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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.

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) nonfat 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