

The Major Conformational IgE-binding Epitopes of Hevein (Hev b6.02) Are Identified by a Novel Chimera-based Allergen Epitope Mapping Strategy*

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A novel approach to localize and reconstruct conformational IgE-binding epitope regions of hevein (Hev b6.02), a major natural rubber latex allergen, is described. An antimicrobial protein (AMP) from the amaranth *Amaranthus caudatus* was used as an immunologically non-IgE-binding adaptor molecule to which terminal or central parts of hevein were fused. Hevein and AMP share a structurally identical core region but have different N-terminal and C-terminal regions. Only 1 of 16 hevein-allergic patients showed weak IgE binding to purified native or recombinant AMP. Chimeric AMP with the hevein N terminus was recognized by IgE from 14 (88%) patients, and chimeric AMP with the hevein C terminus was recognized by IgE from 6 (38%) patients. In contrast, chimeric AMP containing the hevein core region was recognized by IgE from only two patients. When both the N-terminal and C-terminal regions of hevein were fused with the AMP core, IgE from all 16 patients bound to the chimera. This chimera was also able to significantly inhibit (>70%) IgE binding to the native hevein. On the contrary, linear synthetic peptides corresponding to hevein regions in the AMP chimeras showed no significant IgE binding capacity in either enzyme-linked immunosorbent assay or inhibition enzyme-linked immunosorbent assay. These results suggest that the IgE binding ability of hevein is essentially determined by its N-terminal and C-terminal regions and that major IgE-binding epitopes of hevein are conformational. The chimera-based epitope mapping strategy described here provides a valuable tool for defining structural epitopes and creating specific reagents for allergen immunotherapy.

To help understand the molecular basis of Type I allergic immune responses, identification of conformational IgE-binding epitopes on the surface of allergen molecules is required. This knowledge is crucial when designing tools and strategies for allergen-specific immunotherapy or vaccination.

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Natural rubber latex (NRL)¹ allergy has become increasingly common during the last decade, and a large proportion of the general population is regularly exposed to NRL. In particular, health care workers (up to 17%) and children with a history of multiple surgeries (up to 60%) are reported to be sensitized to NRL products (1, 2). Although several NRL allergens have recently been characterized at the molecular level, consensus exists that hevein (Hev b6.02) is one of the most important NRL allergens (3, 4). Hevein (4.7 kDa, 43 amino acids) is the most predominant protein in liquid latex obtained from the rubber tree *Hevea brasiliensis*. Hevein is involved in latex coagulation, and it may play a key role as a defense protein in the protection of wound sites by inhibiting the growth of chitin-containing fungi. Hevein has been found to elute in large quantities from NRL products (5). The importance of hevein as a latex allergen is supported by the findings that >70% of latex-allergic patients have hevein IgE antibodies (6) and that hevein elicits positive skin prick test reactions in the great majority of latex-allergic patients (1). Hevein is therefore a useful tool in the diagnosis of latex allergy as well as a potential candidate for immunotherapy.

Treatment options for allergic diseases are currently based mainly on allergen avoidance and the use of corticosteroids and antihistamines to control inflammation and asthma. Whereas allergen-specific immunotherapy using authentic IgE-binding allergens is effective in controlling some allergies, the high risk of systemic reactions during therapy remains a serious problem. Recently, attempts have been made to eliminate IgE-binding sites of allergens while retaining their T-cell-stimulating sites (7, 8). In order to design a safe reagent for allergen-specific immunotherapy, it is crucial to characterize in detail the IgE-binding epitopes of the allergen. To date, in most studies, identification of IgE-binding epitopes has been performed using short synthetic overlapping peptides that cover the whole allergen sequence. Although this approach can be very helpful in identifying linear T-cell epitopes, its value is limited in B-cell epitope mapping. This is due to the fact that most, if not all, IgE epitopes are reported to be conformational and thus cannot properly be studied using linear peptides (9, 10). An inherent

¹ The abbreviations used are: NRL, natural rubber latex; AMP, antimicrobial protein; ELISA, enzyme-linked immunosorbent assay; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; HPLC, high pressure liquid chromatography; HSA, human serum albumin.

TABLE I
Primers used

Forward primers are indicated by (F), and reverse primers are indicated by (R). Nucleotides coding hevein residues are shown in lowercase letters, restriction enzyme sites (*Bam*HI, *Hind*III, *Eco*RI, and *Sal*I) are underlined, and thrombin cleavage sites are in *italic*.

Name (F/R)	Sequence
Prohev10 (F)	TTATTAGGATCCCTGGTGCCTAGAGGAAGCgagcaatgtggtcg
Prohev2 (R)	AACACAAGCTTCTTAgctctttgcaattgctt
AMP1/ <i>Eco</i> RI (F)	TTATTAGAATTCGTGGGAGAGTGCCTGAGAGGAAGATGCCCTTCCGAATGTGCTGC
AMP2/ <i>Sal</i> I (R)	AACACGTCGACTTTATCTTCCGAGTACTTAGGTCCCTTCCGAGTATCCGAATGGGAGCAGCACATTCCGGA
AMP5' Thr (F)	ACTAGTGGATCCCTGGTGCCTAGAGGAAGCGTGGGAGAGTGCCTG
AMP3' (R)	CTCGAGAAGCTTTTATCTTCCGAGTACTT
AMPN (F)	GAGCTCGGATCCCTGGTGCCTAGAGGAAGCgagcaatgtggtcggaagcaggtggcaagAGATGCCCTTCCGAATGTG
AMPC (R)	AACACAAGCTTTTAgctctttgcaattgcttggcagttatgatcaggtgaGCAGTACTTAGGTCC
AMPcoreI (F)	ACTAGTGGATCCCTGGTGCCTAGAGGAAGCgtgggagagtgcgtgagaggaagatgccccataacatgatg
AMPcoreII (R)	AACACAAGCTTTTATCTTCCgcagtattcatcagtgaggccacaccacccccactgggtacaacataggtattggggca

requirement for studying conformational epitopes is knowledge of the three-dimensional structure of the allergen.

In the present study, we describe a novel allergen epitope mapping strategy for the characterization of conformational IgE-binding epitopes of hevein. By combining protein engineering and modeling-based design, we were able to transfer hevein-specific IgE binding activity to a nonallergenic adaptor protein. Finally, by using this approach, N-terminal and C-terminal regions of hevein were shown to contain the major conformational IgE-binding epitopes of hevein.

EXPERIMENTAL PROCEDURES

Sequences, Structures, and Computational Analyses—Hevein-like domains in the Swiss-Prot Protein Sequence Database were identified by using the advanced BLAST search service provided by the National Center for Biotechnology Information. On the basis of the multiple sequence alignment by Clustal X (11), the structures of hevein-like domains were also obtained from the National Center for Biotechnology Information service and used for additional studies. The three-dimensional structures of hevein from the rubber tree *H. brasiliensis* (hevein, Protein Data Bank access code 1HEV, Ref. 12) and an antimicrobial protein (AMP) from the amaranth *Amaranthus caudatus* (Protein Data Bank access code 1MMC, Ref. 13), both determined using NMR, were obtained from the Protein Data Bank (14). The most representative NMR structure in the ensemble was selected using criteria from WHATCHECK (15).

Structural Modeling of AMP Chimera—The structural alignment of hevein and AMP was performed using VERTAA (16) in the BODIL modeling package (www.abo.fi/fak/mnf/bkf/research/johnson/bodil.html).² VERTAA produces rapid automated structural comparisons based on C- α atoms. MODELLER 4.0 (17) was used to construct three-dimensional model structures of the four chimeras. All four chimera models were energy-minimized using the GROMOS96 43a1 force field and deepest descent method in GROMACS2.0 (18). After minimization, the structures were further refined with 400 ps of molecular dynamics simulation to obtain better packing of the chimeras and to identify any gross problems in the folded model structures. Structure representations were prepared with InsightII 98.0 software (Molecular Simulations Inc., San Diego, CA) and RASTER3D (19).

Synthetic Peptides—Peptides spanning the entire hevein protein were purchased from MedProbe (Oslo, Norway). They include three peptides of 11 (linN), 21 (lincore), and 11 (linC) amino acids in length, corresponding to the hevein N terminus EQCGRQAGGKL (residues 1–11), the hevein core CPNNLCCSQWGWCGSTDEYCS (residues 12–32), or the hevein C terminus PDHNCQSNCKD (residues 33–43), and the corresponding peptides in which each cysteine residue was replaced with a serine.

Production of Recombinant Proteins—The recombinant form of hevein, AMP, and AMP chimeras were produced as chicken avidin (Av) fusion proteins for efficient purification from the insect cells using a baculovirus expression system (Bac-To-BacTM Baculovirus Expression System; Invitrogen) as described previously (20). To introduce a cleavage site for thrombin and the selected region of hevein (Table I), the PCR primers were designed and checked with the Amplify2 program.

Primers were purchased from TAGC (TAG Copenhagen A/S, Copenhagen, Denmark).

Recombinant AMP and AMPcore were constructed by PCR by multiplying the two annealed oligonucleotides (AMP1/*Eco*RI and AMP2/*Sal*I, AMPcoreI and AMPcoreII) in the absence of a template. Recombinant AMP was used as a template for the AMPN and AMPC constructs. Recombinant hevein was amplified from hevein cDNA in the PIIProhev vector with the forward Prohev6 and reverse Prohev2 primers. All PCR reactions were conducted in a volume of 50 μ l containing the template (10 ng), deoxynucleotide triphosphates (200 μ M each), the primers (100 nmol each) (Table I), 10 $\times$ *Pfu* polymerase buffer, and 2 units of *Pfu* DNA polymerase. The following cycling conditions were used in all cases: initial denaturation at 94 $^{\circ}$ C for 5 min following 30 cycles (1 min at 94 $^{\circ}$ C, 2 min at 50 $^{\circ}$ C, and 2 min at 72 $^{\circ}$ C) and final extension at 72 $^{\circ}$ C for 10 min. After the PCR amplification, the products were digested with *Bam*HI and *Hind*III restriction enzymes (Promega, Madison, WI). Digests were extracted from 1.5% agarose gel, ligated into the *Bam*HI/*Hind*III-treated pBacAVs+C vector (20), and transformed into JM109 *Escherichia coli* cells. The recombinant vectors were named AvHev, AvAMP, AvAMPN, AvAMPC, AvAMPN+C, AvAMPcore.

The nucleotide sequence of the constructs was always confirmed by dideoxynucleotide sequencing with an automated Li-Cor DNA sequencer (Li-Cor Biotechnology Division, Lincoln, NE). The vectors were transformed into *E. coli* strain DHC10Bac to construct the recombinant baculo bacmids. After blue/white selection, positive colonies were grown in 5 ml of Luria-Bertani/gentamycin + kanamycin sulfate for bacmid isolation. The viruses were completed during transfection according to the manufacturer's instructions for the Bac-To-BacTM Baculovirus Expression System (Invitrogen).

To produce recombinant proteins, about 4×10^8 Sf9 cells (ATCC CRL 1711) in SF-900 II SFM serum-free culture medium (Invitrogen) depleted of biotin were seeded to a final volume of 200 ml in a 1- or 2-liter Erlenmeyer flask. Recombinant viruses were added to give a multiplicity of infection of 0.1 plaque-forming unit/cell. Infection was allowed to continue at 28 $^{\circ}$ C and 110 rpm agitation for 72 h.

Purification of Recombinant Proteins—Purification of fusion proteins was performed in principle as described previously (20). The cell pellet was resuspended in 80 ml of HilloI buffer (50 mM Tris-HCl, pH 8, 1% Triton X-100, 2 mM EDTA, and 150 mM NaCl). During the purification, a 75- μ l aliquot was taken from the sonicated total cell lysate (sample T), from the residual cell pellet after resuspension in 20 ml of the same buffer and sonication (S), before binding to resin (L₁), after adjusting the pH (L₂), and after binding to resin (L₃). The intracellular fusion proteins bound on the 2-aminobiotin-agarose (Sigma) were washed once with 20 ml of binding buffer (50 mM Na₂CO₃, pH 11, and 1 M NaCl) and twice in the column with 10 ml of the same buffer. 2-Iminobiotin-agarose was used instead of a biotin resin because avidin binds to biotin in an irreversible manner (K_a , $\sim 10^{-15}$ M⁻¹) (21) that prevents the release of the intact fusion protein from the column. Avidin binds to 2-iminobiotin in a pH-dependent manner and can thus be released from the resin by lowering pH (22).

One-ml fractions of constructs were eluted from the column with the elution buffer (50 mM ammonium acetate, pH 4, and 0.5 M NaCl), and 20- μ l aliquots were analyzed in SDS-PAGE gels. AvHev, AvAMP, and all AvChimer fusion proteins were run on 15% denaturing SDS-PAGE and analyzed by immunoblot analysis using an avidin antibody (1:4000) as a primary antibody and goat anti-rabbit antibody (1:2000) as a secondary antibody.

Depending on the method to be used next, the buffer of the fusion

² J. V. Lehtonen, V.-V. Rantanen, D.-J. Still, M. Gyllenberg, and M. S. Johnson, unpublished data.

proteins was changed to fresh 25 or 50 mM Tris buffer (pH 8) with a desalting column HR10/10 (Amersham Biosciences) or Fast Desalting (Amersham Biosciences). Samples were concentrated with an Ultra-free-MC (30,000 NMWL filter unit; Millipore Corp., Bedford, MA) vacuum evaporator or with a MICROSEP (Pall Filtron Corp.). All of the fusion proteins were digested with an excess of thrombin (Amersham Biosciences) overnight at 37 °C.

Characterization of the Recombinant Proteins—Thrombin-cleaved recombinant hevein, recombinant AMP, and chimeras were analyzed by reversed-phase chromatography on a PepRP 5/5 column (Amersham Biosciences) using a linear gradient of acetonitrile (0–40% in 40 min) in 0.1% trifluoroacetic acid at a flow rate of 0.7 ml/min. Eluted fractions were monitored at 214 nm. The separated peptides were collected and further analyzed by mass spectrometry and N-terminal amino acid sequencing (ABI 494 A Procise™ Sequencer; PerkinElmer Life Sciences).

Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry was carried out using a Bruker Biflex II (Bruker-Daltonic, Bremen, Germany) instrument. About 1 pmol (0.5 µl) of the HPLC-purified peptides was applied to a thin-layer matrix preparation (0.5 µl of saturated cyano-4-hydroxycinnamic acid in acetone) and air-dried. External calibration was performed with insulin (Sigma).

Immunological Analyses with Sera from NRL-allergic Patients and Controls—Serum specimens were obtained from 16 NRL-allergic patients (15 women and 1 man; mean age, 46 years; age range, 16–70 years), all of whom were previously shown to have IgE against hevein. All of the patients had positive skin prick test responses to natural hevein. Sera from 19 control subjects without NRL allergy were used in the ELISA studies.

IgE ELISA—ELISA was performed as described previously (6). Purified recombinant hevein, recombinant AMP, or chimeras were diluted in 50 mmol/liter carbonate buffer, pH 9.6, to a final concentration of 1 µg/ml. The proteins were placed onto a polystyrene microtiter plate (100 µl/well; Nunc, Roskilde, Denmark) and incubated at room temperature (20–23 °C) for 3 h or overnight at 4 °C. The wells were emptied, and the remaining protein binding sites were blocked with 1% human serum albumin (HAS; Finnish Red Cross Blood Transfusion Service, Helsinki, Finland) in 50 mM carbonate buffer, pH 9.6, at room temperature for 1 h. After rinsing three times, 100 µl of serum (diluted 1:20 with phosphate-buffered saline containing 0.02% Tween 20 and 0.2% HSA) from a NRL-sensitive individual or from a negative control person was added to the well and incubated at room temperature for 2 h. The wells were washed again, and biotinylated goat anti-human IgE (Vector Laboratories, Burlingame, CA; diluted 1:1000 with phosphate-buffered saline containing 0.02% Tween 20 and 0.2% HSA) was added and incubated at room temperature for 1 h. After washing again, streptavidin-conjugated alkaline phosphatase (Bio-Rad; diluted 1:3000) was added and incubated for 1 h. The substrate, *p*-nitro-phenyl phosphate (Sigma), was added, and the absorbance was read at 405 nm by using an automated ELISA reader (Titertek Multiskan, Eflab, Finland). The mean ± 3 S.D. of the controls was chosen as the threshold value for a positive result.

Inhibition ELISA—The inhibition ELISA was performed as described previously (6), with the following modifications. Hevein or chimeras (AMPN, AMPC, and AMPN+C) were applied to microtiter plates at a concentration of 2 µg/ml. The ELISA inhibition was performed with eight different inhibitors (AMPN, AMPC, AMPN+C, linN, lincore, linC, pool of linN and linC, and hevein) at four different concentrations (0.0001, 0.01, 1, and 10 µg/ml for all inhibitors and also 100 µg/ml for the linear peptides). Sera (diluted 1:10) were obtained from six patients that recognized AMPN and/or AMPC chimeras. Streptavidin-conjugated alkaline phosphatase (Zymed Laboratories Inc.) was diluted 1:1000 (with phosphate-buffered saline containing 0.02% Tween 20 and 0.2% HSA) and used as a secondary antibody. Substrate-produced color was read at 405 nm using an automated ELISA reader (Multiskan MS, Labsystems, Helsinki, Finland).

RESULTS

Hevein and the Amaranth AMP Have a Structurally Identical Core Region but Different N and C Termini—Allergen structures involved in IgE binding can be indirectly analyzed by comparing the IgE binding abilities of structurally related proteins. To find hevein homologues that could be used in the analysis of conformational IgE-binding regions, we first analyzed hevein-related proteins by a sequence alignment program (Clustal X). The central area of hevein showed the most exten-

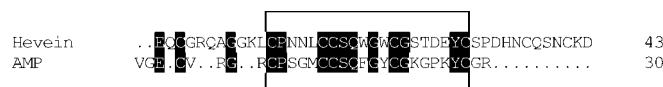


FIG. 1. Structure-based alignment of amino acid sequences of AMP and hevein. Black shading indicates identical residues. Gaps (dots) were introduced for optimal alignment. The box identifies the core regions.

sive conservation between the homologues (data not shown). Because knowledge of the three-dimensional structure of the allergen is essential for the study of conformational epitopes, we analyzed further only those hevein homologues whose three-dimensional structure had been solved (23).

Although hevein-like domains commonly occur in a variety of plant proteins, AMP (30 amino acids) from the amaranth *Amaranthus caudatus* was found to be the only monomeric hevein homologue with a known three-dimensional structure and a size relatively close to that of hevein (hevein, 43 amino acids). In the sequence comparison, the core regions of AMP (residues 9–28) and hevein (residues 12–31) are highly conserved (Fig. 1). In contrast, both the N terminus (residues 1–8) and C terminus (residues 28–30) of AMP differ markedly from the corresponding regions in hevein (residues 1–12 and 32–43, respectively).

Because sequence comparison gives only indirect structural information, we compared the three-dimensional structures of hevein and AMP. The Protein Data Bank file contains six alternative NMR structures for hevein (12). Structure 4 was used for all the comparisons because the majority of its residues had acceptable conformations according to WHATCHECK. The same procedure was used to select AMP NMR structure 16 (Protein Data Bank access code 1MMC) (13). The structural alignment of hevein and AMP was performed using VERTAA in the BODIL modeling environment (Fig. 2). The superimposed three-dimensional structures show that the backbone α -carbons of hevein (between residues 12 and 31) and AMP (between residues 19 and 28) occupy very similar positions in space relative to each other (Fig. 2). However, substantial differences in the structures were observed at the N-terminal and C-terminal ends of the two proteins, which is in good agreement with the results obtained from the sequence alignment.

Native AMP Is Not Recognized by Hevein-specific IgE—To study the immunological properties of AMP, it was extracted from amaranth seeds and purified by affinity chromatography on a chitin column and reversed-phase HPLC as described previously for hevein (24). The molecular mass (3184.8 Da) of the purified protein corresponded to that calculated from the AMP sequence with three disulfide bonds (3183.7 Da). The analyzed N-terminal sequence of 20 residues was identical to the known AMP sequence (AMP2, Swiss-Prot: P27275).

The ability of purified AMP to bind hevein-specific IgE was analyzed using sera obtained from NRL-allergic patients with hevein IgE antibodies. Only 1 of 16 patients and 0 of 19 controls showed IgE binding to native AMP in ELISA (data not shown).

Design and Molecular Modeling of Hevein-AMP Chimeras—To test the IgE binding capacity of the core and terminal regions of hevein, four hevein-AMP chimeras (AMPN, AMPC, AMPN+C, and AMPcore) were designed, as shown in Fig. 3. On the basis of their structural alignment, three-dimensional models of the chimeras were constructed, energy-minimized, and subjected to molecular dynamics simulation to improve side chain packing and identify bad contacts. No bad contacts were observed, and residues with poor geometry were identified with WHATCHECK (WHATIF, Ref. 25) and corrected. The schematic representations of the model structures and their surface models are shown in Fig. 3.

FIG. 2. Superimposition of the NMR structures of the C- α atoms of hevein (blue) on AMP (green), showing the similarities of the core region (red). Disulfide bonds: hevein, yellow; AMP, gray.

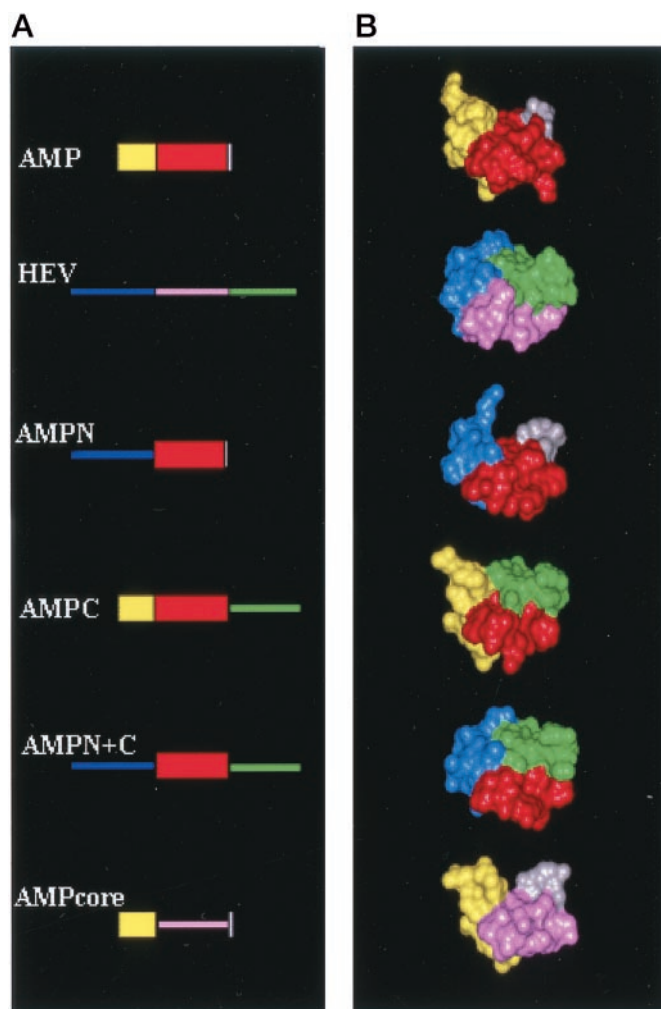
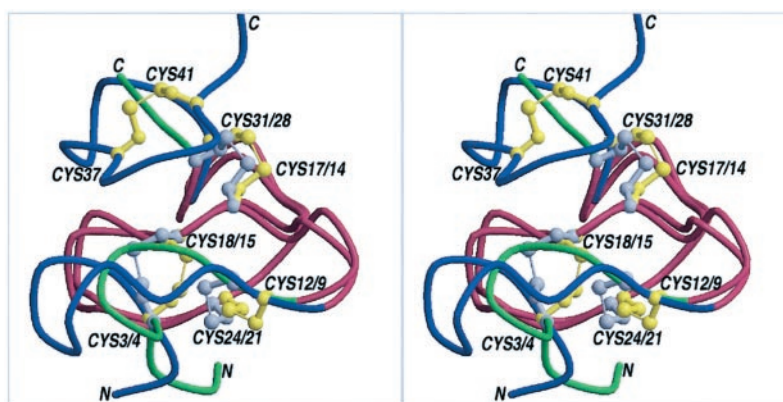


FIG. 3. A, schematic presentation of the designed chimeras. B, modeled structures of the chimeras. Three-dimensional structures correspond to parts of the chimera derived from AMP (yellow, gray, and pink) or hevein (blue, green, and red). The coloring is the same in both A and B.

The modeled structures were used to evaluate the viability of the chimeric constructs. No problems with either packing or side chain interactions were seen when the four chimeras were compared with hevein and AMP. Major differences occur in the N-terminal sequences of hevein and AMP, but these differences were not expected to interfere with the stable folding or packing of the chimeric constructs. Both proteins have a cysteine residue within this segment that forms a disulfide bond with the core region. The first two amino acid residues of AMP are not present in hevein; these residues do not seem to have any

critical role in the fold. The core region in each of the chimera models is nearly identical to the structures of hevein and AMP, and moreover, it was possible to build the disulfide bonds in each model in a way similar to those in the NMR structures of hevein and AMP (Fig. 2). The C-terminal part of hevein contains 10 extra amino acids as compared with AMP (Fig. 1). Hevein has one additional disulfide bond (Cys³⁷ to Cys⁴¹), and the amino acids Gln³⁸-Ser³⁹-Asn⁴⁰ contribute an extra strand to the β -sheet (Fig. 2). In addition to the interactions mentioned above, the C-terminal part of hevein forms no internal interactions that would be affected by the chimeric constructs.

Production and Biochemical Characterization of Hevein-AMP Chimeras—All constructs (recombinant AMP, recombinant HEV, and the four chimeras) were then cloned and produced in insect cells using the Bac-To-BacTM Baculovirus Expression System. Chicken avidin was used as an N-terminal fusion partner for easier affinity purification of the recombinant proteins. After purification, the avidin tag was cleaved from the fusion proteins by protease thrombin. To confirm the correct structure and disulfide bridges of the products, all hevein-AMP chimeras were analyzed by N-terminal sequencing and MALDI-TOF mass spectrometry. After thrombin cleavage, all recombinant proteins had the correct N-terminal amino acid sequence. The measured molecular masses of the recombinant proteins corresponded to the calculated ones, suggesting that the correct number of disulfide bridges are present in recombinant hevein and AMP as well as in all of the hevein-AMP chimeras (data not shown).

The IgE Binding Ability of Hevein Is Determined Almost Exclusively by Its N-terminal and C-terminal Regions—To study the immunological properties of the recombinant allergens, ELISA tests were performed for recombinant hevein and AMP, for all of the chimeras, and for the three synthetic linear peptides of hevein. The binding of IgE to recombinant hevein corresponded to that of native hevein (data not shown). In agreement with the results with native AMP, only 1 of the 16 sera from hevein-allergic patients showed weak IgE binding to recombinant AMP (Fig. 4). These results indicated that the IgE binding ability of recombinant hevein and AMP was virtually identical to that of their native counterparts.

Two of the 16 patients (13%) with IgE antibodies against hevein showed IgE binding to the AMP chimera containing hevein core region (AMPcore). This suggests that only a very few hevein-allergic patients develop IgE response to epitopes present in the hevein core region (Fig. 4). The chimeric AMPN with the N terminus of hevein (residues 1–11) was recognized by 14 of 16 (88%) patients, and the chimeric AMPC with residues from hevein C terminus (residues 32–43) was recognized by 6 of 16 (38%) patients. However, when both the N-terminal and C-terminal regions of hevein were fused with AMP

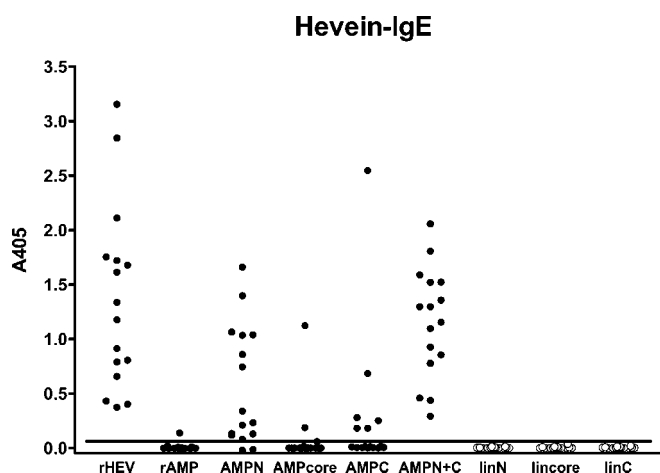


FIG. 4. Occurrence of IgE antibodies to AMPN, AMPC, AMPN+C, and AMPcore by ELISA in sera from NRL patients. It can be seen that of the 16 patients, 88% showed IgE antibodies to AMPN, 38% showed IgE antibodies to AMPC, and 100% showed IgE antibodies to AMPN+C. The AMPcore chimera was recognized in 2 of the 16 NRL patients. No significant binding of IgE antibodies against any of the linear peptides corresponding to hevein regions in the AMP chimeras (linN, lincore, and linC, indicated as open circles) was found. The cut-off level for a positive response (mean optical density $\pm$ 3 S.D. of the control value) is indicated by the horizontal black line.

(AMPN+C), all 16 patients (100%) showed IgE binding against the chimera.

The IgE binding profiles at the individual level revealed that in nine patients, only the N-terminal region of hevein was recognized, and in one patient, only the C-terminal region of hevein was recognized. In four patients, IgE bound simultaneously to both the N-terminal and C-terminal regions. For one patient, the hevein core region was the principal reaction target, and in another patient, IgE bound weakly to both the hevein core and C-terminal regions. Interestingly, in one patient, IgE binding was seen only against the chimera in which both the N terminus and C terminus were present.

Synthetic linear peptides of hevein fragments corresponding to the AMPN, AMPC, and AMPcore chimeras were tested in direct ELISA, but none of them bound IgE from the sera of NRL-allergic patients (linN, lincore, and linC in Fig. 4).

Inhibition ELISA Confirms the Conformational Nature of the IgE Epitopes—Six patients who recognized the N terminus and/or C terminus of hevein in direct ELISA were used for ELISA inhibition analysis. The binding of IgE to solid-phase AMPN, AMPC, or AMPN+C was inhibited with the analogous linear peptides (linN, linC, and linN+linC, respectively) to compare the IgE binding affinities of the chimeras and peptides. No significant inhibition of IgE binding to the chimeras was observed with any of the linear peptides, even at a peptide concentration as high as 100 μ g/ml (data not shown). Furthermore, the pool of the peptides linN and linC was unable to inhibit IgE binding to the solid-phase AMPN+C chimera. In contrast, native hevein completely inhibited the binding of IgE to all chimeras, as expected (data not shown).

To compare IgE binding affinities against native hevein, ELISA inhibition was also performed with the chimeras and peptides as inhibitors. Chimeric AMPN+C remarkably inhibited the binding of IgE to solid-phase hevein in a dose-dependent manner (Fig. 5). The binding of IgE was inhibited 60–97% in five patients (10 μ g/ml), but only 20% inhibition was achieved in one patient. In contrast, AMPC showed only a modest (~30%) inhibition of IgE binding, and no significant inhibition was seen with AMPN (Fig. 5). ELISA inhibition was also performed with the linear hevein peptides (linN, linC, and lincore). No significant inhibition was seen with any of these

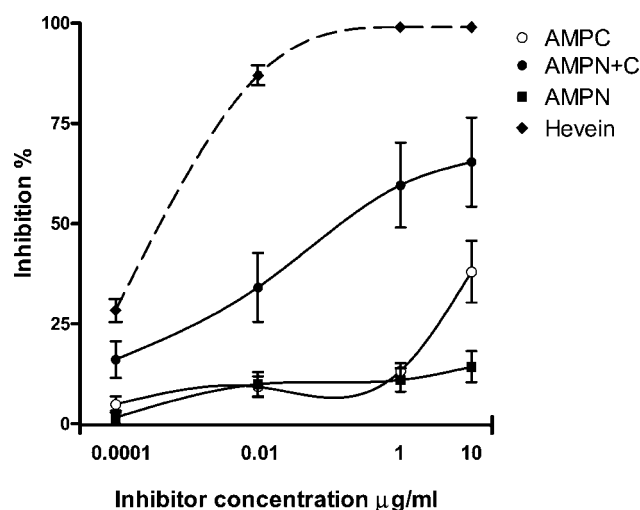


FIG. 5. The average inhibition of IgE binding to solid-phase hevein was detected with AMP chimeras and hevein in sera from six individual NRL-allergic patients. An average of 74% inhibition of IgE binding to solid-phase hevein was found with the AMPN+C chimera (●) at a concentration of 10 μ g/ml. No inhibition was seen with AMPN (■), but a modest inhibition was noted with AMPC (○) at the highest concentration. The inhibition curve with native hevein (◆) as the inhibitor is shown for comparison (indicated as a dotted line).

three peptides, even at the highest peptide concentration used (100 μ g/ml; data not shown).

DISCUSSION

During the last decade, the IgE-binding epitopes of various allergens have been described, as a rule, by overlapping peptide mapping. Because this method enables immunological properties to be studied only in short peptides (usually 8–15 amino acids), it is obvious that only linear IgE-binding epitopes can be identified. However, the currently available data from crystallographic studies suggest that most, if not all, B-cell epitopes on proteins are conformational (9, 10).

Recombinant DNA technology has enabled single in-frame deletions and N-terminal or C-terminal truncations to reduce or hinder antibody-antigen recognition. Tang *et al.* (26) and Tamborini *et al.* (27) have used the deletion system in the characterization of the major allergens from *Aspergillus fumigatus* (Asp f 2) and *Lolium perenne* (Lol p 1). Site-directed mutagenesis is a quite recently described technique in the identification of IgE-binding epitopes (28, 29).

In the present study, a novel chimera-based allergen epitope mapping strategy was developed. The N terminus, core, and C terminus of hevein (Hev b6.02), a major latex allergen, were fused with a nonallergenic hevein homologue (AMP) that served as an immunologically non-IgE-binding adaptor protein. Structure-based modeling confirmed that the designed chimeras could be expressed without structural constraints and with all the possible disulfide bridges formed. IgE from only 2 of 16 hevein-allergic patients bound to the AMP chimera containing the hevein core region (AMPcore), whereas the N-terminal chimera (AMPN) was recognized in 88% of the patients, and the C-terminal chimera (AMPC) was recognized in 38% of the patients. When both the N-terminal and C-terminal regions of hevein were fused with the AMP core, IgE from all patients recognized this chimera (AMPN+C). This chimera was also able to strongly inhibit IgE binding to native hevein. AMPC showed 30% inhibition, most probably due to low affinity binding because it was detected only with a concentration of AMPC 3 or 5 orders of magnitude higher than that of AMPN+C or hevein, respectively. AMPN had no measurable effect in ELISA inhibition. The linear peptides, which corresponded to hevein

regions in AMP chimeras, showed no significant IgE binding capacity in our study. These results strongly suggest that the N-terminal and C-terminal regions of hevein and their correct conformational folding are essential in the formation of viable IgE-binding hevein epitopes.

Banerjee *et al.* (30) and Beezhold *et al.* (31) have independently reported two reactive areas for IgE antibody interactions with hevein, containing amino acid residues 19–24 and 25–37 and 13–24 and 29–36, respectively. These regions are located mainly in the core region, which did not seem to be directly involved in IgE binding in our AMP-hevein chimeras. Differences in IgE epitope mapping approaches may explain part of the discrepancy. Banerjee *et al.* (30) and Beezhold *et al.* (31) used a solid-phase system and an overlapping peptide mapping strategy, whereas, in the present study, synthetic peptides were examined both in a solid phase (direct ELISA) and in a liquid phase (inhibition ELISA). Moreover, in the present study, a chimera-based IgE epitope mapping strategy was used to identify conformational IgE-binding epitopes. Another reason for the discrepant results may be that patients from different geographical areas may have developed variable IgE specificities and recognize different epitope areas on the surface of a single allergen. However, no data to reject or accept this hypothesis are currently available.

Recombinant DNA technology can be used in the design of chimeric proteins, including allergens containing structural and functional properties of parent proteins (related or unrelated) in a single molecule. Colombo *et al.* (32) and Simon-Nobbe *et al.* (33) constructed truncated allergens fused with the maltose-binding protein and glutathione *S*-transferase, respectively, to identify the smallest part of the protein that is recognized by IgE antibodies from allergic patients. We have also produced a number of substitution and deletion mutants of hevein.³ A deletion mutant lacking the C terminus (amino acid residues 33–43) induced distortion of the tertiary structure and precipitation of the protein, rendering it inappropriate for adequate immunological or biochemical analyses.

Discontinuous B-cell epitopes in vespid allergens were recently studied by King *et al.* (8), who examined hybrids containing different segments of Ves v5 from the yellow jacket (*Vespula vulgaris*) and Pol a5 from paper wasp (*Polistes annularis*). They had fused portions of the “guest” allergen (Ves v5) to large portions of a structurally homologous but poorly cross-reacting scaffold protein (Pol a5). Physicochemical characterization suggested that the hybrids had retained their three-dimensional structures close to the native proteins. Allergenicity of some hybrids obviously lacking critical epitope regions of the native allergen was shown to be drastically reduced. Despite the similar objectives of these studies, there are also differences. The rationale in our study was to use the “gain in function” approach, *i.e.* we wanted to transfer areas of a known allergen to a virtually nonallergenic adaptor to verify the existence of functionally active B-cell epitopes. In addition, we assessed the direct IgE binding reactivity of our chimeras using human sera. The results showed that biologically viable IgE-binding allergens were indeed produced by transferring allergenic regions into a minimally IgE-binding protein homologue. In our system, the three-dimensional structure of both counterparts, *i.e.* hevein and the adaptor protein AMP, were known, which facilitated our interpretation of the results and allowed us to conclude that relative small conformational areas of the tightly packed hevein contain major IgE epitopes.

In conclusion, we describe here a novel allergen epitope

mapping strategy for characterization of conformational IgE-binding epitope areas. In this approach, short regions of hevein, a major NRL allergen, were fused with a nonallergenic hevein homologue (AMP) that served as an immunologically non-IgE-binding adaptor protein. Chimera-based technology, together with molecular modeling facilitating viable construct design, enabled us to locate IgE-binding epitope areas to the N-terminal and C-terminal regions of hevein. This approach may provide new possibilities for defining structural allergenic or antigenic epitopes and creating specific reagents for immunotherapy and vaccine development. Moreover, it should be possible to create chimeric proteins in an even more general fashion for studying the structure-function relationship of multifunctional and multidomain proteins.

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