

Immunologic and Molecular Characterization of *Amb p V* Allergens from *Ambrosia psilostachya* (Western Ragweed) Pollen¹

Balaram Ghosh,^{2*} Thorunn Rafnar,^{*} Michael P. Perry,^{*} Donna Bassolino-Klimas,[†] William J. Metzler,[†] David G. Klapper,[‡] and David G. Marsh^{*}

^{*}Division of Clinical Immunology, Johns Hopkins Asthma and Allergy Center, Johns Hopkins University School of Medicine, 5501 Hopkins Bayview Circle, Baltimore, MD 21224; [†]Department of Macromolecular Structure, Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ 08543; and the [‡]Department of Microbiology and Immunology, The University of North Carolina, Chapel Hill, NC 27599

We have purified and characterized the *Amb p V* allergen (A1 variant) from western ragweed (*Ambrosia psilostachya*) pollen. This allergen was found to be highly cross-reactive with the *Amb a VA1* allergen from short ragweed (*A. artemisiifolia*) pollen in a competitive double-Ab radioimmunoassay (DARIA) and the two allergens showed concordant allergenic potency in histamine-release experiments. We cloned and sequenced several *Amb p V* genes from western ragweed pollen and flowers by direct PCR of genomic DNA. The amino acid sequences deduced from the nucleotide sequences indicated the presence of multiple forms of *Amb p V* that could be broadly classified into two groups: *Amb p VA* and *Amb p VB* variants. The sequences of the *Amb p VA* variants are highly homologous to *Amb a V* (about 90% identity) and very similar to the protein sequence that we obtained. The *Amb p VB* variants share approximately 65% amino acid homology with *Amb a V* and have five to seven cysteine residues as compared with the eight found in *Amb a V* and *Amb t V*. Two cysteine residues that form disulfide bonds in other *Amb Vs* (positions 19 and 43 in *Amb a V*) are replaced by serine and alanine in the *Amb p VB1* and *Amb p VB2* variants. We have generated model structures of *Amb p VA1*, *VA2*, *VA3*, and *VB1* variants from the nuclear magnetic resonance-derived structure of *Amb a VA1* by homology modeling. Comparison of antigenic epitopes predicted for the structures of *Amb p V* variants and *Amb a VA1* explains the observed cross-reactivity of the two ragweed proteins and suggests the epitopes likely to be involved in Ab recognition. *Journal of Immunology*, 1994, 152: 2882.

The *Amb V*³ ragweed pollen allergens provide good model systems to study the genetic and molecular basis of human immune responsiveness and its relationship to immunologic diseases. These allergens are proteins of relatively low molecular mass ($M_r = 4400$ –

5000), and include *Amb a V* (formerly Ra5) from short ragweed (*Ambrosia artemisiifolia*), *Amb t V* from giant ragweed (*A. trifida*), and *Amb p V* from western ragweed (*A. psilostachya*) (1). The immune responsiveness to these *Amb V* proteins is closely associated with the HLA-DR2 and Dw2 specificities in atopic subjects; specifically, it was shown that response to *Amb a V* is restricted by the HLA-DR(α , β 1*1501) heterodimer in Caucasian subjects (2–6). Our recent experiments indicated that *Amb t V* and *Amb p V* proteins do not stimulate the *Amb a V*-specific T cell clones but inhibit, in a dose-dependent fashion, the presentation of *Amb a V* to the T cells (6). It seems likely, therefore, that the *Amb V* proteins have similar, if not identical, agretopes which bind to the same MHC class II molecule.

To study the molecular mechanisms of the interactions of peptide epitopes of the *Amb Vs* with class II molecules

Received for publication September 7, 1993. Accepted for publication December 15, 1993.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ This work was supported by Research Grant AI-19727 from the National Institutes of Health, a Johns Hopkins Institutional Grant (to B.G.) and an American Academy of Allergy and Immunology Presidential Award (to T.R.).

² Address correspondence and reprint requests to Dr. B. Ghosh, Division of Clinical Immunology, Johns Hopkins Asthma and Allergy Center, Johns Hopkins University School of Medicine, 5501 Hopkins Bayview Circle, Baltimore, MD 21224.

³ Abbreviations used in this paper: *Amb p V*, allergen No. 5 from *Ambrosia psilostachya*; *Amb a V*, allergen No. 5 from *A. artemisiifolia*; *Amb t V*, allergen No. 5 from *A. trifida*; DARIA, double-antibody radioimmunoassay.

and TCRs, it is important to characterize these allergens. Previously, the *Amb a V* and *Amb t V* allergens have been purified, characterized, and their amino acid sequences determined (7–12). The three-dimensional solution structures of both *Amb a V* and *Amb t V* have been determined by NMR spectroscopy in our laboratories (13, 14). Recently we have cloned and sequenced the cDNAs encoding these two allergens (15, 16). Here, we report the protein sequence and immunologic properties of a further *Amb V* species, *Amb p V*, together with the molecular cloning and sequencing of the genes that encode two distinct subgroups of *Amb p V* allergens. Comparison of model structures of four *Amb p V* variants with the experimentally determined NMR structure of *Amb a VA1* explains the observed antigenic cross-reactivity and suggests candidate clusters of amino acid residues that are likely to be involved in Ab recognition.

Materials and Methods

Purification of *Amb p V* from western ragweed pollen

By using cross-reactivity with *Amb a V* as an indicator, three *Amb p V* proteins were isolated by a procedure similar to that described for *Amb t V* (12, 13). Briefly, 200 g of western ragweed pollen (Greer Labs, Lenoir, NC; Lot# 57BB58–9) were defatted by extraction with petroleum ether and the proteins were extracted by stirring the pollen for 16 min in 2.4 liter of H₂O. After centrifugation and filtration, the supernatant was passed through an H1P5 Amicon hollow-fiber cartridge with a nominal m.w. exclusion of 5000. The filtrate (containing *Amb p V*) was concentrated by Amicon YM2 membrane filtration and depigmented on a Whatman DE52 column. The *Amb p V*-containing fractions were pooled, concentrated by Amicon YM2 membrane filtration, and passed through a P10 Biogel column (2.5 x 100 cm) equilibrated with 0.1 M ammonium bicarbonate buffer. After concentration by lyophilization, three *Amb p V* variants of the VA family were purified by reverse-phase HPLC (Waters, Deltapack 8 x 10 C18 column) (17). Protein concentration was estimated by measuring absorbance at 280 nm, assuming that *Amb p V* variants have the same coefficient of absorbance as *Amb a VA1*. Throughout the procedure the presence of *Amb p VA* was monitored by testing fractions for antigenic cross-reactivity with *Amb a V*, by using competitive DARIA (see below).

Amino acid sequencing

Amino acid sequence analysis was performed using a Milligen 6625 protein/peptide sequencer (Millipore, Milford, MA). Aliquots of HPLC-purified fractions were extensively reduced and radioalkylated ([³H]iodoacetic acid) and subsequently covalently attached to sequencer membranes (either aryl amine or [1,4] phenylene diisothiocyanate) and subjected to Edman degradation with automated conversion to phenyl thiohydantoin derivatives and direct analysis by reverse-phase HPLC. For each *Amb p V* derivative examined in the sequencer, at least one run used the program COV 50 where 50% of each amino acid released during a cycle is sent to the HPLC for analysis and the other 50% is sent to a fraction collector for scintillation counting.

DARIA

Allergenic cross-reactivity between *Amb a VA1* and *Amb p VA1* was measured by competitive DARIA (11, 16). Briefly, 1 ng of [¹²⁵I]-labeled *Amb a VA1* or *Amb p VA1* was incubated for 4 h at 23°C with 100 µl of 1:2 dilution of individual sera from six *Amb a V*-sensitive subjects in the presence of tenfold dilutions (100, 10, 1, 0.1, and 0.01 ng) of unlabeled *Amb a VA1* or *Amb p VA1*. 50 µl each of goat anti-human IgE antiserum and human IgE myeloma PS (1:250) were added and the mixtures were incubated at 4°C for 18 h. The precipitates were pelleted, washed four times in borate-buffered saline and counted to assess direct Ag binding. The percent inhibition was calculated as described (16).

Histamine-release assays

Histamine-release sensitivities to *Amb p VA1* and *Amb a VA1* were assayed from the basophils of *Amb a V*-sensitive, ragweed-allergic, and control individuals by spectrofluorimetric assay (18).

Direct amplification and sequencing of genomic DNA

One hundred milligrams of western ragweed pollen (Greer Labs, Lot# 57BB58–9) were defatted by petroleum-ether extraction, resuspended in PBS, and dried. 160 µl of sterile water and 40 µl of 5X proteinase K buffer (0.5 mg/ml proteinase K/100 mM DTT/85 µM SDS) were added to 50 mg of the defatted pollen. The pollen suspension was vortexed and incubated at 37°C for 1 h, followed by an incubation at 85°C for 15 min and centrifugation for 15 s. The DNA in the pollen supernatant (7.5 µl) was amplified by PCR in the presence of 20 pmol each of BG30 and BG31 or BG30 and BG32 oligonucleotide primers (Table I) in a total volume of 50 µl as described previously (16). After an initial denaturation step at 95°C for 10 min, *Taq* polymerase (2.5 U) was added and the DNA was amplified for 40 cycles in a thermal cycler (Perkin-Elmer Cetus) as follows: 93°C for 2 min, 37°C for 1 min, and 72°C for 1 min, with a final extension for 10 min at 72°C (19). 2 µl of the PCR products were reamplified under the same conditions. The products were purified, subcloned into the M13 mp10 vector and sequenced by using Sequenase (U.S. Biochemicals, Cleveland, OH). To obtain sequence information from the 5'-untranslated region, the genomic DNA was amplified with primers BG33 and BG32 (Table I). All amplification conditions remained as described above except that the annealing temperature was changed to 42°C, and the final MgCl₂ concentration was increased to 3.0 mM.

RNA isolation from western ragweed pollen

Total RNA was isolated from western ragweed pollen (the same lot of pollen that was used for protein purification and genomic DNA amplification) by using a modified procedure of plant RNA isolation (20). Eight grams of petroleum-ether-extracted pollen was placed into a mortar and precooled with liquid nitrogen. Ten milliliters of homogenizing buffer (400 mM NaCl/50 mM Tris-Cl, pH 9.0/50 mM EDTA, pH 8.0/1% SDS/4 U/ml heparin/1 mM aurointricarboxylic acid in DEPC-treated water) was added to the pollen and the mixture was ground to a paste at 4°C. Ten milliliters of denaturation buffer, a 2:1 mixture of homogenizing buffer (phenol/cresol buffer: 100 g phenol, 0.1 g 8-hydroxyquinoline, 100 ml DEPC-treated water, 30 ml cresol, and 100 ml homogenizing buffer), was then added and the pollen was ground to a paste. This procedure was repeated until a total volume of 75 ml of the denaturation buffer was used. The pollen extract was centrifuged at 10,000 × g for 20 min at 4°C. The supernatants were extracted twice with one-third volume of phenol/chloroform (100g phenol, 0.1 g 8-hydroxy-quinoline, 100 ml DEPC-treated water, 100 ml chloroform, and 50 ml homogenizing buffer). LiCl was

Table 1. Oligonucleotides used for reverse-transcription and/or PCR amplification

Name ^a	Sequence ^b	Derived from <i>Amb a V</i> and <i>Amb t V</i> ends
BG20	5'-GAgaaattcGTCGACATCGA(T) ₁₇	—
BG30	5'-AGgaattcTAGGATCCACAg/AATGAAG	5' end
BG31	5'-GAgaaattcGTCGTTCTTAGTGACGTTCA	3' end
BG32	5'-GAgaaattcGCATTTAAACTGc/tAATTA	3' end
BG33	5'-AGgaattcATTACTTTGTGTTAATTTTA	5' end
Amb V-3	5'-AGgaattcTGCCTCTTAGTGAC	3' end

^a The degenerate nucleotides are shown in small letters. Oligonucleotides were synthesized by the phosphoramidite method using a DNA synthesizer (Milligen/Bioscience) and purified by HPLC at the Johns Hopkins School of Hygiene and Public Health. Amb V-3 was synthesized by Immuno-Dynamics, Inc., La Jolla, CA, by similar methodology.

^b Sequences of oligonucleotides used in this study. *EcoRI* restriction sites are shown in lower-case letters, and "wobbles" are shown by small capitals.

added to a final concentration of 3.5 M and the tubes were incubated on ice for 6 h and centrifuged at $10,000 \times g$ for 20 min at 4°C. The pellets were resuspended in a total volume of 10 ml of 5 mM Tris-Cl, pH 7.5. LiCl was added to a 3 M final concentration and incubated on ice for 16 to 18 h. The RNA was pelleted by centrifugation for 20 min at 4°C and resuspended in 600 μ l of DEPC-treated water. The RNA preparation was extracted once with phenol and two to three times with chloroform/isoamyl alcohol (24:1) and precipitated by the addition of 0.1 volume of 3 M sodium acetate, pH 5.2, and 2 volumes of ethanol. The RNA pellet was washed in 70% ethanol, dried, and resuspended in 100 to 200 μ l of DEPC-treated water.

Reverse transcription and PCR

The procedures for reverse transcription and PCR were performed as described previously (15, 16, 21). Total RNA was reverse-transcribed with a (dT)₁₇-adapter-primer (BG20) and amplified by PCR by using primers BG33 and BG32 (Table I). The putative *Amb p V* fragments were subcloned and sequenced as described above.

Isolation, amplification, and sequencing of genomic DNA from western ragweed flowers

Staminate heads of certified *A. psilostachya* were kindly collected for us from the Nebraska prairie by Dr. Margaret R. Bolick, Professor of Botany, University of Nebraska, Lincoln, Nebraska. Genomic DNA was isolated from this material as described (22), but with the addition of 2% polyvinyl pyrrolidone to the extraction buffer. The genomic DNA was amplified as above with primers BG30 and BG32 or BG33 and AMB-V3 (Table I), with the following exceptions: Tween 20 was added to each reaction to 0.5%, and the DNA was amplified for 45 cycles, with denaturation at 94°C for 2 min, annealing at 37°C for 1.5 min, and extension at 72°C for 2 min, and a final extension for 10 min. The PCR products (3 μ l) were reamplified under the same conditions. The *Amb p V* DNA fragments were subcloned into M13 and sequenced as above. We sequenced at least four independent clones for each variant from several different PCRs to reduce the possibility of PCR artifacts.

Computer modeling and prediction of antigenic sites

The program CONGEN (23) was used to generate three-dimensional models of *Amb p VA1*, *VA2*, *VA3*, and *VB1* by homology modeling with the experimentally determined structure of *Amb a VA1* and conformational sampling. The protocol used has been described in detail previously (23, 24, D. Bassolino-Klimas, D. G. Marsh, and W. J. Metzler, manuscript in preparation) and is summarized here. The sequence and coordinates of the experimentally derived structure of *Amb a VA1* (14) served as the starting point. *Amb a VA1* residues were "mutated" to the corresponding *Amb p VA1* residues, with the side chains being placed by exhaustive sampling of the available conformational space in an iterative manner in the program CONGEN. The best conformations were selected with criteria based on low energies and minimal solvent accessible surface areas. The final structures were subjected to 200 steps of steepest descents minimization to allow the whole molecules to relax. All calculations were performed on an SGI Iris 4D/440VGX computer.

Previous studies have shown that for a variety of proteins, including myoglobin, lysozyme, cytochrome c, and myohemerythrin, peaks in the contact surface area profile of a protein correlate well with the location of the experimentally determined antigenic epitopes (25). To estimate the antigenic potential of a particular region of *Amb a V* and *Amb p V*, we calculated their contact surface areas by determining the van der Waals' surfaces contacted by a spherical probe of 10 Å by using the Lee and Richards' algorithm (26). The contact areas should not be confused with solvent accessibilities. Solvent accessibility is defined by the envelope formed by the center of a probe as it rolls over the surface of a protein. The contact surface is a set of patches representing portions of the surface that are in actual contact with the probe. Residue-contact areas were obtained by summing the computed atom-contact areas.

Results

Purification and amino acid sequencing of *Amb p VA* variants from western ragweed pollen

Previous preliminary studies in our laboratory have suggested that western ragweed pollen contains a protein component, *Amb p V*, that has similar physical characteristics and is antigenically and allergenically cross-reactive with *Amb a V* (all known variants). We purified three distinct, but antigenically closely related *Amb p V* components by gel filtration and HPLC, by using their cross-reactivity with *Amb a V* as an indicator. Subsequent partial sequencing (10 residues) revealed that these *Amb p VA* components had identical N-terminal sequences (LLP CAWAGNV) which were highly homologous to *Amb a V*. Further sequencing of 41 residues of one of these components, *Amb p VA1*, showed sequence identity with *Amb a VA1*, except for the following substitutions: Lys 14 of *Amb a VA1* or A2 is replaced by Phe in *Amb p VA1*; Trp 28 is replaced by Pro; Lys 41 is replaced by Gln; Lys 42 is replaced by Asn (see Fig. 4). The Leu 2 residue of *Amb p VA1* is also observed in *Amb a VA2*, but not *Amb a VA1*. The identity of the three amino acids at the C-terminus and residue 40 were ambiguous in *Amb p VA1*.

Allergenic cross-reactivity between *Amb p VA1* and *Amb a VA1*

To determine the degree of allergenic cross-reactivity between *Amb p VA1* and *Amb a V* (HPLC-purified A1 variant) we performed inhibition DRIA with individual sera from six *Amb a V*-allergic individuals (two representative subjects, EK and WS, are shown in Fig. 1). In separate experiments, unlabeled *Amb a VA1* or *Amb p VA1* were used as inhibitors for IgE binding to ¹²⁵I-labeled *Amb a VA1* or ¹²⁵I-labeled *Amb p VA1*. In all individuals tested, the binding of human IgE antibody to ¹²⁵I-labeled *Amb a VA1* could be strongly inhibited (80–90%) by unlabeled *Amb p VA1* (Fig. 1, *a* and *b*). In the reciprocal experiments, the binding of human IgE antibody to ¹²⁵I-labeled *Amb p VA1* was similarly inhibited (about 80%) by unlabeled *Amb a VA1* (Fig. 1, *c* and *d*). These results, therefore, demonstrate a strong cross-reactivity between *Amb p VA1* and *Amb a VA1*, as recognized by IgE Abs from allergic subjects.

Histamine release from basophils induced by *Amb p VA1* and *Amb a VA1*

The allergenic activities of *Amb p VA1* and *Amb a VA1* allergens were examined further by comparing their effectiveness in releasing histamine from the basophils of *Amb a V*-sensitive, ragweed-allergic, and control individuals. Basophils from two ragweed-sensitive individuals, AP and DS, released histamine equally efficiently in response to

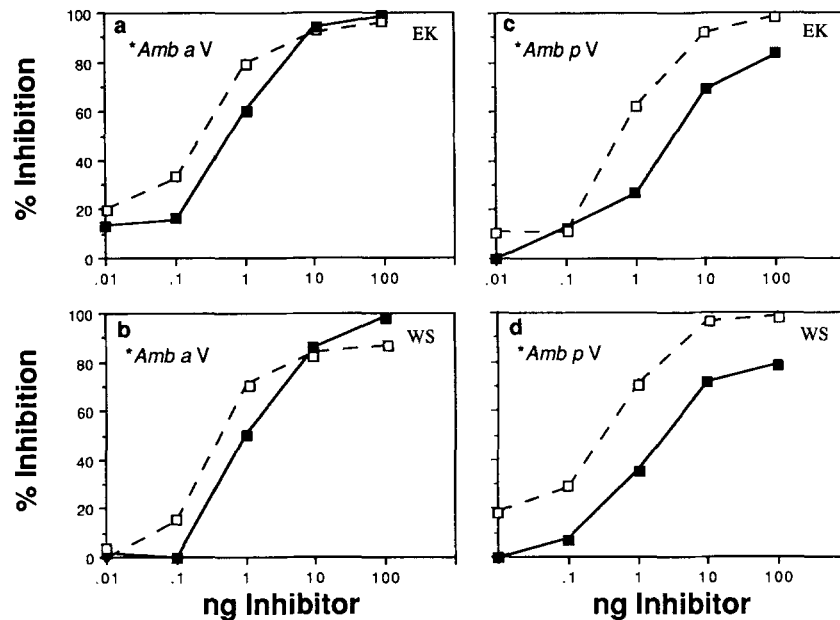


FIGURE 1. Competitive inhibition of ^{125}I -labeled *Amb a* VA1 or *Amb p* VA1 binding to human IgE Abs from two representative *Amb a* V-allergic individuals (EK and WS), by using unlabeled *Amb a* VA1 or *Amb p* VA1 as inhibitors. In *a* and *b*, inhibition of binding of ^{125}I -labeled *Amb a* VA1 to IgE with *Amb a* VA1 (solid lines) or with *Amb p* VA1 (dashed lines) is shown. In *c* and *d*, inhibition of binding of ^{125}I -labeled *Amb p* VA1 to IgE with *Amb a* VA1 (solid lines) or with *Amb p* VA1 (dashed lines) is shown.

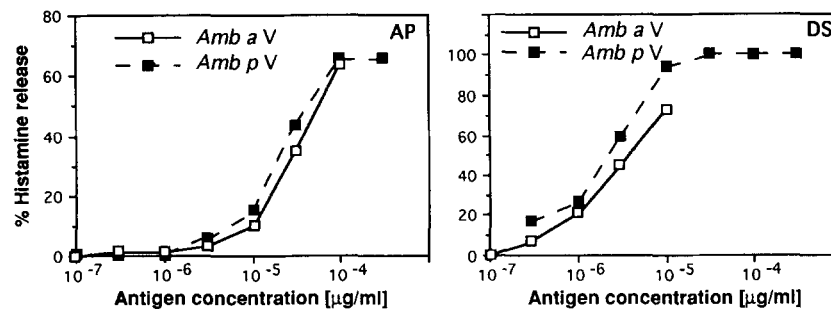


FIGURE 2. Histamine release from basophils of two *Amb a* V-allergic individuals (AP and DS) by *Amb a* VA1 and *Amb p* VA1.

Amb p VA1 and *Amb a* VA1 (Fig. 2). In contrast, basophils from two control individuals, TR (ragweed-allergic but not sensitive to *Amb a* V) and MB (nonallergic), released no histamine in response to either of these allergens (data not shown). It has been noted both in Figures 1 and 2 that the dose-response curves for *Amb p* VA1 are higher than that of *Amb a* VA1. We anticipate that this could be a result of the difference in the absolute concentrations of two proteins.

Cloning of *Amb p* V genes and determination of their nucleotide sequences

Purification of the *Amb p* V proteins by HPLC suggested the presence of several *Amb p* V variants, which we investigated by cloning and sequencing their genomic DNAs and cDNAs from western ragweed pollen and flowers (see

Materials and Methods). A comparison of the *Amb a* V and the *Amb t* V nucleotide sequences that we had previously obtained, unexpectedly revealed that the 5' and 3' ends are highly homologous (15, 16). Primers corresponding to the homologous regions were synthesized and the cDNAs and genomic DNAs encoding *Amb p* V were amplified directly from pollen by PCR. We obtained a total of five sequences for *Amb p* V (Fig. 3). The sequences obtained from cDNAs agreed with the genomic sequences, confirming the data obtained separately in each of the cloning experiments (Fig. 3). No introns were found in *Amb p* V genomic DNA. Comparison of the various *Amb p* V sequences derived from DNA and amino acid sequencing reveals that the *Amb p* V variants fall into two subgroups of highly homologous sequences, *Amb p* VA and VB. The three *Amb p* VA sequences are all very

		10	20	30	40	
Amb a VA1		<u>attactttt</u> <u>gttaatttt</u> <u>tatat</u>	ATG	AAT	AAT	G*A AAA AAT GTC TCG TTT
Amb p VA2		---	---	---	---	---
Amb p VA3		---	---	---	---	---
Amb p VB1		---	---	---	---	---
Amb p VB2		---	---	---	---	---
Amb p VB3		---	---	---	---	---
	50	60	70	80	90	
Amb a VA1	GAA TTT	<u>ATA GGA TCC ACA</u>	<u>GAT GAA</u>	GTC	GAT GAA ATA AAA TTA	GTG
Amb p VA2	---	---	---	---	---	---
Amb p VA3	---	---	---	---	---	---
Amb p VB1	---	---	---	---	---	---
Amb p VB2	---	---	---	---	---	---
Amb p VB3	---	---	---	---	---	---
	100	110	120	130		
Amb a VA1	CCT TGT GCC TGG GCG GGG AAC GTT	TGT	GGT	GAA AAG CGT	GCA	TAT
Amb p VA2	---	---	---	---	---	---
Amb p VA3	---	---	---	---	---	---
Amb p VB1	G--	TA- GC-	---	TC- A--	---	---
Amb p VB2	G--	TA- GC-	---	TC- A--	---	---
Amb p VB3	G--	TA- GC-	---	TC- A--	---	---
	140	150	160	170	180	
Amb a VA1	TGT TGT AGC GAC CCA GGG CGG TAC	TGT	CCT	TGG	CAA GTG GTT	TGT
Amb p VA2	---	---	---	---	---	---
Amb p VA3	---	---	---	---	---	---
Amb p VB1	---	-C-	---	---	---	---
Amb p VB2	-C-	-C-	---	---	---	---
Amb p VB3	---	-C-	A--	---	---	---
	190	200	210	220		
Amb a VA1	TAC GAA TCC AGC GAA ATA TGC AGC	AAA AAA	TGC	GGC AAA	ATG	CGG
Amb p VA2	---	---	---	---	---	---
Amb p VA3	---	---	---	---	---	---
Amb p VB1	---	---	---	---	---	---
Amb p VB2	---	---	---	---	---	---
Amb p VB3	---	---	---	---	---	---
	230	240	250	260	270	
Amb a VA1	<u>ATG AAC</u>	<u>GTC ACT</u>	<u>AAG AAC</u>	<u>GCA ATA</u>	<u>TAA aattaattgcagtttaaatgc</u>	
Amb p VA2	---	---	---	---	---	---
Amb p VA3	---	---	---	---	---	---
Amb p VB1	---	---	---	---	---	---
Amb p VB2	---	---	---	---	---	---
Amb p VB3	---	---	---	---	---	---

FIGURE 3. Comparison of nucleotide sequences of *Amb p V* and *Amb a V* genes. The numbers in parentheses after the asterisks indicate the numbers of clones sequenced from *A. psilostachya* pollen genomic DNA, pollen cDNA, and certified flower genomic DNA. The presumed translation-initiation codon (ATG) and termination codon (TAA) are shown in italics, adding an extra base (*) at position 33 to keep translation in-frame (see *Discussion*). The underlined sequences were used to design primers for amplifying the genes. The GenBank accession numbers for *Amb p* VA2, A3, B1, B2, and B3 nucleotide sequences are L24465, L24466, L24467, L24468, and L24469, respectively.

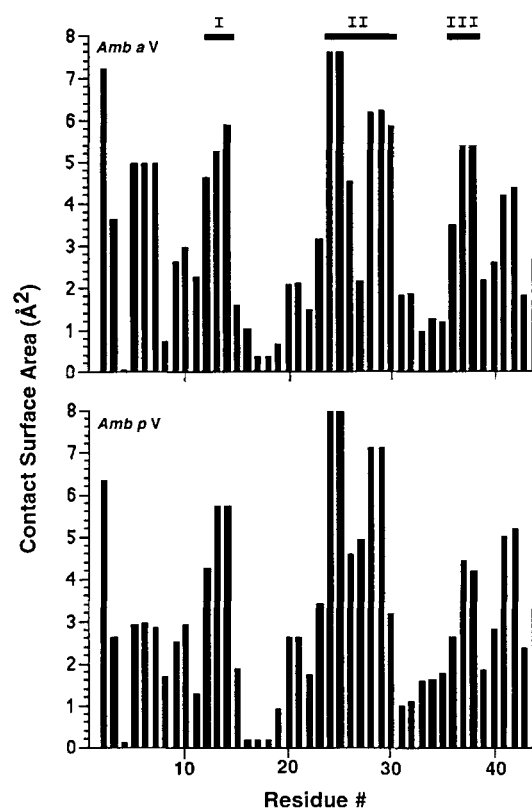
similar and show about 90% homology with *Amb a V*, whereas the three *Amb p* VB variants are only approximately 65% homologous with *Amb p* VA1 and *Amb a* VA1 (Fig. 4). *Amb p* VB1 has six cysteine residues instead of the eight found in *Amb a* VA, *Amb t V*, and *Amb p* VA. Interestingly, the two cysteine residues (positions 19 and 43) that are substituted in *Amb p* VB1 by Ser and Ala, respectively, form a disulfide bond in *Amb a V* and *Amb t V* (13, 14). *Amb p* VB2 and VB3 have five and seven cysteine residues, respectively.

Comparison of predicted antigenicity of *Amb a V* and *Amb p V* variants

To gain insight into the cross-reactivity between the *Amb a V* and *Amb p V* variants, we predicted the antigenic sites of *Amb a* VA1, the *Amb p* VA variants, and *Amb p* VB1. The availability of the experimentally determined three-dimensional structure of *Amb a* VA1 by NMR spectroscopy (14) provided a platform on which model structures of *Amb a* VA1, *Amb p* VA1, VA2, VA3, and

		-20					-15					-10					-5				
		M	N	N	E	K	N	V	S	F	E	F	I	G	S	T	D	E	V	D	
Amb a	VA1																				
Amb a	VA2																				
Amb p	VA1																				
Amb p	VA2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Amb p	VA3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Amb p	VB1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	N	-	-	-	
Amb p	VB2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	N	-	-	-	
Amb p	VB3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	N	-	-	-	
Amb t	V																				
						1				5					10					15	
Amb a	VA1	E	I	K		L	V	P	C	A	W	A	G	N	V	C	Q	E	K	R	A
Amb a	VA2					L	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Amb p	VA1					L	-	-	-	-	-	-	-	-	-	-	-	F	-	-	
Amb p	VA2	-	-	-	-	L	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Amb p	VA3	-	-	-	-	L	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Amb p	VB1	-	-	-	V	M	A	-	Y	A	-	-	S	I	-	-	-	-	-	G	
Amb p	VB2	-	-	-	V	M	A	-	Y	A	-	-	S	I	-	-	-	-	-	G	
Amb p	VB3	-	-	-	V	M	A	-	Y	A	-	-	S	I	-	-	-	-	-	G	
Amb t	V				D	D	G	L	-	Y	...	E	-	T	N	-	-	K	V	G	K
						20				25					30					35	
Amb a	VA1	Y	C	C	S	D	P	G	R	Y	G	P	W	Q	V	V	C	Y	E	S	
Amb a	VA2																				
Amb p	VA1	-	-	-	-	-	-	-	-	-	-	-	P	-	-	-	-	-	-	-	
Amb p	VA2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Amb p	VA3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Amb p	VB1	-	-	S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Amb p	VB2	-	-	S	S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Amb p	VB3	-	-	-	T	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Amb t	V	-	-	-	P	I	-	K	-	...	...	...	...	...	...	-	-	-	D	-	
						40				45					50					55	
Amb a	VA1	\$	E	I	C	S	K	K	C	G	K	M	R	M	N	V	T	K	N	A	I
Amb a	VA2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Amb p	VA1	-	-	-	-	-	Q	N	-	-	-	-	-	-	-	-	-	-	-	-	
Amb p	VA2	-	-	-	-	-	Q	-	-	-	-	-	-	-	-	-	-	-	-	T	
Amb p	VA3	-	K	-	-	-	Q	-	-	-	-	-	-	-	-	-	-	-	-	T	
Amb p	VB1	R	K	-	-	A	-	N	A	A	-	-	-	-	-	-	-	-	-	T	
Amb p	VB2	R	K	-	-	A	-	N	A	A	-	-	-	-	-	-	-	-	-	T	
Amb p	VB3	R	K	-	-	A	-	N	A	A	-	-	-	-	-	-	-	-	-	T	
Amb t	V	K	A	-	-	N	-	N	-	T	-	-	-	-	-	-	-	-	-	-	

Novotny and coworkers have shown that the contact surface area of a protein accessible to spherical probes of a size comparable to Abs (10 Å radius) may be an especially reliable indicator of the location of antigenic epitopes on a protein (25, 27–29), with peaks in the contact surface profile corresponding to prominent epitopes. In Figure 5, large probe contact surfaces were shown for the model structure of *Amb p* VA1 and the experimentally-determined structure of *Amb a* VA1. The regions of high solvent accessibility are indicated by the horizontal black bars in Figure 5. Essentially identical contact surface profiles were found for *Amb p* VA2, *Amb p* VA3, and *Amb p* VB1 (data not shown). Similar regions of the proteins



(Sites I, II, and III) are predicted to be potentially antigenic (30). Sites I and II contain at least one residue that has been found to be among the most antigenically important for Ab binding, namely, Glu 13 in Site I and Arg 24 in Site II. A potentially important Glu 37 is conserved in Site III only in *Amb a* VA, *Amb p* VA1, and *Amb p* VA2 but not in *Amb p* VA3 and *Amb p* VB variants (see below). One or more of these residues are likely to be prominent elements for observed cross-reactivity between the allergens.

Discussion

By using competitive DARIA we have shown that the *Amb p* VA1 allergen from western ragweed (*A. psilostachya*) pollen is highly cross-reactive with *Amb a* VA1 from short ragweed (*A. artemisiifolia*) pollen. These two allergens also showed concordant allergenic potency in histamine-release experiments. We determined the sequences of ten N-terminal amino acid residues of three of the *Amb p* VA allergens and 41 amino acids of the *Amb p*

VA1 subcomponent. This amino acid sequence information was consistent with the sequences of *Amb p* VA genomic DNA and cDNA (see below). To obtain the sequences of other *Amb p* V variants, we cloned and sequenced the *Amb p* V cDNAs and genomic DNAs from western ragweed pollen and flowers, which revealed two distinct *Amb p* V subgroups, VA and VB, and several variants within these subgroups. There was a high degree of homology within members of each subgroup (~93%) and a lower degree of homology between the VA and VB subgroups (~65%). Also, a high degree of homology (~90%) was found between *Amb a* VA and *Amb p* VA, and a lower degree of homology between *Amb a* VA and *Amb p* VB (~65%).

The *Amb p* V genes are similar to the *Amb a* V and *Amb t* V genes previously studied in that they contain no introns (15, 16). Furthermore, *Amb p* V cDNAs, like their *Amb a* V homologues, do not have in-frame ATG translation-initiation codons (16). Because sequencing was performed on PCR products amplified in multiple independent PCRs of both genomic and cDNA of *Amb p* Vs, it is unlikely that the absence of the initiation codon is a result of PCR artifacts. Rather, either an RNA-editing mechanism or a translational frame-shifting mechanism may be in operation in this plant species (31–33). Alternatively, an initiation codon other than ATG may be used (34). It is also interesting to note that, like *Amb a* V, the amino acid sequences of *Amb p* V variants deduced from cDNA sequences have ten amino acids at their C-termini that are absent in the proteins isolated from pollens, possibly resulting from posttranslational cleavage (Fig. 4).

By using homology modeling we predicted the antigenic sites for *Amb a* VA, *Amb p* VA variants, and *Amb p* VB1. Because B cell epitopes are usually found on the surfaces of proteins (35, 36), we determined the contact surface area profiles of these proteins to a 10 Å probe, which suggested the location of three putative antigenic sites (Fig. 5). The conserved residues in these sites are likely to be responsible for the observed cross-reactivity between *Amb p* VA and *Amb a* VA. It is noteworthy that Glu 13 in Site I, and Arg 24 and Tyr 25 in Site II are conserved in *Amb a* VA and all of the *Amb p* V variants. In contrast, Glu 37 in Site III is conserved only in *Amb a* VA, *Amb p* VA1, and *Amb p* VA2. In the *Amb p* VB variants, Glu 37 in Site III is replaced by Lys (lysines are rarely important for Ab binding (30)). In addition, Ser 36 in Site III is replaced by Arg. These latter substitutions may be expected to alter the antigenic character of Site III, and suggest significant differences in cross-reactivity between *Amb a* VA and the *Amb p* VB variants. This is consistent with no *Amb p* VB variants being detected during protein purification, where the presence of *Amb p* V was monitored via cross-reactivity with *Amb a* V. Interestingly, Glu 13, Arg 24, and Glu 37 are not conserved in *Amb t* V, an *Amb* V homologue which has essentially no cross-reactivity with *Amb a* V (12). Our failure to detect the *Amb p* VB protein variants, however, could also be a

result of inadequate expression of these variants in pollen. Indeed, we do not know the relative levels of expression of the *Amb p* V variants in pollen at this time.

Given the fact that both the *Amb p* VA and VB variants are obtained from certified western ragweed flowers and also from western ragweed pollen collected in a different area, it seems likely that these two *Amb p* V forms are part of the natural variation within the *A. psilostachya* species. It has been shown that *A. psilostachya* exhibits polyploidy and can form hybrids with related ragweed species (37, 38), which may account for the quite different *Amb p* VA and VB sequences.

Acknowledgments

We thank Mary Brummet for expert technical assistance and Dr. Margaret R. Bolick, Curator of Botany, University of Nebraska State Museum, for providing us with certified western ragweed flowers and information concerning polyploidy in western ragweed.

References

- Marsh, D. G., P. Zwollo, S.-K. Huang, B. Ghosh, and A. A. Ansari. 1990. Molecular studies of human response to allergens. *Cold Spring Harbor Symp. Quant. Biol.* 54:459.
- Marsh, D. G., S. H. Hsu, M. Roebber, E. Ehrlich-Kautzky, L. R. Freidhoff, D. A. Meyers, M. K. Pollard, and W. B. Bias. 1982. HLA-Dw2: a genetic marker for human immune response to short ragweed allergen Ra5. I. Response resulting primarily from natural antigenic exposure. *J. Exp. Med.* 155:1439.
- Marsh, D. G., D. A. Meyers, L. R. Freidhoff, E. Ehrlich-Kautzky, M. Roebber, P. S. Norman, S. H. Hsu, and W. B. Bias. 1982. HLA-Dw2: a genetic marker for human immune response to short ragweed allergen Ra5. II. Response after ragweed immunotherapy. *J. Exp. Med.* 155:1452.
- Zwollo, P., E. Ehrlich-Kautzky, A. A. Ansari, S. J. Scharf, H. A. Erlich, and D. G. Marsh. 1991. Molecular studies of human immune response genes for the short ragweed allergen, *Amb a* V. Sequencing of HLA-D second exons in responders and non-responders. *Immunogenetics* 33:141.
- Huang, S.-K., P. Zwollo, and D. G. Marsh. 1991. Class II MHC restriction of human T cell responses to short ragweed allergen, *Amb a* V. *Eur. J. Immunol.* 21:1469.
- Huang, S.-K., and D. G. Marsh. 1991. Human T cell responses to ragweed allergens: *Amb* V homologues. *Immunology* 73:363.
- King, T. P., P. S. Norman, and J. J. Connell. 1964. Isolation and characterization of allergens from ragweed pollen. *Biochemistry* 3:458.
- King, T. P., P. S. Norman, and L. M. Lichtenstein. 1967. Isolation and characterization of allergens from ragweed pollen. *Biochemistry* 6:1992.
- Underdown, B. J., and L. Goodfriend. 1969. Isolation and characterization of an allergen from short ragweed pollen. *Biochemistry* 8:980.
- Mole, L. E., L. Goodfriend, C. B. Lapkoff, J. M. Kehoe, and J. D. Capra. 1975. The amino acid sequence of allergen Ra5. *Biochemistry* 14:1216.
- Roebber, M., D. G. Klapper, and D. G. Marsh. 1982. Two isoallergens of short ragweed component Ra5. *J. Immunol.* 129:120.
- Roebber, M., D. G. Klapper, L. Goodfriend, W. B. Bias, S. H. Hsu, and D. G. Marsh. 1985. Immunochemical and genetic studies of *Amb t* V (Ra5G), an Ra5 homologue from giant ragweed pollen. *J. Immunol.* 134:3062.
- Metzler, W. J., Valentine, K., Roebber, M., Friedrichs, M., Marsh, D. G. & Mueller, L. 1992. Determination of the three-dimensional solution structure of ragweed allergen *Amb t* V by nuclear magnetic resonance spectroscopy. *Biochemistry* 31:5117.

14. Metzler, W. J., K. Valentine, M. Roebber, D. G. Marsh, and L. Mueller. 1992. Proton resonance assignments and three-dimensional solution structure of the ragweed allergen *Amb a V* by nuclear magnetic resonance spectroscopy. *Biochemistry* 31:8697.
15. Ghosh, B., M. P. Perry, and D. G. Marsh. 1991. Cloning the cDNA encoding the *Amb t V* allergen from giant ragweed (*Ambrosia trifida*) pollen. *Gene* 101:231.
16. Ghosh, B., M. P. Perry, T. Rafnar, and D. G. Marsh. 1993. Cloning and expression of immunologically active recombinant *Amb a V* allergen of short ragweed (*Ambrosia artemisiifolia*) pollen. *J. Immunol.* 150:5391.
17. Rafnar, T., B. Ghosh, W. J. Metzler, S.-K. Huang, M. P. Perry, L. Mueller, and D. G. Marsh. 1992. Expression and analysis of recombinant *Amb a V* and *Amb t V* allergens: comparison with native proteins by immunological assays and NMR spectroscopy. *J. Biol. Chem.* 267:21119.
18. May, C. D., M. Lyman, R. Alberto, and J. Chang. 1970. Procedures for immunochemical assay of the histamine release from leukocytes with small volumes of blood. *J. Allergy* 46:12.
19. Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 239:487.
20. Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl. 1991. *Current Protocol in Molecular Biology*. John Wiley & Sons, New York.
21. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning: A Laboratory Manual*, 2nd Ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
22. Dellaporta, S. L., J. Wood, and J. B. Hicks. 1983. A plant DNA miniprep: version II. *Plant Mol. Biol. Rep.* 1:19.
23. Bruccoleri, R. E., and M. Karplus. 1987. Prediction of folding of short polypeptide segments by uniform conformational sampling. *Biopolymers* 7:165.
24. Bruccoleri, R. E., and J. Novotny. 1992. Antibody modeling using the conformational search program CONGEN. *Immunomethods* 1:96.
25. Novotny, J., M. Handschumacher, E. Haber, R. E. Bruccoleri, W. B. Carlson, D. W. Fanning, J. A. Smith, and G. D. Rose. 1986. Antigenic determinants in proteins coincide with surface regions accessible to large probes (antibody domains) *Proc. Natl. Acad. Sci. USA* 83:226.
26. Lee, B. K., and F. M. Richards. 1971. The interpretation of protein structures: estimation of static accessibility. *J. Mol. Biol.* 55:379.
27. Novotny, J., M. Handschumacher, and R. E. Bruccoleri. 1987. Protein antigenicity: a static surface property. *Immunol. Today* 8:26.
28. Granier, C., J. Novotny, J.-C. Fontecella-Camps, P. Fourquet, M. ElAyeb, and E. Bahraoul. 1989. The antigenic structure of a scorpion toxin. *Mol. Immunol.* 26:503.
29. Novotny, J. 1991. Protein antigenicity: a thermodynamic approach. *Mol. Immunol.* 28:201.
30. Jin, L., B. M. Fendly, and J. A. Wells. 1992. High resolution functional analysis of antibody-antigen interactions. *J. Mol. Biol.* 226:851.
31. Brierley, I., M. E. G. Boursnell, M. M. Binns, B. Bilimoria, V. C. Blok, T. D. D. Brown, and S. C. Inglis. 1987. An efficient ribosomal frame-shifting signal in the polymerase-encoding region of the coronavirus IBV. *EMBO J.* 6:3779.
32. Hoffman, M. 1991. RNA editing: what's in a mechanism? *Science* 253:136.
33. Kozak, M. 1990. Downstream secondary structure facilitates recognition of initiator codons by eukaryotic ribosomes. *Proc. Natl. Acad. Sci. USA* 87:8301.
34. Mehdi, H., E. Ono, and K. C. Gupta. 1990. Initiation of translation at CUG, GUG, and AUG codons in mammalian cells. *Gene* 91:173.
35. Benjamin, D. C., J. A. Berzofsky, I. J. East, F. R. N. Gard, C. Hunnum, S. J. Leach, E. Margoliash, J. G. Michael, A. Muller, E. M. Prager, M. Reichlin, E. E. Sarcarz, S. J. Smith-Gill, P. E. Todd, and A. C. Wilson. 1984. The antigenic structure of proteins: a reappraisal. *Annu. Rev. Immunol.* 2:67.
36. Berzofsky, J. A. 1991. Mechanisms of T cell recognition with application to vaccine design. *Mol. Immunol.* 28:217.
37. Payne, W. W. 1962. Biosynthetic studies of four widespread weedy species of ragweeds (*Ambrosia*: Compositae). Doctoral dissertation, University of Michigan, Ann Arbor, MI.
38. Payne W. W., P. H. Raven, and D. W. Kyhos. 1964. Chromosome numbers in Compositae. IV. *Ambrosieae*. *Am. J. Bot.* 51:419.