Structure
Article

Mapping the Interactions between a Major Pollen Allergen and Human IgE Antibodies

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SUMMARY

The interaction of specific IgE antibodies with allergens is a key event in the induction of allergic symptoms, thus representing an important target for therapeutic interventions in Type I allergies. We report here the solution NMR structure of Art v 1, the major mugwort pollen allergen. Art v 1 is the first protein structure with an allergenic defensin fold linked to a polyproline domain, which has not been identified in any reported allergen structure in the PDB. Moreover, the direct interaction of polyclonal IgE antibodies from an allergic patient has been mapped on the surface of an allergen for the first time. The data presented herein provide the basis for the design of tools for safe and effective vaccination against mugwort pollen allergy.

INTRODUCTION

Allergic diseases such as allergic rhinitis or hay fever, allergic asthma, food allergy, allergic skin inflammation, and anaphylaxis affect up to 25% of the population in industrialized countries, and their incidence is continuously rising, particularly in children and young adults (Finkelman and Vercelli, 2007). Type I allergy is characterized by an overwhelming expansion of allergen-specific T helper (TH)2 cells resulting in class switching of B cells to produce polyclonal IgE antibodies specific to common environmental allergens originating from various sources including pollen of grasses, weeds, or trees, spores of molds, foods, mites, cockroaches, and dander from pets and other domestic animals (Parronchi et al., 2000). Although numerous allergens have been characterized at the structural level, common features that would definitely predict the allergenic potential (allergenicity) of a protein are still unknown (Thomas et al., 2009). Therefore, the identification of IgE epitopes is crucial for better understanding the phenomenon of allergenicity and has direct implications for the improvement of allergen-specific therapeutic approaches. Bioinformatics (Stadler and Stadler, 2003) and peptide-based approaches (Lin et al., 2009) have been successfully applied to reveal linear or sequential IgE epitopes but are less suited for the identification of conformational or discontinuous epitopes, which have been studied in most cases by mutational analysis of allergens (Ferreira et al., 1998). Thus, 3D structure determination of the antigen-antibody interaction remains the most accurate method to identify IgE-binding sites on allergens. Such approaches have been successful in revealing epitopes recognized by monoclonal antibodies directed against mite (Li et al., 2008; Naik et al., 2008), bee venom (Padavattan et al., 2007), food (Niemi et al., 2007), as well as pollen (Mirza et al., 2000; Padavattan et al., 2009; Spangfort et al., 2003) allergens. However, to date no structural information on the interaction between allergens and polyclonal human IgE antibodies of allergic patients has been reported. To investigate the interaction of human IgE and allergens at the atomic level of resolution, we have focused on Art v 1, the major allergen of *Artemisia vulgaris* (mugwort) pollen. The herb mugwort can be considered one of the main causes of pollen allergy in late summer and autumn in Europe, Northern America, and parts of Asia (Dedic et al., 2009). Among the allergic population, 10%–14% are sensitized to mugwort and more than 95% of these patients display specific IgE antibodies to the major allergen Art v 1. At the primary structure level, Art v 1 appears as a modular glycoprotein composed of two domains: an N-terminal domain with high homology with plant defensins and a hydroxyproline-rich C-terminal part (Himly et al., 2003). The Art v 1 carbohydrate moiety which comprises up to 30% of the molecular weight has been characterized in detail (Leonard et al., 2005). The hydroxyproline-rich domain carries two types of glycosylation: (1) single adjacent hydroxyproline-linked β-arabinofuranoses, and (2) a large type III arabinogalactan composed of a short β1,6-galactan core substituted by a variable number (5–28) of α-arabinofuranoses. The immune response against Art v 1 is characterized by IgE antibodies that are mainly directed against the disulfide-bond stabilized defensin domain (Dedic et al., 2009; Himly et al., 2003). Interestingly, T cell recognition of Art v 1 is conspicuously restricted to a single immunodominant epitope located in the defensin domain (Art v 1_{25-36}) and is

associated with the expression of the HLA-DRB1*01 phenotype (Jahn-Schmid et al., 2005, 2008). In addition, Art v 1 was considered by Aalberse et al. (2001) as an intriguing and clinically highly relevant cross-reactive system.

In this study, we report the solution NMR structure of an allergenic defensin linked to a polyproline domain and the identification of conformational IgE-binding epitopes using antibodies isolated from the peripheral blood of a mugwort-pollen allergic patient. We identified two IgE-binding patches on the surface of the Art v 1 defensin domain that are crucial in triggering allergic reactions in mugwort pollen-sensitized patients. These epitope patches are predominantly positively charged and are highly conserved among Art v 1-homologous allergens in ragweed and sunflower pollen. Thus, the data presented herein provide a basis for the design of novel tools for safe and effective vaccination against mugwort pollen allergy.

RESULTS

Art v 1 Consists of a Defensin Domain Linked to a Proline-Rich Segment

The solution structure of Art v 1, the major allergen from *Artemisia vulgaris* pollen, was solved by NMR. Recombinant isotopically ^{15}N- and ^{13}C-labeled Art v 1 was produced in *Escherichia coli* and used for data collection. Chemical shift assignments were deposited in the BMRB (entry 16111) (Razzera et al., 2009). An ensemble of 100 structures was calculated from 627 distance constraints using a simulated annealing method within the program CYANA (Guntert et al., 1997). All structures are in agreement with the experimental constraints showing only minor deviations from the idealized covalent geometry (Table 1). The 20 lowest energy structures had no violations of either distance restraints greater than 0.25 Å or dihedral angles greater than 3.0° and were chosen to represent the solution structure of Art v 1 (deposited in the PDB under the assigned ID code 2kpy). The ensemble of the calculated structures superposed over the backbone is illustrated in Figure 1A; a cartoon representation is shown in Figure 1B. The backbone rmsd of the 20 refined Art v 1 structures is 0.49 Å for residues involved in the secondary structure elements of the defensin domain and 9.71 Å for the full-length polypeptide chain. Considering all atoms, the defensin domain or the entire polypeptide presented a rmsd of 1.12 or 9.85 Å, respectively. The calculated structure revealed that Art v 1 displays three distinct regions: (1) a defensin domain, (2) a transitional region, and (3) a C-terminal polyproline segment. The N-terminal domain (residues 1–56) shows the typical α/β fold of plant defensins (Thomma et al., 2002) composed of an α helix from residues 20–29 and three antiparallel β strands formed by amino acid residues 4–9, 34–40, and 44–53, respectively. Moreover, this fold is stabilized by eight disulfide-linked cysteines, with disulfide bonds linking C6–C53, C17–C37, C22–C47, and C26–C49. These data are in accordance with the structural motif of the cysteine-stabilized α/β fold of other plant defensins: two consecutive cysteine residues spaced by a tripeptide sequence (C-X-X-X-C) in the α helix and by a C-X-C in the β strand (Thomma et al., 2002). A comparison of 3D structures using Dali analysis (Holm et al., 2008) showed that the Art v 1 defensin domain is closely related to a radish (*Raphanus sativus*) seed defensin (PDB: 1ayj) that has antifungal activity. In contrast, the Art v 1 proline-rich segment (residues 57–108) seems to be unique in the PDB database and has not been identified in any structure reported so far. Using PONDR, the poly-proline region was predicted to be unstructured. However, several long-range NOEs between the defensin domain and the transitional region (residues 57–70) were observed, restricting the calculated structure of this region to 1.7 Å rmsd. For the identification of long-range NOEs, each decision of the network anchoring algorithm in CYANA was manually verified when transitional NOEs were added.

Table 1. Summary of the Conformational Restraints and the Statistical Analysis

Experimental Restraints	
NOE distances[a]	627
Intraresidues (i, i)	164
Sequential (i, i ± 1)	221
Medium range (i + 2, i + 3)	63
Long range (>i + 3)	179
Hydrogen bonds	24
Dihedral angles	58
Φ (°)	29
Ψ (°)	29
Rmsd from the average structure	
Structured defensin domain (3–56)	
Backbone (Å)	0.49 ± 0.07
Heavy atoms (Å)	1.12 ± 0.12
Quality Check	
Ramachandran plot (PROCHECK)[b]	
Most favored regions (%)	89.5
Additional allowed regions (%)	10.0
Generously allowed regions (%)	0.5
Disallowed regions (%)	0.0
CYANA "target function" (Å^2)	2.97 ± 0.39

[a] Derived from 627 assigned NOESY cross-peaks, including intraresidue peaks.
[b] For secondary structure elements.

The defensin domain was the first to be calculated, excluding all NOEs from the transitional and the polyproline region. In a first step, calculations were done without restraints for disulfide bonds. Once the structure is allowed to unambiguously attribute all disulfide bonds, this pattern was included in the subsequent calculations. Next, the list containing the NOEs for the defensin domain was fixed and manually given beforehand. Following the manual inclusion of the sequential NOEs (αN(i,i+1) and NN (i,i+1)) of the transitional and C-terminal region, and those defining the stereochemistry of the prolines, i.e., $\alpha\delta$(i,i+1) for *trans* and $\alpha\alpha$(i,i+1) for the *cis* prolines, the entire protein was calculated by CYANA. In this way, we could clearly identify NOEs between the defensin and the transitional region, suggesting some degree of order in the transitional region. It should be noted that the heteronuclear NOE goes from ~0.8 in the well-ordered defensin domain to ~−0.5 in the flexible C terminus, while the transitional region displays intermediate values. In addition, we observed two residues (G66 and A67) with

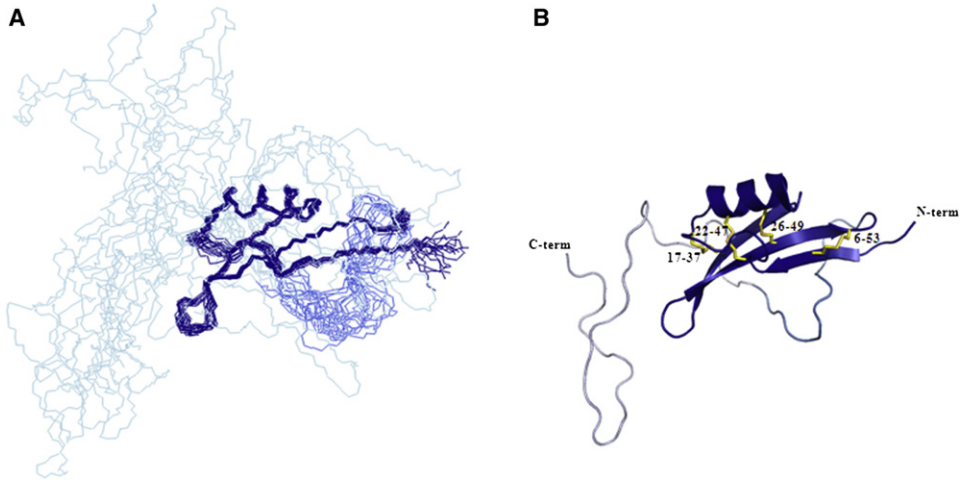

Figure 1. NMR Solution Structure of the mugwort Pollen Major Allergen Art v 1

(A) Backbone superposition of 20 lowest energy Art v 1 structures. The defensin domain is shown in dark blue, the intermediate region in blue, and the C-terminal part in light blue.

(B) In the ribbon illustration, disulphide pairings Art v 1 are indicated by numbers and yellow sticks (PDB ID code 2kpy).

heteronuclear NOEs of ~0.2 indicating a higher order in the transitional region. The order parameters for this region also showed values indicative of higher order for the same residues G59 and A60 (S2 ~0.6) and G66 and A67 (S2 ~0.45). Notably, we observed that residues displaying higher order are intercalated with more flexible regions. The presence of structural order in the transitional region is supported by the α- and β-carbon chemical shift values, which did not indicate random coil (Razzera et al., 2009). This view agrees with observations that all proline residues were in *trans* conformation, with the exception of P95 and P103 at the C terminus of Art v 1 which were primarily found as *cis* isomers. No *trans*-conformers of these residues were observed, which is consistent with the notion that *cis/trans* isomerization is much more probable in highly flexible nonstructured regions of proteins.

The interaction of the defensin and the transitional region was initially defined by two nonambiguous NOEs identified between E7 HN and A63 HN and between C6 HA and A63 HN. The inclusion of these two NOEs in the calculation largely limited the range of the conformational space for interaction between the transitional region and the defensin domain. This space restriction led to the identification of six other NOEs (C6 HA - A63 HB, C6 HA - P62 HB2, E7 HN - A63 QB, E7 HN - P62 HA, E7 HN - P62 HB2, K4 QD - P62 HA) confirming the interaction between the defensin domain and the transitional region. All these NOE assignments were manually checked.

Interestingly, we observed that the C terminus loops back to the defensin domain and makes contact with residues 6/7 and 62/64. One reason for this could be the presence of the disulfide bond between C6 and C53, which might be an important element for constraining this interaction. The absence of proline *cis-trans* isomerization in the more flexible part of the C terminus (P95 and P103) may also contribute to the restricted conformational space. The latter is corroborated by data on the dynamical behavior of Art v 1. In the heteronuclear NOE experiments as well as in the calculated order parameter, we observed an oscillation between higher and lower order, with a periodicity of about 7 residues. These results could be interpreted as a tendency to form a typical poly-proline extended segment. Notably, residues displaying higher orders (G59, A60, G66, A67, G74, G83, G84, S94, G99, G100, S106) are mostly glycines. The last two C-terminal residues showed a remarkable drop in their heteronuclear NOE values, an oscillation pattern that was also observed for chemical shift deviation from random structures (ΔδC_α – ΔδC_β) (Razzera et al., 2009).

Art v 1 Relaxation Parameters Reveal Three Distinct Regions of Different Flexibility

To evaluate the backbone dynamics of Art v 1, we measured ^{15}N longitudinal and transversal relaxation times (R1 and R2) as well as ^{15}N-^{1}H heteronuclear NOEs (see Table S1 available online). The relaxation properties of the backbone ^{15}N atoms in the defensin domain behaved as expected for a folded protein exhibiting heteronuclear NOE values around 0.8, with exception of the N-terminal ^{15}N atoms, which displayed negative values. Residues 14 and 15 and 41–44 forming loops 1 and 2, respectively, showed slightly lower values around 0.4 (Figure 2A). In contrast, the Art v 1 C-terminal segment was flexible and displayed heteronuclear NOE values of –1.8 for residues 107 and 108. The transitional region (residues 57–70) located between the folded domain and the highly flexible C terminus showed intermediate values of relaxation parameters. Due to overlapping amide resonances, residues G2, T12, G83, G92, G84, G100, G75, G93, and S54 were not included in the analysis (Razzera et al., 2009). Residues K39 and S94 were also not included as their signal intensities in the ^{15}N-HSQC spectrum were too weak to allow analysis.

The Lipari and Szabo model free formalism (Lipari and Szabo, 1982a, 1982b) was used for further interpretation of the relaxation data and to gain insights into the magnitudes and the timescales of the backbone motion (Figures 2B and 2C). We used the TENSOR2 software for analysis (Dosset et al., 2000) and data fitted best to anisotropic tumbling in solution. The calculated correlation time (τ_c) of Art v 1 was 5.87 ± 0.02 ns and was used to estimate internal mobility parameters: order parameter (S^2)

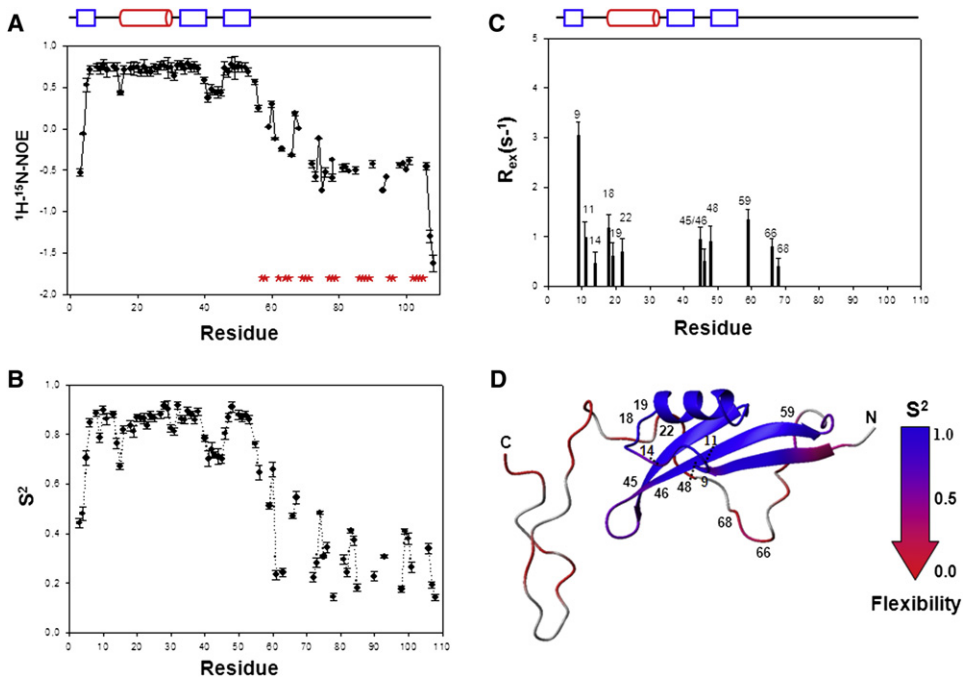

Figure 2. Backbone Dynamics of Art v 1

(A) Heteronuclear NOE data reveal three regions of Art v 1 movements. Proline residues are represented by red asterisks.

(B) Pico-nanosecond timescale backbone dynamics are represented by order parameters (S^2).

(C) Milli-microsecond chemical exchange values [$R_{ex}(s^{-1})$] are plotted as bars as a function of the residue number.

(D) Art v 1 residues are colored according to their order parameter (S^2): (i) blue in the well-folded defensin domain, (ii) purple in the transitional region, and (iii) red in the C terminus. Prolines and nonobserved residues are indicated in gray. Residues involved in conformational exchange are highlighted by numbers in the structure. Error estimates were calculated by the program Modelfree.

and conformational exchange rates (R_{ex}). Residues located in the loops and the poly-proline region displaying values higher than one standard deviation from the mean of R2/R1 were excluded from the τ_c calculation (Table S2 and Figure S1). The mean value of S^2 was 0.81 for the defensin domain and 0.2 for the C-terminal region. The C-terminal residues from amino acid 71–108 were flexible exhibiting thermal movements that occurred in the ps-ns timescale. The transitional region displayed intermediate values of S^2 showing a complex motion, including some degree of thermal mobility and conformation exchange in the μs-ms timescale ($R_{ex} > 0$) (Figure 2C).

Despite the fact that these residues showed small R_{ex} values and the R_{ex} estimation error in the Lipari-Szabo fitting model, the raw data R2/R1 is more suitable to interpret the data because it is independent of the diffusion model and fitting. As shown in Figure S1, R2/R1 values obtained for residues T9, K11, D18, G66, and G68 are above the average, which is in agreement with conformational exchange. Notably, most of the residues involved in conformational exchange were found to be located in the first and second loop of the Art v 1 defensin domain (T9, K11, S14, D18, N19, C22, E45, S46, F48) as well as in the transitional region (G59, G66, G68) (Figure 2D).

Domain Interactions in Hydroxylated and Glycosylated Natural Art v 1

Art v 1 was purified from *Artemisia vulgaris* pollen and natural abundance spectra were collected by $^1H/^{13}C$ HSQC experiments. Chemical shift differences between the natural and the recombinant protein were measured for Cα and Hα (Figure 3). Differences in chemical shifts were small and the assignment of nArt v 1 could be obtained through direct comparison with values obtained for rArt v 1. Carbon chemical shifts of the carbohydrate moiety were assigned based on previous studies by Leonard et al. (2005). In Figure 3A, observed differences are presented according to the residue number of Art v 1. Figure 3B shows the $^1H/^{13}C$ HSQC spectrum with the assigned Hα/Cα of cysteines, as an example. The only residues that could not be assigned in the natural molecule were the prolines. Instead of separated peaks, prolines clustered in three different regions of the spectrum (Figure 3B). Therefore, the differences in chemical shift could not be measured, although there were clear changes. Presumably, the hydroxylated and glycosylated proline residues in the C-terminal region of the natural molecule (Leonard et al., 2005) would account for the more pronounced differences ($>\Delta\delta + \sigma$). Interestingly, some residues in the N terminus, such as C6 and K8, also showed significant differences. In addition, other residues in the defensin domain exhibited shifts greater than the mean value ($\Delta\delta = 0.04$), i.e., H34 in the second β sheet, A42 in the second loop, Y50 in the third β sheet, and C53 which forms a disulfide bond with C6. Figure 3C highlights the residues with significant shifts in the structure. Amino acids in the defensin domain with significant changes were located at the surface of the β sheet and in the transitional region. These

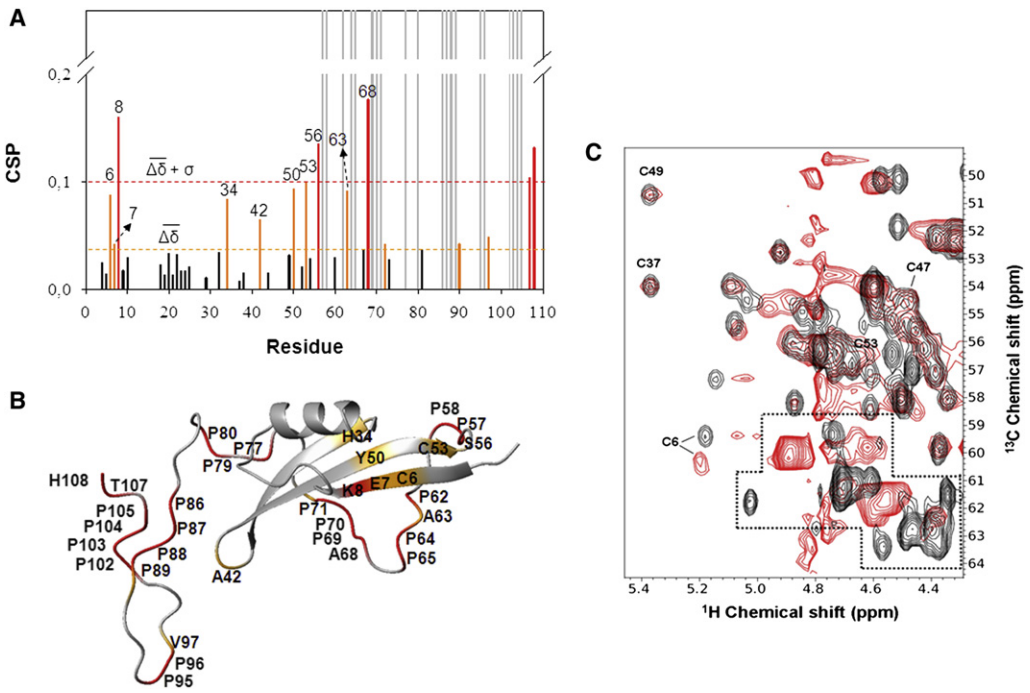

Figure 3. NMR Analysis of Natural Art v 1 Based on Natural Abundance ^{13}C-HSQC Spectra

(A) Chemical shift deviations between natural and recombinant Art v 1 α-hydrogen and carbons are shown for individual amino acids. The average (Δδ) and one standard deviation from the average (Δδ + σ) cutoffs are represented by orange and red dashed lines, respectively. Bars for corresponding residues are shown in the same color; prolines are represented as gray bars.

(B) Shifted amino acids are highlighted in orange (>Δδ) and red (>Δδ + σ) in the structure of Art v 1 (2kpy).

(C) Resonances in the Hα/Cα region of the ^{1}H/^{13}C HSQC spectrum are illustrated in black for the recombinant protein and in red for the natural counterpart. Proline resonances (highlighted by the dotted box) clearly changed but could not be assigned by comparison with values from the recombinant protein.

observations support the view that in the natural Art v 1 allergen the transitional region interacts with the defensin domain.

IgE Epitopes of Art v 1 Are Predominantly Located on the defensin Domain

In the presence of IgE antibodies, most of the residues did not change, yet some showed small but significant chemical shift perturbations (CSP) (Figure 4A). In more detail, residues S3, K4, E45, K55, S56, A63, S85, and H108 displayed CSPs higher than two standard deviations from the mean (Δδ + 2σ) and residues K8, S14, D18, R40, E41, S46, Y50, G74, G75, and G93 showed minor perturbations (>Δδ = 0.0027).

Figure 4B displays sections of the spectrum showing residues with high CSPs upon interaction with Art v 1-specific IgE antibodies. At least three residues with major perturbations, i.e., E45, S56, and A63 exhibited significantly reduced signal amplitudes in the presence of IgE antibodies (Figure 4B). As shown in Figures 4C and 4D, most of the observed shifts could be localized in two distinct surface patches, presumably constituting individual conformational IgE epitopes. One epitope was formed by residues S14, R40, E41, E45, and S46 located in the β1-α and β2-β3 loops and β3 sheet and thus entirely on the surface of the Art v 1 defensin domain. The second identified IgE epitope was composed of amino acids S3, K4, K55, S56, and A63 and encompassed both, the defensin domain and the structured transitional region of the proline-rich segment. Interestingly, analysis of the electrostatic surface potential showed that the IgE interaction sites are predominantly positively charged (Figure 4E).

The observed chemical shift differences are directly related to changes in the chemical environment of the observed nuclei. The results showed that the complex is in fast exchange regime, since Art v 1 was in 25-fold molar excess to IgE (2 μM IgE and 50 μM Art v 1). A broad range of IgE affinities (0.0358–291 nM) has been previously reported using a repertoire of recombinant IgE antibodies specific for the major house dust mite allergen (Christensen et al., 2008). This study convincingly demonstrated that, in the presence of single high affinity IgE, medium to low affinity antibodies can efficiently cause crosslinking and effector cell degranulation. In our experiments, interactions were probed in the medium to low affinity ranges (>10^{-8} M), which were shown to be of relevance in triggering allergic symptoms.

In order to validate the results obtained by chemical shift mapping, the binding activity of serum IgE from the patient enrolled in this study was tested with a set of eight Art v 1 mutants (Gadermaier et al., 2010). These single cysteine variants were designed to target all disulfide bonds stabilizing the Art v 1 defensin domain. Complete loss or only marginal IgE reactivity was observed upon disruption of disulfide bonds C22–C47 and C26–C49, or C17–C37, respectively (Figure 5). However, targeting the cysteine residues in the N- and C-terminal region of the defensin domain (C6, C53) did not

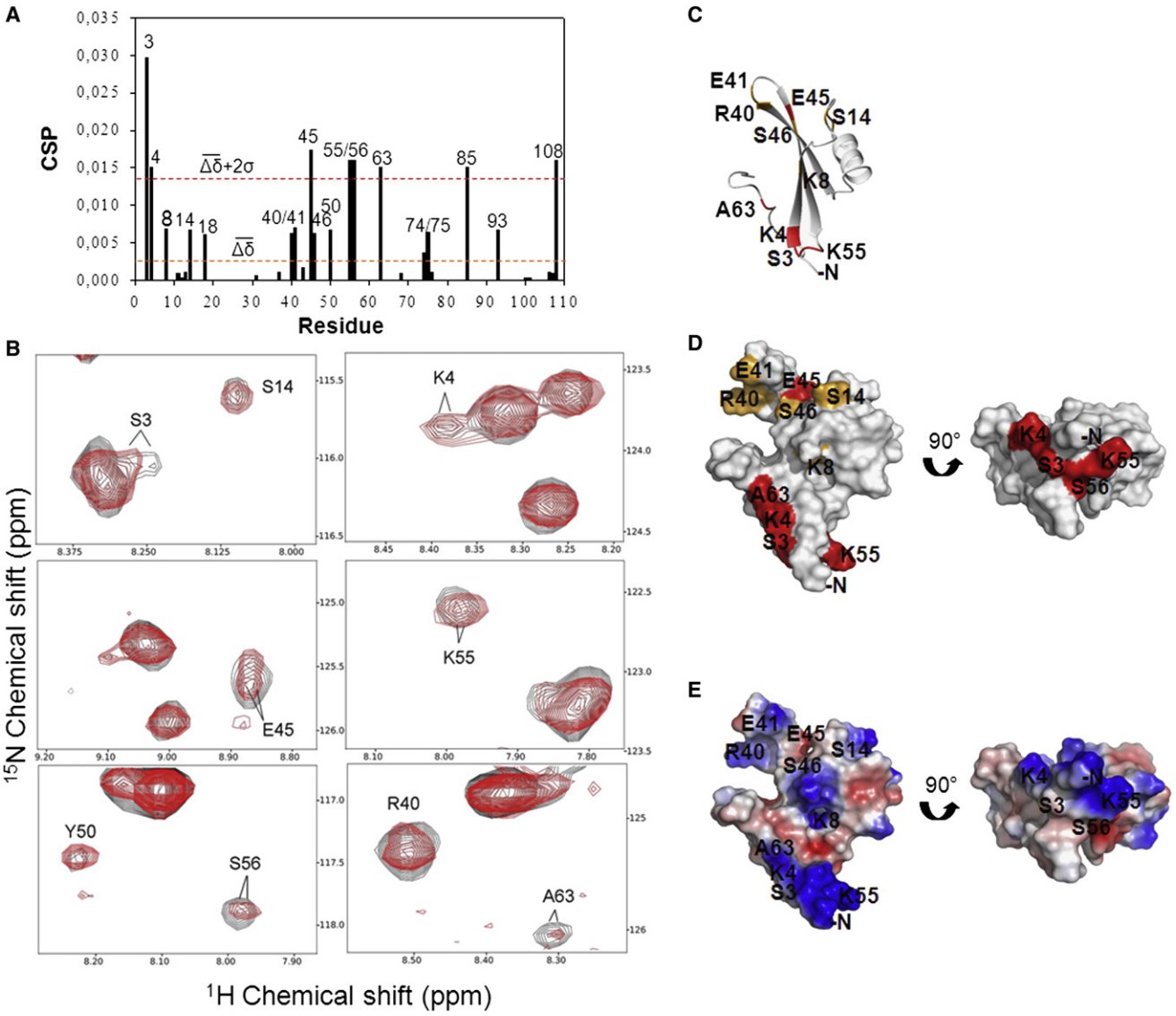

Figure 4. Art v 1 IgE Epitopes

HSQC of ^{15}N labeled rArt v1 were acquired in the absence and presence of specific human IgE purified from the peripheral blood of a mugwort pollen-allergic patient. All experiments were measured in 50 mM citrate buffer (pH 7.8).

(A) For distinct amino acids chemical shift perturbations (CSP) observed upon IgE interaction.

(B) Sections of the actual spectrum showing changes in specific amino acids, in black free Art v1 and in red in the presence of IgE.

(C and D) Residues with high CSP (>$\Delta\delta + \sigma$) are represented in red; those with lower CSP (>$\Delta\delta$) are in orange.

(E) The charge distribution on the Art v 1 surface is depicted in blue and red for positive and negative charges, respectively. Residues 71–108 displaying an unrestricted structure were excluded from the figures.

alter the reactivity of IgE antibodies, implying that the identified epitopes could be correctly formed. Since no chemical shifts were observed for the Art v 1 cysteine residues upon interaction with IgE, we can conclude that each of the disulfide bonds formed between C22–C47, C26–C49, and C17–C37 is crucial for the stabilization of the antibody-binding patches identified by chemical shift mapping. It should also be mentioned that none of the IgE-interacting residues described here were previously shown to be involved in other posttranslational modifications described for the Art v 1 molecule (Leonard et al., 2005).

In addition to amino acids contributing to the epitope patches described above, residues G74, G75, S85, G93, and H108 in the C-terminal region also exhibited elevated CSPs. Since those positions are not restricted in the calculated structure and are localized in the more flexible extended region of the molecule, it is not obvious if they could form a contiguous patch and hence their role in IgE binding remains unclear.

In summary, most residues with higher CSPs are located on the surface of the Art v 1 defensin domain and transitional region, forming two distinct IgE-binding epitopes that are predominately composed of positively charged residues.

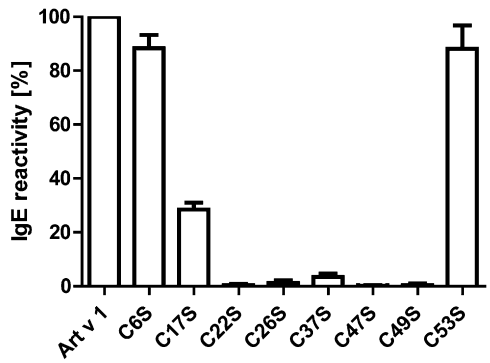

Figure 5. Influence of Cysteine Residues on the Allergenic Property of Art v 1

IgE-binding to Art v 1 and single cysteine mutants, i.e., C6S, C17S, C22S, C26S, C37S, C47S, C49S, and C53 was determined in ELISA using the allergic patient's serum previously applied for structural interaction studies. Measurements at 405 nm were performed in triplicates and presented as percentage of IgE reactivity to Art v 1 (= 100%).

DISCUSSION

In the present study, the three-dimensional structure of the major pollen allergen Art v 1 determined by NMR methods revealed a typical plant defensin fold (Thomma et al., 2002) linked to a novel flexible poly-proline structure. This is the first structure of an allergenic defensin, which is structurally similar to an antifungal defensin from *Raphanus sativus* (PDB: 1AYJ). Sequences of ancient antimicrobial defensin-like peptides fused to proline-rich domains have been previously described. Such defensin chimeras have been proposed to function by anchoring onto plant cell walls and acting as sentinels or direct microbicides when proteolytically cleaved by predators (Silverstein et al., 2007).

Although there are no data supporting a role for the Art v 1 defensin domain in plant defense, its involvement in IgE-mediated allergic reactions is unquestionable: (i) the IgE-binding activity of the defensin domain is similar to the full-length molecule (Dedic et al., 2009), (ii) destabilization of the defensin fold by reduction and alkylation of disulfide bonds (Himly et al., 2003) or by site-directed mutagenesis of cysteine residues (Gadermaier et al., 2010) abolishes IgE reactivity, and (iii) the capacity to release inflammatory mediators from basophils is restricted to the defensin domain (Dedic et al., 2009).

Crosslinking of allergen-specific IgE antibodies by allergens at the surface of mast cells and basophils trigger the release of preformed inflammatory mediators and the onset of allergic symptoms (Gould and Sutton, 2008). Thus, knowledge on 3D structure and IgE epitopes of allergens is crucial for understanding allergic reactions and has direct implications for the development of improved diagnostic and therapeutic products. At the structural level, B cell epitopes have been mapped by cocrystallization or by NMR interaction experiments of allergens from diverse sources (Li et al., 2008; Naik et al., 2008; Padavattan et al., 2007, 2009; Niemi et al., 2007; Mirza et al., 2000) with murine monoclonal Fab antibody fragments. Even though valuable data on B cell epitopes of allergens have been generated due to partial overlap between binding sites of murine and human antibodies, so far authentic epitopes recognized by polyclonal IgE antibodies from allergic patients have not been reported. In this study, we identified two individual IgE epitopes on the major allergen Art v 1 that are specifically recognized by IgE polyclonal antibodies purified from the peripheral blood of a mugwort pollen-allergic patient. Typical for conformation-dependent epitopes, the amino acids involved in IgE binding were discontinuous in the primary sequence but formed two distinct patches on the surface of the defensin domain. The location of these epitopes is in good agreement with in silico predictions using software that identifies surface exposed epitope fragments based on 3D protein structure (Moreau et al., 2008).

In addition, the relevance of the identified epitope patches is further supported by the results obtained in ELISA experiments using Art v 1 cysteine mutants and serum from the same patient enrolled in this study. Our experiments clearly demonstrated that the epitope patches mapped by chemical shift are primarily stabilized by crosslinking between the α helix and the third β sheet (C22–C47 and C26–C49 bonds) whereas crosslinking of the loop region formed by residues 10–19 and the second β sheet (C17–C37 bond) contributes only partially to their conformational stability. Remarkably, crosslinking between the first and the third β sheets plays no role in the stabilization of these conformational epitopes.

A closer examination of the IgE epitopes revealed a predominance of charged residues (i.e., K4, K8, D18, R40, E41, E45, and K55). This observation is in accordance with previous findings that charged residues (Li et al., 2008; Naik et al., 2008; Padavattan et al., 2007, 2009; Niemi et al., 2007; Mirza et al., 2000; Spangfort et al., 2003), in particular lysines (Chan et al., 2006; Gehlhar et al., 2006; Oezguen et al., 2008), are important for antibody binding. It should be noted that all amino acid residues shown here to be involved in the formation of these IgE epitopes are strictly conserved in all known naturally occurring isoforms of Art v 1 (Dedic et al., 2009). Interestingly, some residues interacting with human IgE (K4, K8, S14, R40, E41, Y50, and K55) are also highly conserved among Art v 1-homologous molecules containing a defensin fused to a proline domain, i.e., ragweed (*Ambrosia artemisiifolia*), sunflower (*Helianthus annuus*), endive (*Cichorium endivia*), groundsel (*Senecio vulgaris*), lettuce (*Lactuca serriola*), and dandelion (*Taraxacum officinale*) (Figure 6A). In particular, Art v 1 homologs in sunflower and ragweed have been previously reported to bind IgE antibodies from mugwort-sensitized patients (Asero et al., 2006; Gruber et al., 2009). Thus, the occurrence of allergic cross-reactions in Art v 1-sensitized patients upon exposure to defensins from other plant sources cannot be excluded. Cross-reactivity is, at least in part, related to phylogenetic relations between organisms that lead to similarity in the primary and tertiary structures (Aalberse et al., 2001). It should also be considered that antibodies undergo conformational diversity that, therefore, can adopt multiple structures. This ability increases the effective repertoire leading to autoimmunity and allergy (James et al., 2003). The identification of interaction hot spots can help in the recognition of cross-reactive binding sites.

The Art v 1 poly-proline domain is composed of a relatively constricted segment, termed the transitional region, followed by a completely unrestricted C-terminal part. Relaxation parameters and model free analysis were compatible with the

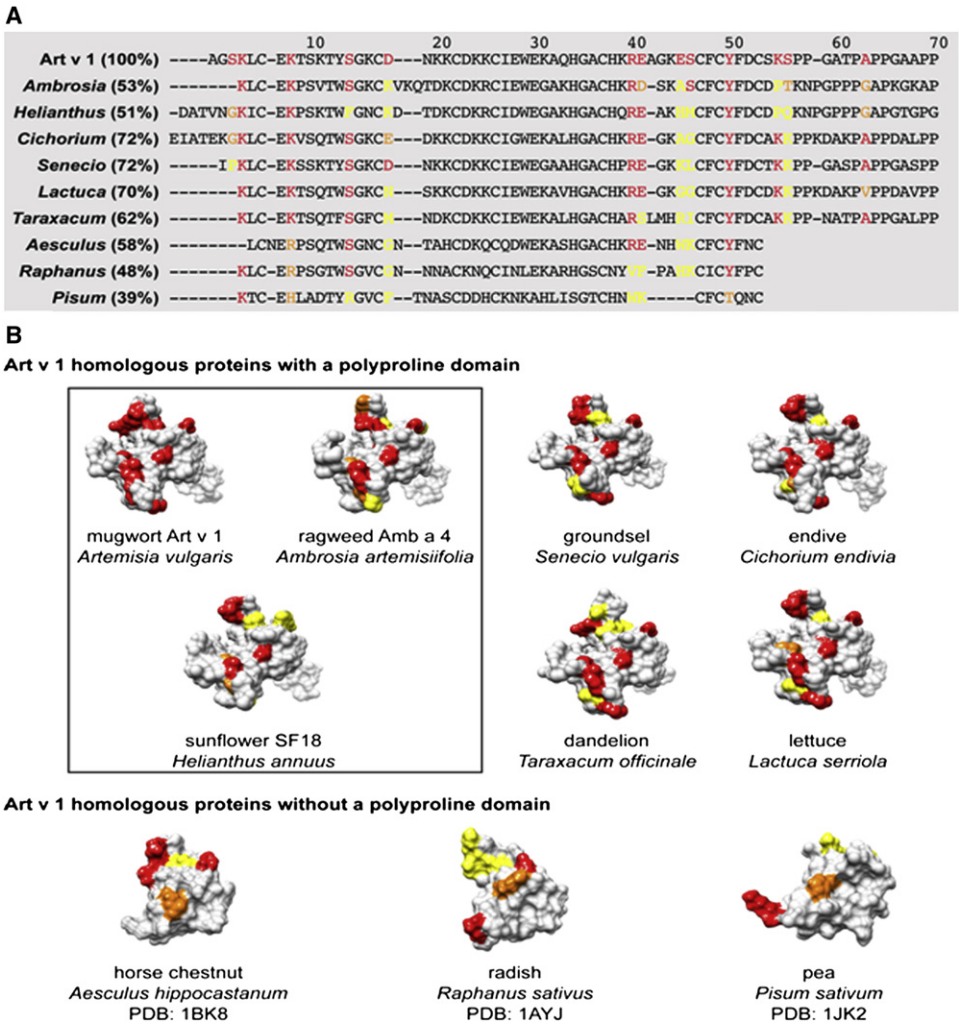

Figure 6. IgE Interacting Amino Acids in Art v 1-Homologous Molecules

Defensin-proline domain fusions displaying more than 35% sequence homology with Art v 1 were identified in the following *Asteraceae* species: *Ambrosia artemisiifolia* (Leonard et al., 2010), *Helianthus annuus* (protein: P22357.1), *Cichorium endivia* (EST: EL366705.1), *Senecio vulgaris* (EST: DY660118.1), *Lactuca serriola* (EST: DW116026.1), and *Taraxacum officinale* (EST: DY835145.1). Structures of homologous plant defensins without proline domain were found in *Aesculus hipocastanum*, *Raphanus sativus*, and *Pisum sativum*.

(A) Sequence alignment of Art v 1 and homologous proteins. Percentages of sequence identity to Art v 1 are given in parenthesis for each molecule.

(B) 3D structure of Art v 1 and homology modeling. The 3D structure models of proteins in ragweed, sunflower, groundsel, and lettuce were generated by homology modeling using the NMR structure of Art v 1 as template (RMS deviations: *Ambrosia artemisiifolia* 2.03 Å; *Helianthus annuus* 1.09 Å; *Cichorium endivia* 0.47 Å, *Senecio vulgaris* 0.55 Å; *Lactuca serriola* 0.48 Å; *Taraxacum officinale* 0.06 Å; *Aesculus hipocastanum* 2.35 Å; *Raphanus sativus* 2.40 Å and *Pisum sativum* 2.75 Å). Amino acids displaying chemical shift perturbations upon IgE interaction are colored in red for conserved residues, orange for residues with similar physicochemical properties, and yellow for residues that differ from those of the Art v 1 template. The boxed structures/models indicate molecules previously shown to bind IgE antibodies from Art v 1-sensitized patients.

structure calculation and NOE pattern, clearly suggesting three distinct regions with different dynamic properties. Previous circular dichroism analysis of the Art v 1 proline domain suggested a poly-proline type II structure (Dedic et al., 2009; Himly et al., 2003). However, the transitional region seems to represent an entirely novel arrangement, since no similar structure was found in the PBD based on Dali and Blast analyses. The chemical shifts observed for natural glycosylated Art v 1, when compared with the recombinant nonglycosylated protein, indicated that carbohydrates in the polyproline region affect to some extent the defensin domain in the natural molecule. This is of interest because a small subgroup of mugwort-allergic patients has been reported to produce IgE antibodies recognizing both the defensin domain and the Art v 1 O-linked glycans (Leonard et al., 2005). The nature of the epitopes recognized by such antibodies is unclear and might comprise carbohydrate (hydroxyproline-linked β-arabinoses and adjacent residues) as well as protein structures. This would explain the chemical shift perturbations observed for several residues in proximity to potentially glycosylated hydroxyproline residues in the Art v 1 C terminus upon interaction with patient's IgE antibodies. However, the clinical relevance of IgE antibodies specific for

carbohydrates and, in particular, for the O-glycans of Art v 1, remains unclear. Moreover, their involvement in IgE cross-reactivity (Altmann, 2007) seems to be very limited due to restricted distribution of hydroxyproline-linked β-arabinoses within plant proteins.

At the T cell level, the immune response to Art v 1 is restricted to a single immunodominant epitope (Jahn-Schmid et al., 2008). The Art v 1_{25-36} T cell epitope is highly conserved among defensins displaying a C-terminal poly-proline domain, with identical anchor positions (I27, E30, A32, and G35) for binding the groove of the HLA-DR molecule (Figure S2A). Exposure to plant defensins might therefore boost the production of allergen-specific IgE molecules outside the mugwort flowering season through activation of cross-reactive Art v 1-specific T cells (Schimek et al., 2005). Further, the immunodominant T cell epitope appears to be surface exposed and thus, might be accessible for binding to HLA-DR prior to proteolytic degradation of Art v 1 during antigen processing (Sercarz and Maverakis, 2003) (Figure S2B).

In summary, the direct interaction of polyclonal IgE antibodies from an allergic patient was mapped for the first time by analyzing the chemical shifts induced upon antibody binding in a fast exchange regime on the timescale of the NMR parameters. We identified two distinct patches on the surface of Art v 1 defensin domain interacting with IgE antibodies that are of paramount importance in triggering allergic reactions in mugwort pollen-sensitized patients. The data presented herein provide valuable insights on the immune recognition of allergenic proteins and may be utilized to improve allergy vaccines. A major problem associated with allergy vaccination, the only curative approach for allergy, is the occurrence of IgE-mediated side effects. To avoid such undesirable reactions, current allergy research focuses on the development of novel allergen vaccines using molecules with reduced allergenicity (IgE-binding capacity) but retained immunogenicity (T cell activation potential) (Ferreira et al., 1998; Egger et al., 2009; Larche et al., 2006; Wallner et al., 2007). Recently, a hypoallergenic fold variant of the birch pollen major allergen Bet v 1 has been designed and shown to effectively reduce birch pollen-induced allergic symptoms in a double-blind, placebo-controlled clinical trial (Kettner et al., 2007). Previous knowledge regarding the sequence of the immunodominant T cell epitope together with data uncovered in this study on the topography of IgE-epitopes may permit rational design of hypoallergenic Art v 1 variants to create safer and more efficient vaccines for mugwort pollen allergy.

EXPERIMENTAL PROCEDURES

Production of Recombinant Art v 1

Mature rArt v 1 (accession number AF493943) was produced as nonfusion protein in *Escherichia coli* as recently described (Razzera et al., 2009). For structure solution purified isotopically ^{15}N and $^{15}N^{13}C$ labeled molecules (1.0–1.2 mM) were prepared in 10 mM sodium acetate (pH 5.5) containing 10% D_2O, and 0.03% sodium azide which appeared to be optimal regarding sample stability and spectra quality.

NMR Experiments for Assignment and Structure Calculation

NMR data were acquired at 298 K using Bruker Avance DRX 600 MHz or Avance III 800 MHz spectrometers equipped with a three-axis gradient 5 mm triple resonance probe. A set of 3D triple-resonance spectra were collected for sequential backbone and side-chain resonance assignments, respectively (Razzera et al., 2009, BMRB 16111). ^{13}C and ^{15}N NOESY spectra used for distance restrains during structure calculation were acquired with 120 ms mixing time (Sattler et al., 1995). All spectra were processed using Topspin 2.0 and analyzed with CARA 1.8.4 (Keller, 2004). Semiautomated NOE cross-peak assignments were assessed using the ATNOS/CANDID module in the Unio08 software (Herrmann et al., 2002a, 2002b). Structural calculations were performed with the CYANA 2.1 software using a simulated annealing protocol with torsion angle molecular dynamics (Guntert et al., 1997). A total of 100 random conformers were annealed in 10,000 steps for seven cycles, and 20 structures with the lowest target function after the final cycle were selected to represent Art v 1. Additional dihedral angle restraints were predicted from backbone chemical shifts using the program TALOS (Cornilescu et al., 1999) and structural geometry was analyzed with the PROCHECK-NMR, version 3.5.4 (Laskowski et al., 1996).

Art v 1 structure was first calculated without any restraints for disulfide bridges. Once the folding was good enough to unambiguously attribute the disulfide bridge pattern, we explicitly included this pattern in the subsequent calculations. In addition, the disulfide bridges pattern were also verified by the presence of Hβ-Hβ NOEs between cysteine pairs.

The presence of sequential NOEs αδ(i,i+1) for *trans* isomer and αα(i,i +1) for *cis* isomer were essential. Chemical shifts of the Cβ and Cγ from the proline residues were used to differentiate between *trans* and *cis* isomers, with *cis* isomers defined as Cβ-Cγ >8. Secondary structure elements were identified using the "Protein Structure Validation Suite" (PSVS http://psvs-1_4-dev.nesg.org). Structure coordinates were deposited in PDB under the code 2kpy and stereo view is available in Figure S3.

Art v 1 ^{15}N Backbone Dynamics

^{15}N relaxation experiments were performed on a Bruker Avance 600 MHz spectrometer as detailed in Supplemental Information. Experimental parameters were analyzed using Lipari-Szabo model-free analysis (Lipari and Szabo, 1982a, 1982b; Clore et al., 1990) and performed with the TENSOR 2 program (Dosset et al., 2000; Farrow et al., 1994).

Structural Analysis of Natural Art v 1

Natural Art v 1 was purified from aqueous *Artemisia vulgaris* pollen extracts according to Himly et al. (2003), with minor modifications. In brief, purification was performed by cation exchange and gel filtration chromatography. Pure, lyophilized aliquots of Art v 1 were suspended in 10% D_2O and natural abundance ^{13}C–HSQC 1024 × 200 and 1024 scans were collected at Avance III 800 MHz.

Purification of Human Art v 1-Specific IgE

Allergic patients were selected on the basis of typical case history, as well as positive skin prick and in vitro IgE tests to *Artemisia vulgaris* pollen and Art v 1. Experiments with human material were approved by the Ethics Committee of the Medical University of Vienna, Austria (no. EK497/2005) and informed written consents were obtained. For purification of polyclonal allergen-specific antibodies, human serum was loaded onto an NHS-activated Sepharose column (GE Healthcare, Chalfont St. Giles, UK) coupled with rArt v 1. After washing with PBS, bound immunoglobulins were eluted with citrate buffer (pH 2.3) and immediately neutralized by addition of sodium phosphate buffer (pH 7.8). IgG antibodies were removed by protein G affinity chromatography (GE Healthcare) according to standard protocols. Art v 1-specific IgE antibodies were recovered in the flow-through, analyzed for purity by sodium dodecyl sulfate polyacrylamide gel electrophoresis, and tested in enzyme-linked immunosorbent assays. For interaction studies purified Art v 1-binding IgE antibodies were concentrated using a 10 kDa cutoff ultracentrifugation device (Millipore, Milford, MA).

Interaction of Art v 1 with Human IgE

Allergen-antibody interactions in solution were monitored by $^{1}H/^{15}N$ HSQCs experiments (1024 × 256 points) using 2 μM of unlabelled purified human IgE antibodies and different concentrations of ^{15}N-labeled Art v 1, titrated from 0 to 150 μM. Evaluation of IgE binding was based on chemical shift deviations compared with a control sample without IgE. To avoid any interference from the medium, all experiments were conducted in exactly the same buffer conditions. ELISA experiments with previously designed

single cysteine mutants of Art v 1, i.e., C6S, C17S, C22S, C26S, C37S, C47S, C49S, and C53 were performed as published (Gadermaier et al., 2010). The chemical shift perturbations (CSPs) from the 2D ^{15}N-^{1}H HSQC NMR experiments were used to identify the IgE epitopes using the following equation: $CSP = [(\Delta NH)^2 + (\Delta^{15}N/10)^2]^{1/2}$, where ΔNH is the difference between free and bound ^{1}H amide chemical shifts (ppm) and Δ^{15}N is the difference between free and bound ^{15}N chemical shifts (ppm).

ACCESSION NUMBERS

Coordinates have been deposited in the PDB with accession code 2KPY.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and two tables and can be found with this article online at doi:10.1016/j.str.2010.05.012.

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