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Mapping of a conformational epitope on the cashew allergen Ana o 2: A discontinuous large subunit epitope dependent upon homologous or heterologous small subunit association☆

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ABSTRACT

The 11S globulins are members of the cupin protein superfamily and represent an important class of tree nut allergens for which a number of linear epitopes have been mapped. However, specific conformational epitopes for these allergens have yet to be described. We have recently reported a cashew Ana o 2 conformational epitope defined by murine mAb 2B5 and competitively inhibited by a subset of patient IgE antibodies. The 2B5 epitope appears to reside on the large (acidic) subunit, is dependent upon small (basic) subunit association for expression, and is highly susceptible to denaturation. Here we fine map the epitope using a combination of recombinant chimeric cashew Ana o 2-soybean Gly m 6 chimeras, deletion and point mutations, molecular modeling, and electron microscopy of 2B5-Ana o 2 immune complexes. Key residues appear confined to a 24 amino acid segment near the N-terminus of the large subunit peptide, a portion of which makes direct contact with the small subunit. These data provide an explanation for both the small subunit dependence and the structurally labile nature of the epitope.
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1. Introduction

Tree nuts are major etiological agents of food allergy affecting 0.2% children and 0.4% adults in the USA (Sampson, 2004). Allergy to cashew ranks second among the tree nut allergies (Sicherer et al., 2003) and has been reported to cause allergic responses in sensitive individuals exceeding those observed for peanut (Clark et al., 2007). The major classes of tree nut allergens include 7S globulins (vicilins), 11S globulins (legumins), and 2S albumins, all of which are classified as food storage proteins (Roux et al., 2003; Sathe et al., 2005). The native and recombinant cashew homologues of each of these proteins have been characterized and are designated Ana o 1, 2 and 3, respectively (Teuber et al., 2002; Wang et al., 2002, 2003; Robotham et al., 2005). Of the cashew allergens, Ana o 2, the 11S globulin, is the best characterized (Wang et al., 2003; Barre et al., 2007; Robotham et al., 2009).

Ana o 2, like other 11S globulins, is synthesized as a large proprotein in the developing seed and is posttranslationally cleaved into large (acidic, 257 aa) and small (basic, 171 aa) subunits that remain associated via disulfide bonds (Staswick et al., 1984; Wang et al., 2003). The proproteins typically form trimers which, upon maturation to the cleaved form, dimerize face-to-face to form hexamers (Adachi et al., 2003; Shewry et al., 2004). The atomic structures of the native 11S globulin of soybean and, more recently, of peanut and almond have been reported and share considerable structural homology (Adachi et al., 2003; Jin et al., 2009a,b).

We have previously described a series of linear epitopes recognized by patients with severe allergic reactions upon cashew nut ingestion (Wang et al., 2003). Homology modeling of cashew 11S globulins revealed that the linear epitopes are dispersed over the large and small subunits and display a range of exposure to solvent (Barre et al., 2007; Robotham et al., 2009).

Screening for the presence of specific conformational epitopes, using patients' serum IgE, and their subsequent characterization is technically challenging compared to screening for linear epitopes.

Abbreviations: mAb, monoclonal antibody; TBS, tris-buffered saline; aa, amino acid.

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One approach is to screen mouse monoclonal antibodies, (mAbs) raised against the allergen, for the ability to recognize conformational epitopes and then to determine if patient IgE competes with the mAb for binding to the identified epitopes. We previously reported the characterization of one such mAb (2B5) which defines an epitope on the cashew 11S globulin large subunit that is conformationally dependent upon the association of the large subunit with the small subunit of the cashew allergen for its expression (Venkatachalam et al., 2008; Robotham et al., 2010). Interestingly, association with the small subunit of the soybean homologue, Gly m 6, can also foster 2B5 epitope expression on the cashew large subunit (2B5 does not bind Gly m 6). This epitope is expressed in both the recombinant cashew proprotein and the enzymatically cleaved mature protein. The 2B5 epitope is highly susceptible to loss of immunoreactivity upon protein denaturation, further attesting to its conformational nature. In addition, mAb 2B5 partially inhibits the binding of human IgE to Ana o 2 and patient's IgE fully inhibits the binding of 2B5 to Ana o 2 indicating the likelihood of at least partial overlap of one or more epitopes recognized by IgE with the 2B5 epitope.

In the present study, we have attempted to fine map the 2B5 epitope by first probing a series of cashew–soybean chimeric proteins to locate the target region necessary for epitope expression, and then, using molecular modeling and alanine mutagenesis to identify residues in the epitope. Our results showed that the specific residues residing in an area spanning amino acids (aa) 20–43 of the large subunit, a region of mixed secondary structure, are primarily responsible for 2B5 binding.

2. Methods

2.1. Antibody production

Ana o 2-reactive mAbs were generated in the Hybridoma Core Facility at Florida State University according to standard procedures as previously described (Venkatachalam et al., 2008). Briefly, BALB/c mice were immunized with 25 μg of whole cashew extract in RIBI adjuvant (Corixa Inc., Hamilton, MT) and boosted with 15–20 μg of antigen in the RIBI adjuvant system 3 weeks later. Spleen cells were fused with NS-2 myeloma cells and screened for reactivity to whole cashew protein extract and reactivity to native (n) and recombinant (r) Ana o 2 by ELISA. A total of 20 monospecific Ana 2-binding mAbs were obtained, one of which was identified as recognizing a conformational epitope (Robotham et al., 2010).

2.2. Cloning and expression of chimeric molecules

In addition to rAna o 2 and Gly m 6, 28 Ana o 2/Gly m 6 chimeric and truncated genes were constructed by blunt end ligation of phosphorylated PCR fragments as described in Robotham et al. (2010). Point mutations were made using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, USA) as directed by the manufacturer. The various constructs were ligated into a modified version of the maltose-binding protein (MBP) fusion expression vector pMAL-c2 (New England BioLabs Inc., Beverly, Mass) and the cloning, expression, and purification of the chimeric-MBP fusion proteins were carried out as previously described for rAna o 2 (Wang et al., 2003; Robotham et al., 2010).

2.3. Dot blot probing of chimeric molecules with monoclonal antibody

Two micrograms of the purified proteins were dotted onto 0.45 μm nitrocellulose membranes (Shleicher & Schuell). The membranes were blocked by Tris-buffered saline (TBS)-T (20 mM Tris, 137 mM NaCl, 0.2% Tween 20, pH 7.6) containing 5% (w/v) non-fat dry milk for 1 h at room temperature and then reacted with mAb 2B5 at 4 °C overnight. The membranes were then washed three times by TBS-T and incubated at 4 °C overnight with horseradish peroxidase-labeled goat-anti-human IgE (Biosource Intl., Camarillo, CA) diluted 1:2000 (v/v) in TBS-T. Membranes were visualized by a 5-min incubation in ECL+ (Amersham Pharmacia) and subsequent exposure to Kodak XAR film (Kodak Molecular Imaging, New Haven, CT, USA). Recombinant Ana o 2 was used as a positive control and the Ana o 2 large subunit (ALG) and small subunit (ASM) were used as negative controls as previously described (Robotham et al., 2010).

2.4. Molecular modeling

Molecular modeling was performed using PyMOL (http://www.pymol.org) by decorating the atomic structures corresponding to homologous segments and residues of Ana o 2 using the soybean glycinin homohexamer (PDB ID: 1OD5) A3B4 (Adachi et al., 2003) and the soybean proglycinin (PDB ID: 1fxz) A1aB1b homotrimer (Adachi et al., 2001) structures as templates. Solvent accessible surface areas were calculated using VADAR (http://redpoll.pharmacy.ualberta.ca/vadar).

2.5. Electron microscopy

Ana o 2 was purified from an aqueous cashew nut protein extract as previously described (Sathe et al., 1997) and subjected alone or in complex with mAb 2B5 (1:1 molar ratio) to negative stain electron microscopy using previously described methods (Roux, 1989, 1996). Briefly, soluble proteins and complexes were allowed to spontaneously adhere to carbon membranes and were subsequently stained with 1% (w/v) uranyl formate. Electron micrographs were recorded at 100,000× magnification at 100 kV on a JOEL JEM 1200 electron microscope.

3. Results

3.1. Chimeric constructs

Various chimeras composed of cashew 11S globulin Ana o 2 substituted with homologous segments of soybean 11S globulin Gly m 6 (Fig. 1A) were iteratively produced, expressed, purified, and probed with mAb 2B5 in dot blot assays (Fig. 2) in an effort to define the minimal Ana o 2 segment(s) required for 2B5-reactivity. All recombinant proteins included fusion partners (MBP) as attempts to cleave and remove the fusion partner typically resulted in insoluble aggregation. For simplicity, the numbering of the aa residues in both constructs begins at 1 and the descriptions of the chimeras are presented in the text with reference to the Ana o 2 numbered residues (a codon deletion at position 3 of Gly m 6 shifts its numbering by one with respect to that of Ana o 2, Fig. 1).

3.2. Defining the 2B5-reactive segment

Dot blot probing of rAna o 2 gave a strong positive signal (+++) whereas none was detected with rGly m 6 (−) (Figs. 1A and 2A). The location of the 2B5 epitope was initially narrowed to rAna o 2 segment 1–87 by demonstrating a similarly strong signal when this segment was associated with Gly m 6 87–289 of the large subunit and either the Ana o 2 (C1, +++) or Gly m 6 small subunit (C2, +++). The "opposite" construct, Gly m 6 1–87/Ana o 2 88–257/Ana o 2 SM (C3, −), was non-reactive as was the N-terminal truncated construct Ana o 2 88–257/Ana o 2 SM (C4, −).

Several constructs were produced in an attempt to define the N-terminal boundary of the 2B5-reactive segment(s). The results

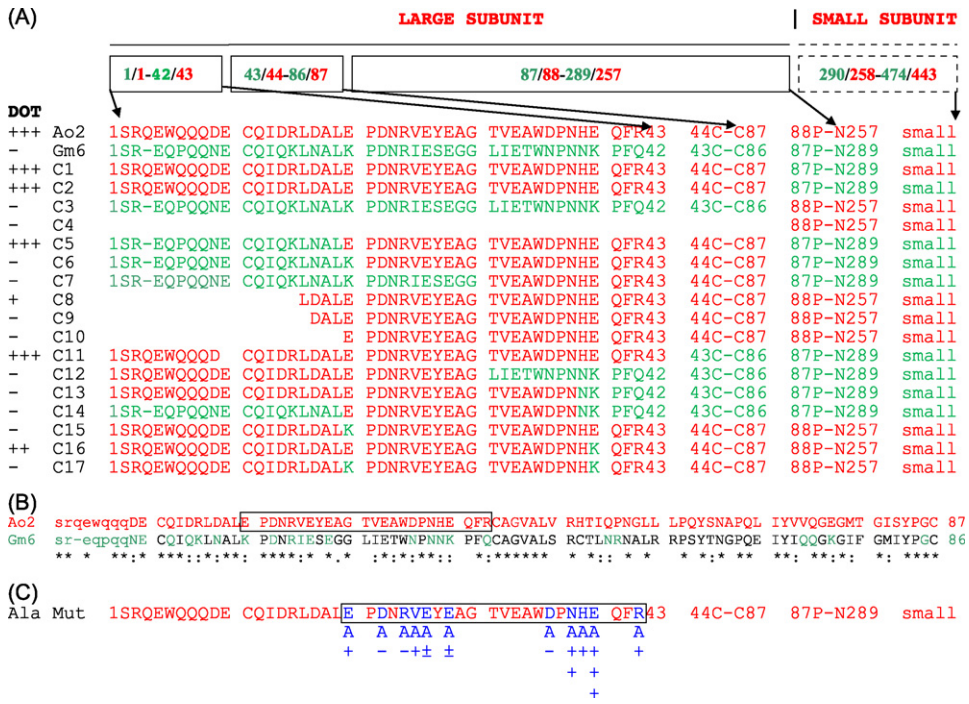

Fig. 1. Chimera constructs, mAb 2B5-reactivity, and sequence comparison. (A) Diagram and aa sequences of constructs (C1–C17) showing Ana o 2 (Ao2, red font) and Gly m 6 (Gm6, green font) segments. Closed rectangles: segments of the large subunit. Dotted rectangle: the small subunit. The degree of dot blot reactivity with mAb 2B5 (DOT: – to +++) shown to the left of each construct. (B) Alignment of Ana o 2 aa 1–87 (red) and Gly m 6 aa 1–86 (green). Boxed segment: 2B5-reactive sequence. Black font: amino acids buried within the Gly m 6 homohexamer crystal structure (i.e., not accessible for mAb interaction). Amino acids in lowercase are not present in the Gly m 6 crystal structures. Asterisk (*) denotes aa identity, colon (:) indicates aa similarity. (C) Alanine mutants (Ala Mut) shown in blue font with 2B5-reactivity (– to +++) below each mutated residue. Each alanine substitution represents a different construct. Boxed segment: 2B5-reactive sequence. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

demonstrated that the chimeras could remain reactive upon Gly m 6 substitutions for Ana o 2 residues 1–19 (C5, +++) but not for substitution of the first 20 (C6, –) or 30 (C7, –) aa. A weak signal was produced upon deletion of the N-terminal 15 aa (C8, +) of Ana o 2 but not for deletions of the N-terminal 16 or 19 aa (C9, –; C10, –).

We also sought to determine if a C-terminal boundary of the 2B5 epitope could be defined. We previously demonstrated that the entire Ana o 2 small subunit could be replaced by the Gly m 6 homologue (Robotham et al., 2010) and thus focused our attention on the C-terminus of the Ana o 2 large subunit. A Gly m 6 homologous substitution for the Ana o 2 segment aa 88–257 (C2, +++) did not diminish the 2B5 binding signal, as indicated above, nor did an aa 44–257 substitution (C11, +++). However, an aa 31–257 Gly m 6 substitution eliminated binding (C12, –) as did a less intrusive N-terminal substitution for Ana o 2 aa 39–257 (C13, –). A similarly negative signal was observed for a construct (C14, –) which combined the C-terminal 39–257 (as in C13, –) and the above described N-terminal 1–19 substitution (C5, +++).

Taken together, these data indicate that full expression of the 2B5 epitope requires an intact 11S globulin structure displaying the Ana o 2 sequence within the E20-R43 segment. Some N-terminal truncation of the Ana o 2 sequence (S1-R15, C8, +) is also tolerated for epitope display.

3.3. Identification of key amino acids and molecular modeling

In examining the primary sequence of the Ana o 2 aa 20–43 peptide in comparison to the Gly m 6 counterpart, we noted two positions with oppositely charged residues (Ana o 2 E20/Gly m 6 K19 and Ana o 2 E40/Gly m 6 K39). Since such differences could have profound effects on epitope expression, especially since they are surface exposed, we generated constructs in which one or both residues were switched. An E20K substitution (C15, –) prevented binding of 2B5 whereas the E40K substitution (C16, ++) had relatively little effect. Not surprisingly, no binding was observed when both substitutions were made (E20K and E40K, C17, –). E20 therefore appears to be a critical aa for epitope recognition whereas E40 has little if any effect. With respect to E20, these data are consistent with the observation described above in which the N-terminal 19 aa of Ana o 2 could be substituted with the homologous Gly m 6 segment (see C5, +++) but substitution for Ana o 2 aa 1–20 greatly inhibited binding (C6, –). Note that E20 sits in an extended sequence which is otherwise identical between Ana o 2 and Gly m 6 (18ALE/KPDNR24).

An atomic model of Gly m 6 is presented in Fig. 3. In the top panel, two of the visible monomeric subunits on one of the IA (solvent-exposed) faces are indicated in different shades of gray. The third is subdivided into the small subunit (blue), the 2B5-reactive segment homologue of the large subunit (red, aa 20–43 using the Ana o 2 homologous numbering), and the remainder of the large subunit (yellow, aa 1–19 segment and 44–257) (for interpretation of the references to color in this sentence, the reader is referred to the web version of the article). The bottom panel shows a side view wherein the second trimer layer is shown in white.

To further fine map the 2B5 epitope, surface-exposed residues within the modeled Ana o 2 E20-R43 segment (Fig. 4A–F) were screened by alanine mutagenesis (Figs. 1C and 2B). The results show that of the 11 residues screened, three completely abolished 2B5-reactivity (D22A, R24A, and D36A), six greatly reduced binding (E20A, V25A, E26A, E28A, H39A and R43A), and two had little to no effect (N38A and E40A) (Fig. 1C). Fig. 4A and B shows a surface map wherein the critical residues are colored magenta, the less critical residues light pink and the unmutated, less exposed residues green (for interpretation of the references to color in this sentence, the

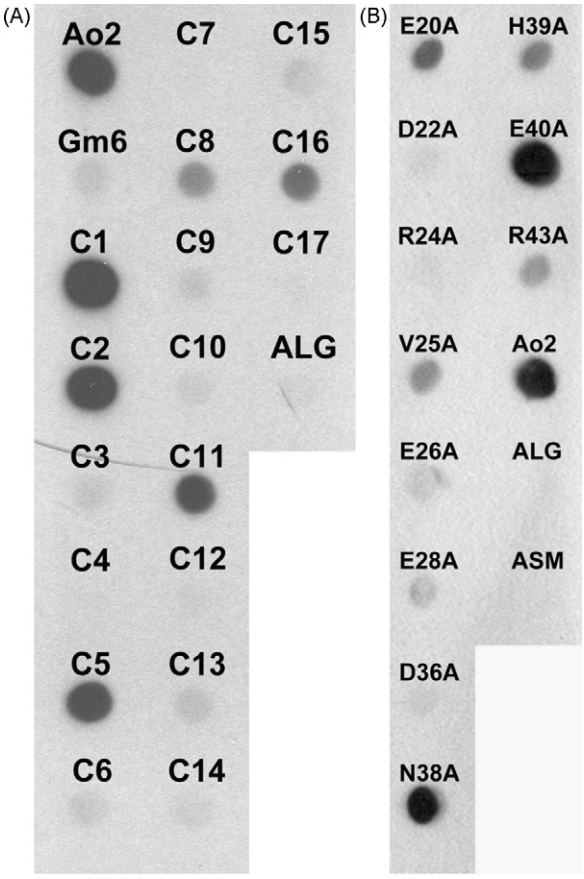

Fig. 2. Dot blot analysis of mAb 2B5-reactivity with (A) Ana o 2 (Ao2), Gly m 6 (Gm6), chimeric constructs (C1–C17) and (B) alanine point mutants. ALG, large subunit of Ana o 2; ASM, small subunit of Ana o 2.

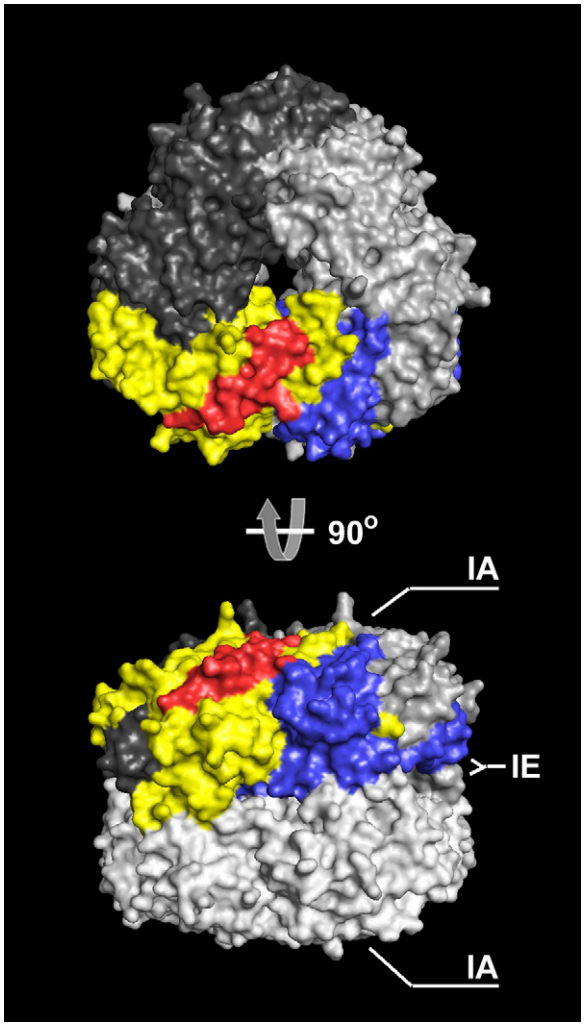

Fig. 3. Atomic model of 11S globulin homohexamer showing relative positions of subunits and 2B5 epitope. "Top" view showing the solvent-exposed IA face (top) and "side view" (bottom) showing the face-to-face (IE-IE) stacked trimer conformation. Two of the monomeric subunits in the top view are in shades of gray and the third monomer is subdivided into the small subunit (blue) and the large subunit (yellow) including the amino acids of glycinin corresponding to the 2B5 epitope-bearing segment (aa 20–43 in red) of Ana o 2. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

reader is referred to the web version of the article). For simplicity, only one trimer layer is shown.

An analysis of the α-carbon backbone structure of the 2B5-reactive segment homologue reveals that it contains two β-strands connected by a β-turn along with segments of random coil and a small portion (single turn) of an α-helix (Fig. 4C and D, red segment). We noted that several residues in the 2B5 segment (W36, P42, F43) appear to be in direct contact with residues of the small subunit (Fig. 4F). In addition, a conserved disulfide bond between C44 of the α-helix and C11 provide additional stability. Mapping of the aa involved in 2B5 expression shows that most of the surface-exposed residues are critical for binding (Fig. 4E). Only N38A and E40A near the C-terminus were permissive of alanine mutations. The inability of the E40K substitution (C16 ++), described above (Fig. 1A), to block binding is consistent with this pattern.

Although the Ana o 2 segment identified by the chimera analyses and the specific residues identified by mutagenesis likely represent the core of the epitope, it is possible that additional key contact or otherwise structurally important residues may lie in adjacent regions. To investigate this possibility, we mutated several surface-exposed aa in topologically adjacent regions of the large and small subunits through alanine substitutions as shown on the homology model (Fig. 4G). Eight constructs, representing 12 alanine substitutions, were screened (R15A, H178-R181AAAA, D212-N213AA, R214A, N346A, E352A, E355A, and Q357A). Interestingly, none of the mutations had an effect on 2B5 epitope expression (data not shown) suggesting that the above identified core segment constitutes the entire epitope.

3.4. Electron microscopy of mAb 2B5-Ana o 2 immune complexes

The location of the epitope on the surface of the subunits of the nAna o 2 hexameric structure suggest that the Fab arms of 2B5 would bind such that the pseudo-two-fold axis of the bound Fabs would protrude from the planar surface of the IA (solvent accessible) face at an angle projecting about 30° away from the 3-fold axis of symmetry, assuming a flush contact between the two surfaces. To test this hypothesis, we performed negative stain transmission electron microscopy (EM) on nAna o 2 and nAna o 2-2B5 immune complexes and analyzed the resulting images. In the absence of mAb, nAna o 2 assumed two predominant orientations with respect to the carbon membrane support. One motif showed a slightly triangular configuration typically with a central stain-filled void (top row, Fig. 5A). We interpreted this configuration as representing molecules being viewed "face on" where the planar faces of the molecules are parallel to the carbon support and the 3-fold axis of symmetry is at 90° to the carbon as depicted in Fig. 5D. The other predominant molecular configuration was square in profile, often having some stain appearing to divide the square into

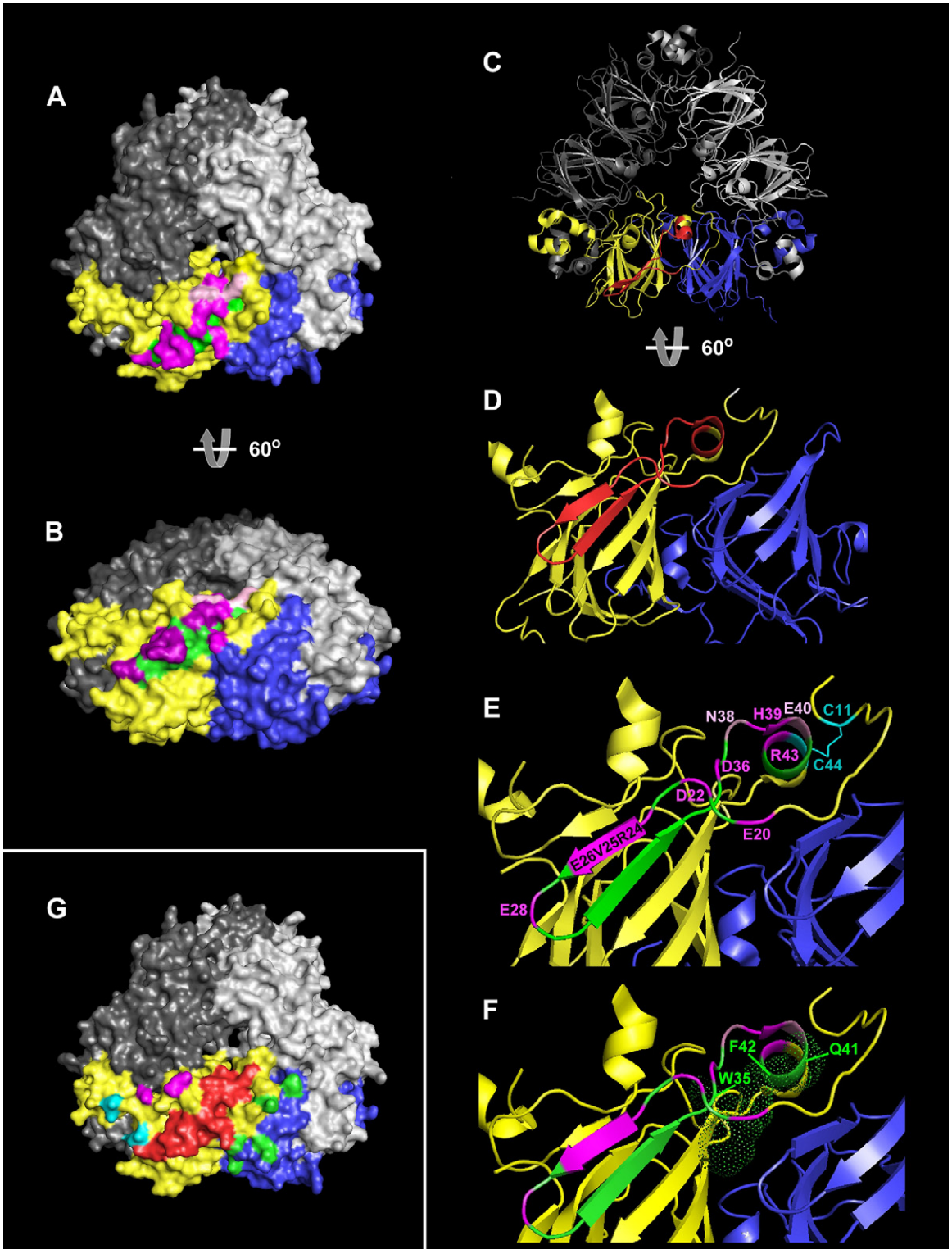

Fig. 4. Gly m 6 trimer model showing surface and internal structure of 2B5 epitope region and locations of alanine substantiation mutations. (A–F) 2B5 epitope and mutations. Large and small subunits colored as in Fig. 3. Red: 2B5 epitope region. Magenta: surface residues which significantly weaken or eliminate 2B5-reactivity when mutated. Light pink: surface residues with relatively little or no effect on 2B5-reactivity when mutated. Green: residues largely buried and not assessed for effect on 2B5-reactivity. Aqua in E: intrachain disulfide bond. Dots in F: 2B5 segment residues in contact with small subunit. (G) Mutations outside of 2B5 epitope. Green: residues subjected to alanine substantiations. Magenta and aqua: connecting residues for two aa segments which are not resolved in the Gly m 6 crystal structure (PDB ID: 1FXZ) but which contain alanine mutations. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

two halves (Fig. 5A bottom row). We interpret this to representing molecules being viewed "on edge" as depicted in Fig. 6E. The 2B5 epitopes in the models are shown in red to assist in interpretation.

Coincubation of 2B5 mAb with nAna o 2 generated a variety of complexes (Fig. 5B and C). Most immune complexes included a single Ana o 2 molecule and one or more mAbs (Fig. 5B rows one and two and the right-hand three panels in row three). Most of these

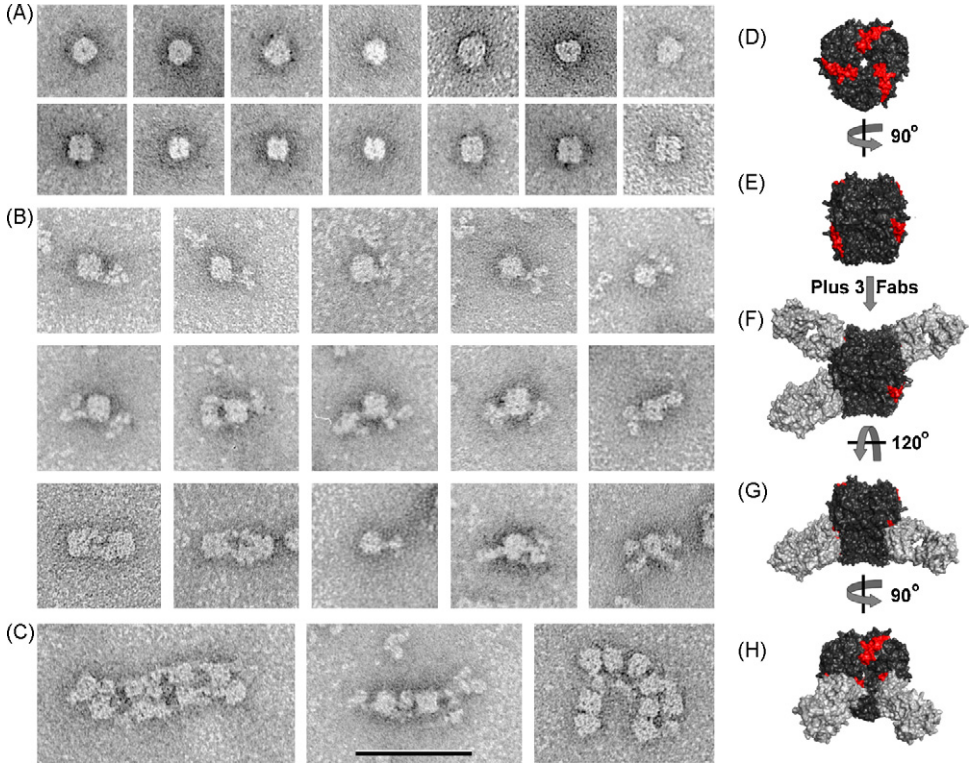

Fig. 5. Negative stain electron micrographs (A–C) and interpretive homohexamer models (D–H) of nAna o 2 (A) and nAna o 2-2B5 immune complexes (B and C). Red surface patches = 2B5 epitope. Bar = 50 nm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

were interpreted to show the Ana o 2 in the on edge view (Fig. 5B rows one and two as depicted in F and G). For simplicity, the modeled complexes in Fig. 5F–H show only the Fab arms of antibodies binding to three of the six available epitopes. The "face on" orientation of complexed Ana o 2 was also observed for a minority of complexes (Fig. 5B bottom row, right three panels as depicted in H). Larger complexes composed of two or more Ana o 2 molecules in complex with multiple mAbs were also observed, typically showing the allergens "on edge" (Fig. 5B bottom row, left two panels and C). Overall, the EM results were fully consistent with the modeling predictions in that the mAb bound to the IA face of the hexamer near the periphery at an angle of ~30° from the 3-fold axis of symmetry.

3.5. Comparative mapping of the 2B5 conformational epitope and linear epitopes on Ana o 2

To gain a sense of the relative location of conformational epitope 2B5 with respect to the previously mapped linear Ana o 2 patient IgE-reactive epitopes (Wang et al., 2003; Barre et al., 2007; Robotham et al., 2009), all were mapped to homologous positions on the Gly m 6 atomic model (Fig. 6). Of the 12 mapped linear Ana o 2 epitopes (3 others were on segments not resolved in the atomic structure), 4 were substantially mapped to the solvent-exposed IA face of the nAna o 2 hexamer; the remainder mapped to the lateral edge or are occluded on the IE face (in the hexameric form) (4) or mostly buried within the trimer (4).

4. Discussion

The analysis of allergen epitopes is essential for a full understanding of issues relating to allergy induction, effector cell triggering, allergen cross-reactivity, and the development of hypoallergens for safer immunotherapy (Aalberse, 2000; Lorenz et al., 2001; Bannon, 2004; Linhart and Valenta, 2005; Niederberger and Valenta, 2006; Untersmayr and Jensen-Jarolim, 2006). By probing overlapping synthetic peptides, corresponding to the entire length of the sequence in question, with allergic patient sera, linear (continuous) epitopes can be readily identified on allergens (Geysen et al., 1984; Reineke et al., 2001; Frank, 2002). Other epitopes, typically described as being conformational, are only efficiently recognized when presented in the context of the native protein. Such epitopes may represent a contiguous segment that is held in a specific configuration by the general protein scaffold or may represent discontinuous segments similarly stabilized (Barlow et al., 1986; Van Regenmortel and Pellequer, 1994; Schramm et al., 2001; Roggen, 2006; Gershoni et al., 2007). The technology for identifying and characterizing conformational epitopes is technically more challenging and varied than that for identifying linear epitopes (Gershoni et al., 2007). The most direct method for conformational epitope analysis is through co-crystallization of allergens with epitope-specific Fab (Mirza et al., 2000; Padavattan et al., 2007, 2009; Niemi et al., 2008). However, the difficulty in obtaining homogenous allergen-specific IgE antibody for co-crystallization with allergens and the generation of suitable crystals hinders such research.

We have been developing a model system for the study of food allergen epitopes using the clinically important cashew allergen, Ana o 2 (Wang et al., 2003; Venkatachalam et al., 2008; Robotham et al., 2009, 2010). Ana o 2, an 11S globulin, is representative of a class of plant proteins (cupins) which have been identified as important allergens in a variety of nuts and seeds (Roux et al., 2003; Breiteneder and Radauer, 2004). As a major seed (nutrient) storage protein, the 11S globulins are often found in abundance in nuts and seeds. They are generally considered resistant to digestion which is a characteristic of class I allergens (Metcalfe et al., 1996; Taylor and Hefle, 2001; Breiteneder and Mills, 2005), though this point, with respect to the 11S globulins, is controversial (van Boxtel et al., 2008). Cupins are so named because of a prominent β-barrel structural feature which, in the 11S globulins, is found in both the large (acidic) and small (basic) subunits. Thus far, linear epitope

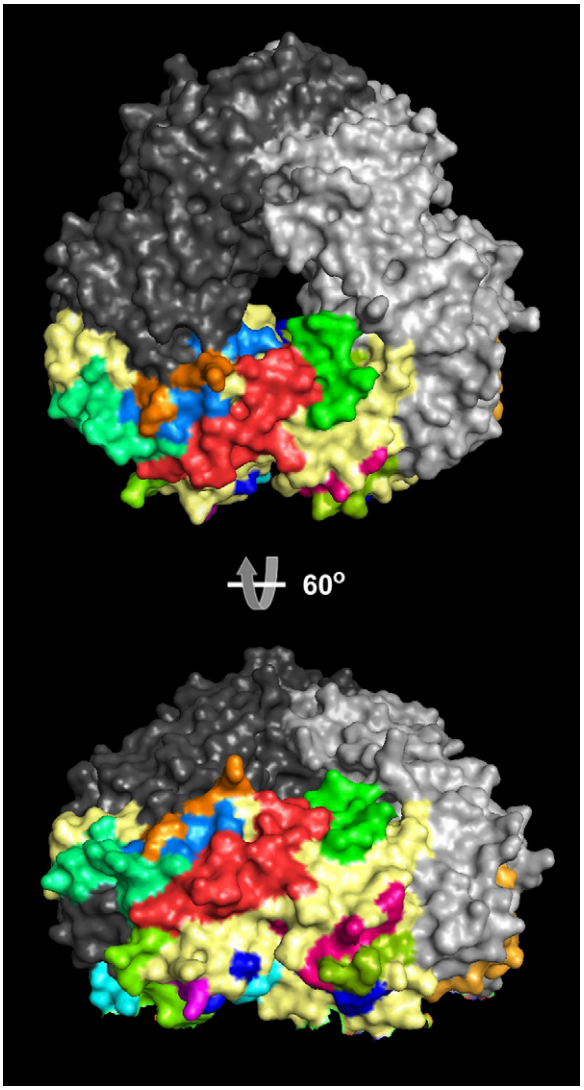

Fig. 6. Surface model showing the location of the 2B5 epitope (red) in relation to those of the previously reported linear Ana o 2 epitopes (other variously colored regions) (Wang et al., 2003). Segments in the large and small subunits of the selected monomer that are not identified as containing epitopes are in yellow. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

maps have been generated for 11S globulins of cashew (Wang et al., 2003), walnut (Robotham et al., 2009), hazelnut (Robotham et al., 2009), peanut (Rabjohn et al., 1999; Rouge et al., 2009), and soybean (Zeece et al., 1999; Helm et al., 2000), however, no conformational epitope maps have been published for these allergens.

Our Ana o 2 conformational mapping stems from the fortuitous identification of a mouse IgG mAb (2B5) which recognized a conformational epitope. This mAb competes with a subset of IgE Abs from cashew-allergic patients for binding to Ana o 2. The conformational nature of the epitope was demonstrated by its expression on the cashew 11S large subunit of Ana o 2 only when associated with a small subunit (Robotham et al., 2010). Interestingly, in addition to being expressed when associated with the Ana o 2 small subunit, the 2B5 epitope is fully expressed when associated with the homologous small subunit from the soybean 11S globulin, Gly m 6, an allergen in its own right but not one that is recognized by our cashew-allergic patients or by mAb 2B5. Additional evidence for the conformational nature of the 2B5 epitope was provided by assays demonstrating the loss of 2B5 binding following a variety of protein denaturation treatments (Robotham et al., 2010).

In the present study, we generated a set of chimeric proteins in which segments of the cashew Ana o 2 were substituted with homologous segments of soybean Gly m 6, and assessed them for binding with mAb 2B5 in dot blot assays. The data from these chimeras, as well as from N-terminal deletion mutants of rAna o 2, narrowed the epitope to a 24 aa segment (E20-R43) (Fig. 1). Analysis of the homologous Gly m 6 crystal structure showed that this segment is comprised of two β-strands and a connecting loop of the large subunit β-barrel, N-terminal and C-extended strands, and a short helical segment (Fig. 4D). Alanine scanning mutagenesis of 11 surface-exposed residues, identified by molecular modeling, showed that 9 were critical for expression (Figs. 1C and 4A, B and E). The 9 key residues were scattered throughout the 24 aa segment with the exception of the C-terminal β-strand, which is largely buried. Similar mutations in surrounding surface-exposed residues and unmodeled loops, including residues in the adjacent small subunit, did not adversely influence 2B5 binding (Fig. 4G). Though it is still formally possible that some contact may be made with topologically neighboring residues, it appears likely that the identified segment is largely responsible for binding to mAb 2B5. The solvent accessible surface of the segment (~890 excluding the two non-contact residues) is in line with that of a typical protein epitope (~750) (Sheriff et al., 1987).

Having identified the likely position of the 2B5 epitope on rAna o 2, we performed negative stain EM on purified nAna o 2 with and without 2B5 mAb. This technique has been applied to a wide variety of proteins and complexes (Roux, 1996) but, to our knowledge, not to the analysis of allergens. The uncomplexed images were easily interpretable based on correspondence with the atomic structure of previously crystallized native 11S globulins (Adachi et al., 2003; Huber et al., 2009; Jin et al., 2009b). The "face on" configuration showed a rounded triangular configuration with a central stain-filled hole. The "on edge" view showed a squared off shape with hints of two protein layers in some images. The immune complexes typically showed one or more 2B5 mAbs binding obliquely to the outer edge of the planar surface as would be predicted from a flush interaction between Fab and the 2B5 peptide segment as modeled (Fig. 5). The multivalency of hexameric Ana o 2, as visualized in the EMs, and of the 11S globulins in general would favor extensive Ig cross-linking during both the induction and effector phases of an allergic response (Untersmayr and Jensen-Jarolim, 2006).

Both linear (Niemi et al., 2008; Padavattan et al., 2009) and conformational (Mirza et al., 2000; Padavattan et al., 2007) allergen epitopes have thus far been identified by X-ray crystallography. In both cases of linear epitope recognition (birch pollen Bet v 1 and honey bee venom Api m 2), murine IgG Fabs bound to prominent protruding epitopes. In contrast, the two conformational epitopes were situated on relatively flat surfaces (grass pollen Phl p 2 and bovine β-lactoglobulin) and were recognized by patient IgE Fabs. The epitope recognized by our murine IgG mAb 2B5 more closely resembles those of the human IgE Abs in that it binds a non-protruding surface. Also, the 2B5 epitope is technically discontinuous (conformational) even though it resides within a relatively short (24 aa) peptide. For comparison, the Phl p 2 IgE Fab makes contact with residues in four strands of a β-sheet, essentially binding the sheet "face on". In contrast 2B5 appears to make contact with a single β-strand (R24-E26) of the outer rim of the large subunit β-barrel, as well as some additional residues on a connecting loop, extended strands, and a helical component (Fig. 4E).

Two interesting features of the 2B5 epitope, namely the absolute dependence upon small subunit association for expression and its susceptibility to destruction by a variety of denaturants (Robotham et al., 2010), can be explained by its structural features. Whereas the two β-strands of the 2B5 segment (R24-E26 and G30-E33) and the connecting loop probably remain intact when expressed in the absence of the small subunit, other portions of the peptide

make direct contact with the small subunit and likely misfold in its absence. Residues W35, Q41 and F42 of the 2B5 peptide (W35 and F42 are conserved between Ana o 2 and Gly m 6) and others outside of the peptide make direct contact with the small subunit (Fig. 4F). This region may also be susceptible to denaturation though complete disordering of the non-β-strand regions of the 2B5 peptide region is unlikely since it should be at least partially stabilized by the adjacent C44–C11 disulfide bond (Fig. 4E). Reduction would eliminate this stabilizing feature.

Our observation that 2B5 mAb reacted similarly with the recombinant pro and mature 11S globulin forms is predicted from a comparison of the two crystallized Gly m 6 homologues (Adachi et al., 2001, 2003). The peptide cleavage of the pro form, with its concomitant peptide rearrangement that facilitates dimerization upon maturation, occurs on the IE face of the molecule. In contrast, a comparison of the crystal structures of the 2B5 homologous segments and neighboring peptide regions, which are located on the opposite (IA) face of the proglycinin trimer, with the mature glycinin hexamer reveal that they are structurally quite similar (data not shown).

The known Ana o 2 linear epitopes (Wang et al., 2003) map to both exposed and occluded surfaces and to internal sites (Robotham et al., 2009). With respect to those on the exposed surfaces, the four IA-situated and one of the edge-situated linear epitope peptides make direct surface contact with the 2B5 conformational epitope peptide segment forming a large nearly contiguous patch on the monomeric solvent-exposed surface (Fig. 6). It is worth noting that even though the mapped Ana o 2 linear epitopes do not overlap with the 2B5 epitope segment, linear epitopes have mapped to segments homologous to the C-terminal half of this region (i.e., the region of the 2B5 epitope topologically proximal to the 3-fold axis of symmetry, near the central hole in the trimer/hexamer) for peanut Ara h 3 (Rabjohn et al., 1999), walnut Jug r 4 (Robotham et al., 2009), and hazelnut Cor a 9 (Robotham et al., 2009) (see Fig. 1 in Robotham et al., 2009).

There is a general consensus that the entire surface of a protein may be targeted by antibodies, especially if unglycosylated (Berzofsky, 1985; Van Regenmortel, 1996). If so, then perhaps the region of the Ana o 2 solvent-exposed monomer surface not showing mapped epitopes (i.e., the region to the right of the 2B5 epitope in Fig. 6, top image) is shielded by segments of the 11S globulin that were not resolved in the atomic structure of Gly m 6 used in our modeling and/or they may contain other, as yet, unidentified linear or conformational epitopes.

In summary, we have used a molecular genetic approach to generate chimeras composed of cashew Ana o 2, which expresses the labile 2B5 conformational epitope, in combination with a non-target (soybean) 11S globulin homologue, Gly m 6, as well as Ana o 2 deletion and point mutation constructs, to fine map the 2B5 discontinuous epitope. The position of the epitope, as predicted by molecular modeling, was confirmed by electron microscopy of mAb 2B5-nAna o 2 immune complexes. The surface location and structural characteristics of the 2B5 epitope provide likely explanations for its unique attributes. The use of murine IgG mAbs to probe for and identify conformational epitopes on allergens is a useful approach but further assessment using patient IgE directly will be needed to fully define the nature of conformational epitopes on this and other tree nut allergens.

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