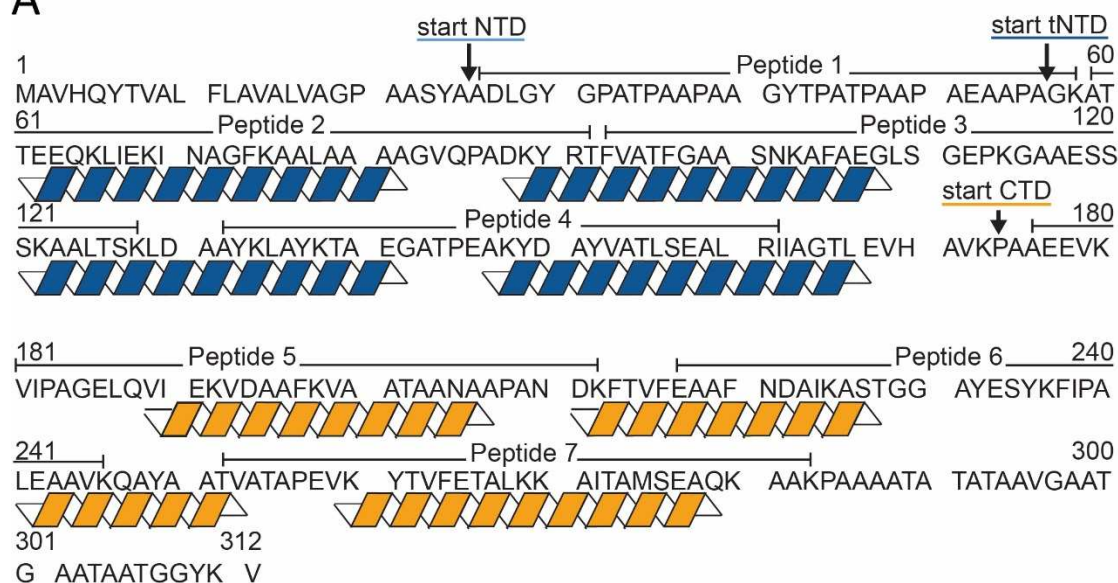
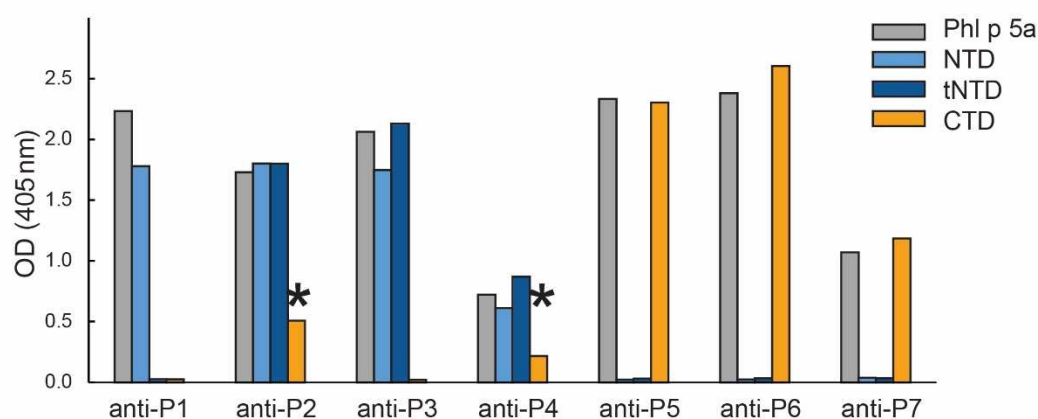
A**B**

FIG E5

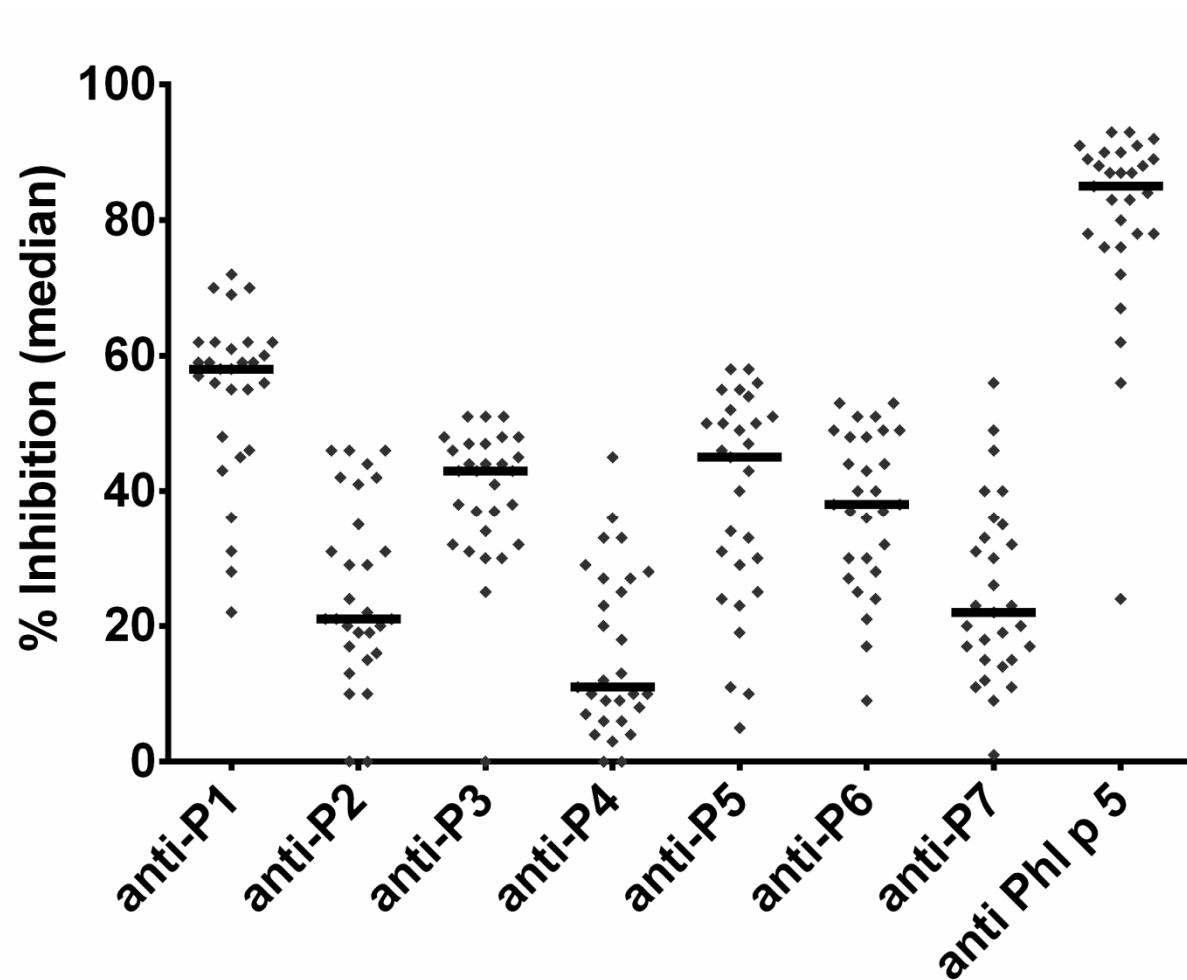


FIG E6

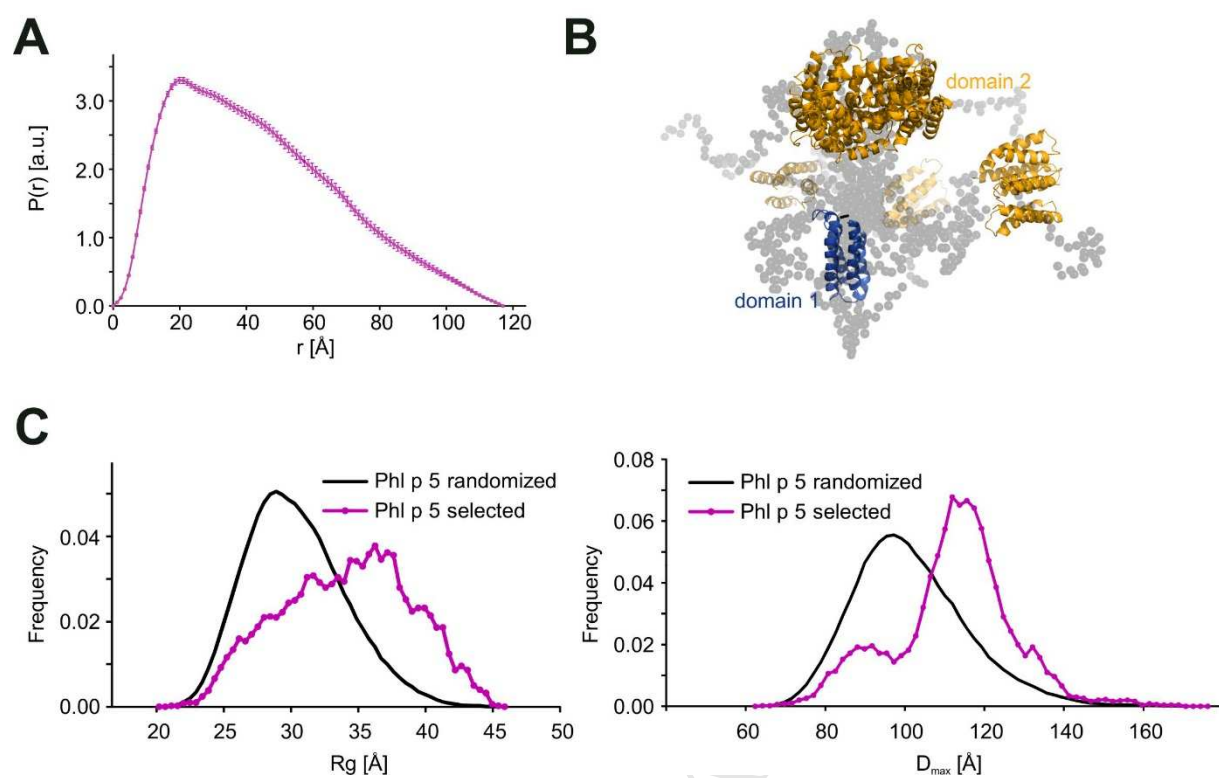


FIG E7

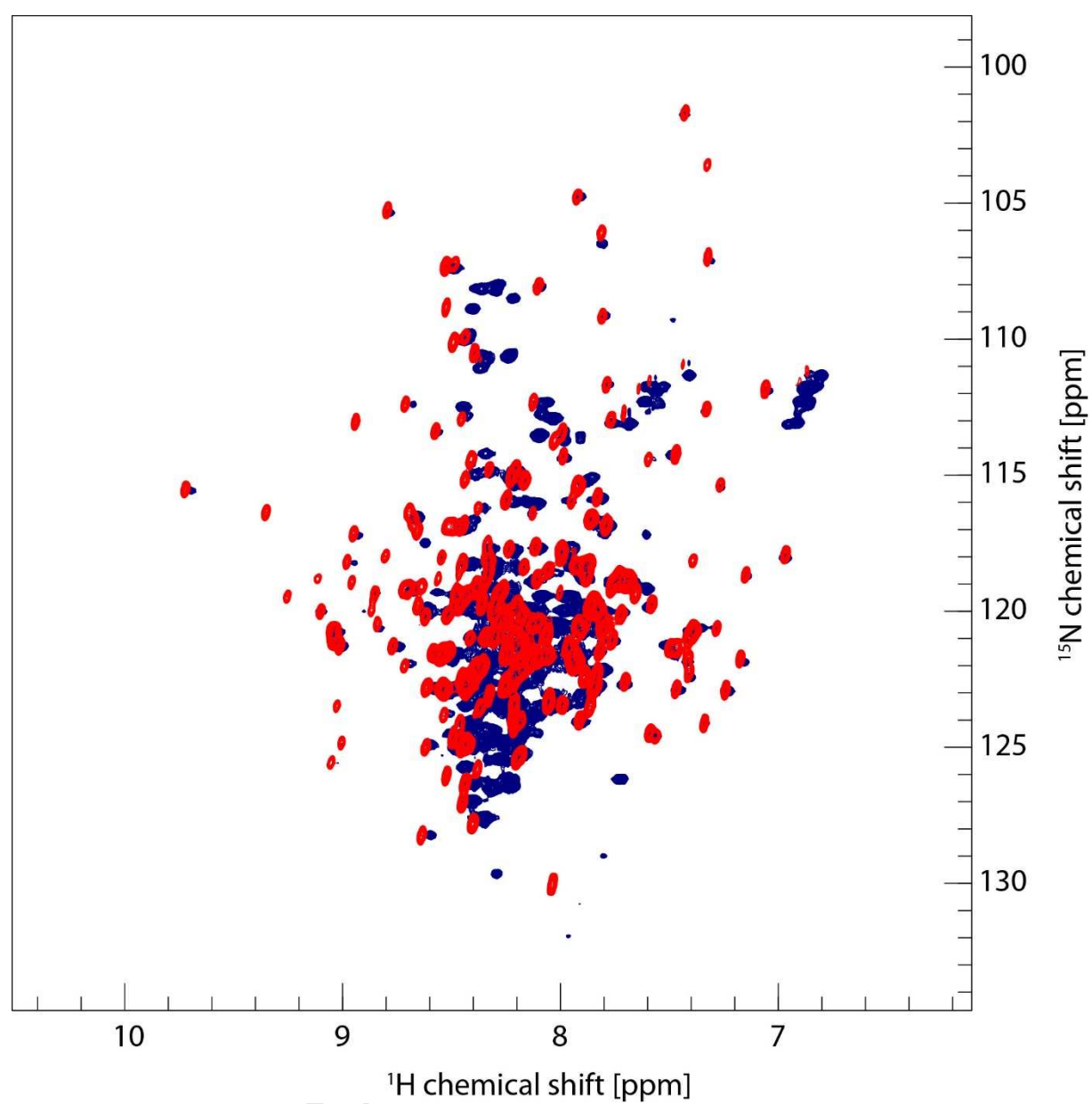


FIG E8

