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Characterization of a cashew allergen, 11S globulin (Ana o 2), conformational epitope

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ABSTRACT

Both linear and conformational epitopes likely contribute to the allergenicity of tree nut allergens, yet, due largely to technical issues, few conformational epitopes have been characterized. Using the well studied recombinant cashew allergen, Ana o 2, an 11S globulin or legumin, we identified a murine monoclonal antibody which recognizes a conformational epitope and competes with patient IgE Ana o 2-reactive antibodies. This epitope is expressed on the large subunit of Ana o 2, but only when associated with an 11S globulin small subunit. Both Ana o 2 and the homologous soybean Gly m 6 small subunits can foster epitope expression, even when the natural N-terminal to C-terminal subunit order is reversed in chimeric molecules. The epitope, which is also expressed on native Ana o 2, is readily susceptible to destruction by physical and chemical denaturants.

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1. Introduction

Immunoglobulin (Ig)-binding epitopes on proteins are typically divided into two categories, linear (continuous) and conformational (discontinuous) though some epitopes appear to be a blend of the two forms (Barlow et al., 1986; Van Regenmortel and Pellequer, 1994; Schramm et al., 2001; Roggen, 2006; Gershoni et al., 2007), and even the shortest linear epitopes probably adopt, at least transiently, a defined configuration upon interaction with an antibody complementarity-determining region (CDR) (Aalberse, 2000). Linear epitopes typically derive from short contiguous 8–10 amino acid (aa) segments whereas conformational epitopes, as the name implies, require a defined 3D peptide conformation dependent upon the scaffolding of the native protein which often (though not necessarily) is comprised of discontinuous segments brought

into close proximity as a consequence of peptide/protein folding. The technology for identifying linear epitopes is rather straightforward and is typically performed by reacting the antibodies of interest with enzymatically derived or cloned peptide fragments or sets of short (12–20 aa) overlapping peptides encompassing the sequences of interest (i.e., peptide scanning) (Geysen et al., 1984, 1987; Gershoni et al., 2007). The identification and characterization of conformational epitopes, in contrast, is technically more challenging, requiring large scale mutagenesis, chimeric molecule production, phage display, deuterium exchange footprinting, or ideally, X-ray crystallography of immune complexes (Gershoni et al., 2007). Consequently, scant attention has been paid to the latter even though it is generally believed that the majority of binding sites on protein antigens are conformational (Barlow et al., 1986; Roggen, 2006; Gershoni et al., 2007).

Studies on IgE binding to allergens mimic this pattern with relatively few focusing on conformational epitopes (Aalberse, 2000; Untersmayr and Jensen-Jarolim, 2006). The recent publication of the atomic structure of Phl p 2, a grass pollen allergen, in complex with a human IgE Fab targeting a conformational epitope is a notable exception (Padavattan et al., 2009). It has been suggested that a differential responsiveness to linear versus conformational epitopes in allergic patients is of considerable clinical and diagnostic significance (Nowak-Węgrzyn, 2003; Shreffler et al., 2004). For

Abbreviations: mAb, monoclonal antibody; ALG, Ana o 2 large subunit; ASM, Ana o 2 small subunit; GLG, Gly m 6 large subunit; GSM, Gly m 6 small subunit; RT, room temperature; NC, nitrocellulose; TBS, tris-buffered saline; BSB, buffered saline borate; aa, amino acid; bp, base pair.

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example, children who outgrow allergies to ovomucoid (a major allergen in egg) and casein in cow's milk may develop IgE to mostly conformational epitopes whereas those that remain allergic into adulthood typically target linear epitopes (Cooke and Sampson, 1997; Vila et al., 2001). It has been argued that most food allergen epitopes are linear (Bannon, 2004), however, there are clear exceptions in which linear epitopes, though present, appear to play no role in IgE-mediated effector cell triggering (Albrecht et al., 2009). At a more fundamental level, the process by which the immune system targets specific epitopes is still largely unknown. With regard to allergy, the roles that epitope configuration and presentation to B cells in the induction phase, and IgE-charged mast cells and basophils in the effector phase, play in cell triggering need elucidation (Untersmayr and Jensen-Jarolim, 2006). Certainly, issues relating to epitope stability during food processing, digestion and absorption following consumption, and the ability of IgE to cross-link are key factors that must also be considered (Husby et al., 1986; Sampson, 2004; Untersmayr and Jensen-Jarolim, 2006). However, such studies are predicated on the demonstration of IgE binding to conformational epitopes in the native protein.

In this study we characterize the binding of a murine monoclonal antibody (mAb) targeting a conformational epitope on the cashew nut 11S globulin allergen, Ana o 2 (anacardein, a legumin), and demonstrate that it competes with IgE from cashew-sensitive patient sera. The 11S globulins of the tree nuts display several characteristics which make them a good choice for such studies. They are found in abundance in many tree nuts and are reactive with IgE from a majority of tested nut allergic-patients (reviewed in Roux et al., 2003; Sathe et al., 2005). Moreover, linear epitope maps have been generated for several of these nut 11S globulins, including those from cashew (Wang et al., 2003), walnut (Robotham et al., 2009), and hazelnut (Robotham et al., 2009), as well as from certain oilseed legumes such as peanut (Rabjohn et al., 1999; Rouge et al., 2009) and soybean (Zeece et al., 1999; Helm et al., 2000). The 3D structure of cashew 11S globulin has recently been homology-modeled (Barre et al., 2007; Robotham et al., 2009) based upon extrapolation from the X-ray structure of the soybean 11S globulin (glycinin) (Adachi et al., 2003). The atomic structures of the peanut Ara h 3 and almond prunin-1 homologues have also been recently published and are shown to closely resemble that of soybean (Jin et al., 2009a,b).

The higher order configuration of the 11S globulins presents some interesting features that could impact conformational epitopes. The naturally occurring form of this class of allergens is typically a hexamer of heterodimers, each consisting of a large (acidic) and a small (basic) subunit. The hexamer is arranged as two ring-shaped trimers associated face-to-face (IE face to IE face)

with the two IA faces exposed to the solvent (Shewry et al., 2003; Adachi et al., 2003; Jin et al., 2009b). The mature hexameric protein is derived by enzymatic cleavage of pre-protein consisting of contiguous large (acidic) and small (basic) subunits, typically arrayed in a trimeric configuration. Consequently, it is likely that at least some conformational epitopes are dependent upon the associations between these polypeptide components.

To test this hypothesis we created a set of recombinant molecules in which the large and small subunit genes of cashew Ana o 2 and soybean Gly m 6 were grafted in various combinations. Isolated individual domains were also produced. The recombinant molecules were then tested for reactivity with cashew-allergic patients' serum IgE and a panel of mouse anti-Ana o 2 IgG mAbs in ELISA and dot blot assays under non-denaturing and denaturing conditions. We found that one of the mAbs bound to a conformational epitope expressed on the large subunit Ana o 2. However, expression of this epitope is dependent upon large subunit association with either an Ana o 2 or Gly m 6 small subunit and is susceptible to loss through denaturation. This mAb can compete with patient IgE for binding to Ana o2.

2. Methods

2.1. Experimental design

For reference, an atomic model of native 11S globulin (soybean Gly m 6 (Adachi et al., 2003)) showing the arrangement of the N-terminal large (LG) and C-terminal small (SM) subunits is illustrated in (Fig. 1A). To test for the presence of conformational 11S globulin epitopes that are dependent upon subunit association, a panel of murine anti-Ana o 2 mAbs were used to probe several combinations of large and small subunits from Ana o 2 and its 11S globulin soybean homologue, Gly m 6, as illustrated in Fig. 1B–M. These constructs included intact recombinant (r)Ana o 2 (contiguous N-terminal large (ALG) and C-terminal small (ASM) subunits) (Fig. 1C), similarly arranged Gly m 6 subunits (GLG and GSM) (Fig. 1A), and chimeras of both proteins in the conventional N-terminal LG–C-terminal SM orientation (Fig. 1D, E). In addition, each of the above molecules and chimeras were also expressed wherein N-terminal to C-terminal order of the LG and SM subunits was reversed (Fig. 1F–I) (i.e., SM–LG rather than LG–SM). Unassociated LG and SM subunits from both Ana o 2 and Gly m 6 were also tested (Fig. 1J–M).

The reactivity of these various constructs was also compared to native (n)Ana o 2 and nGly m 6. In all cases, the recombinant molecules were expressed as maltose-binding protein (MBP) fusion molecules since attempts to enzymatically remove the fusion partner lead to irreversible protein aggregation (data not shown).

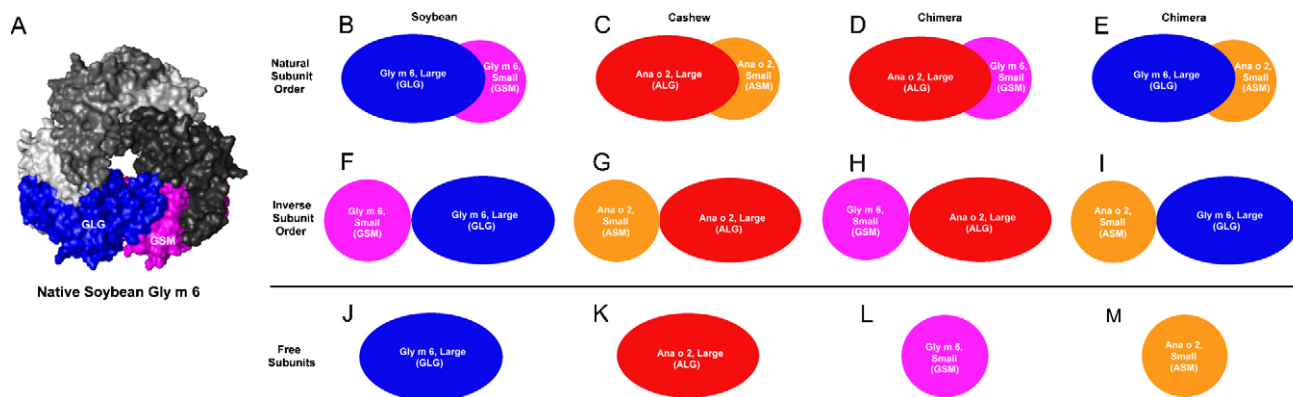


Fig. 1. Atomic model of soybean 11S globulin (Gly m 6, PDB ID# 1od5 (Adachi et al., 2003)) (A) and schematic representation of the various 11S globulin constructs tested (B–M). In (A), one solvent-exposed face of the hexamer (containing three heterodimeric subunits) is shown. One of the three Gly m 6 monomers is color coded (large, (GLG) subunit, blue; small subunit (GSM) mauve) and the large subunits are dark gray and the small subunits are light gray on the remaining two monomers. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

2.2. Generation of recombinant genes

A previously described (Wang et al., 2002), recombinant pMAL-c2-His vector containing the full-length Ana o 2 gene was used as a template for PCR-based cloning of the large and small subunit genes. The target coding sequences were modified by the addition of restriction sites at the 5' and 3' ends during the PCR reactions using *PfuTurbo* DNA polymerase (Stratagene, Cedar Creek, TX, USA) and the appropriate primers (Table 1). This same amplification strategy was employed using the soybean Gly m 6 11S globulin gene (GenBank accession #X15121), graciously provided in a pET28 bacterial expression vector (Invitrogen Corp., Carlsbad, CA, USA) by Dr. Rudolf Jung (Pioneer Hi-Bred Intl. Inc., Johnston, IA), to generate full-length rGly m 6 protein as well as its independent large and small subunits. Stop codons were introduced into both the Ana o 2 and Gly m 6 large subunit reverse primers (Table 1; A2L-R and G6L-R) where appropriate. All PCR products were digested using PstI and HindIII and the appropriate reaction buffers (Invitrogen) according to the manufacturer's instructions. Digested products were purified using a QIAquick Gel Extraction Kit (Qiagen Inc., Valencia, CA, USA) and ligated with T4 DNA Ligase H.C. (Invitrogen) to their respective sites of the pMAL-c2-His expression vector.

The various Ana o 2/Gly m 6 chimeric constructs and deletion mutants were generated by blunt-end ligation of Platinum *Pfx* DNA polymerase (Invitrogen)-amplified PCR products. These products then served as full-length templates for *Pfu Turbo* DNA polymerase amplification in subsequent PCR reactions.

2.3. Subcloning of constructs

PCR products were purified using the QIAquick PCR Purification Kit (Qiagen) and digested using PstI and HindIII and the appropriate reaction buffers (Invitrogen) according to the manufacturers instructions. Digested products were purified using a QIAquick Gel Extraction Kit (Qiagen) and ligated with T4 DNA Ligase H.C.

(Invitrogen) to their respective sites of the restriction enzyme-cut pMAL-c2-His expression vector. Sequences were checked for identity to their respective templates using the BLAST program after both strands of the transfected vectors were sequenced as previously described (Wang et al., 2002).

2.4. Expression and purification of MBP-fusion proteins

Expression and purification of all cDNA/pMAL-c2-His plasmids was carried out as previously described (Wang et al., 2002).

2.5. Monoclonal antibody production

Cashew nut protein-reactive mAbs were generated by immunizing BALB/c mice with soluble protein extract in the Hybridoma Core Facility at Florida State University according to standard procedures as previously described (Venkatachalam et al., 2008). Hybridoma culture supernatants were screened for reactivity to whole cashew extract using ELISAs. Those mAbs found to be reactive were then checked for reactivity to nAna o 2 and rAna o 2 in subsequent ELISAs using plates coated with 5 µg/ml of the protein as previously described (Venkatachalam et al., 2008).

2.6. Human sera

Blood samples were drawn after informed consent from patients with a history of life-threatening systemic reactions to cashew nuts and the sera frozen at −70 °C until use. The study was approved by the institutional review board of the University of California at Davis. The presence of cashew-reactive IgE was confirmed by ImmunoCAP assay (Phadia AB, Uppsala, Sweden) or by Western immunoblotting. Control serum was obtained from a patient with a history of asthma and a high IgE titer who reported tolerance to cashews. The two patients chosen for study were those that reacted most strongly with nAna o 2 and rAna o 2 on Western blot (Wang

Table 1

Primers used for the amplification and subcloning of cashew and soybean 11S globulin genes, constituent subunits, and chimeras.

Gene	Primer Name: Primer Sequence(5'–3')	Amplicon
Ana o 2	A2L-F: AA <u>ACTG</u> CAGTCTCGCCAGGAATGGCAAC A2S-R: CCCAAGCTTTTAAGCATCATCCCTCATGTGG	Full length Ana o 2 (ALG–ASM)
Ana o 2	A2L-F: AA <u>ACTG</u> CAGTCTCGCCAGGAATGGCAAC A2L-R: CCCAAGCTTTTAATGTACGCTGTCCCATC	Ana o 2 large subunit (ALG)
Ana o 2	A2S-F: AA <u>ACTG</u> CAGGGGATTGAAGAGACCATTTGC A2S-R: CCCAAGCTTTTAAGCATCATCCCTCATGTGG	Ana o 2 small subunit (ASM)
Gly m 6	G6L-F: AA <u>ACTG</u> CAGTCCAGAGAGCAGCCTCAGC G6S-R: CCCAAGCTTCTAAGCCACAGCTCTCTCTG	Full length Gly m 6 (GLG–GSM)
Gly m 6	G6L-F: AA <u>ACTG</u> CAGTCCAGAGAGCAGCCTCAGC G6L-R: CCCAAGCTTCTAATTCTCTCTGCTTTGCTTTGGC	Gly m 6 large subunit (GLG)
Gly m 6	G6S-F: AA <u>ACTG</u> CAGGGCAATTGACGAGACCATATGC G6S-R: CCCAAGCTTCTAAGCCACAGCTCTCTCTG	Gly m 6 small subunit (GSM)
Ana o 2	A2L-F: AA <u>ACTG</u> CAGTCTCGCCAGGAATGGCAAC P*-A2L-R: TTAATTGTACAGCTGTCCCCATC	P*-ALG
Gly m 6	P*-G6S-F: GGCAATTGACGAGACCATATGC G6S-R: CCCAAGCTTCTAAGCCACAGCTCTCTCTG	P*-GSM
Gly m 6	G6L-F: AA <u>ACTG</u> CAGTCCAGAGAGCAGCCTCAGC P*-G6L-R: CTAATTCTCTCTGCTTTGCTTTGGC	P*-GLG
Ana o 2	P*-A2S-F: GGGATTGAAGAGACCATTTGC A2S-R: CCCAAGCTTTTAAGCATCATCCCTCATGTGG	P*-ASM
Ana o 2	A2S-F: AA <u>ACTG</u> CAGGGGATTGAAGAGACCATTTGC *P-A2S-R: AGCATCATCCCTCATGTGGTGCTT	*P-ASM
Gly m 6	*P-G6L-F: TCCAGAGAGCAGCCTCAGCAAAAC G6L-R: CCCAAGCTTCTAATTCTCTCTGCTTTGCTTTGGC	*P-GLG
Gly m 6	G6S-F: AA <u>ACTG</u> CAGGGCAATTGACGAGACCATATGC *P-G6S-R: AGCCACAGCTCTCTCTGAGACTCTGA	*P-GSM
Ana o 2	*P-A2L-F: TCTCGCCAGGAATGGCAACAACA A2L-R: CCCAAGCTTTTAATGTACGCTGTCCCATC	*P-ALG

Restriction sites underlined; CTGAG: Pst I, AAGCTT: Hind III. F: forward primer, R: reverse primer. P*: 5'-phosphorylated primers and resultant amplicons used to create naturally ordered Ana o 2 and Gly m 6 chimeras via blunt end ligation. *P: 5'-phosphorylated primers and resultant amplicons used to create inverse ordered Ana o 2 and Gly m 6 chimeras via blunt end ligation.

et al., 2003) and were unreactive with soybean (data not shown). Patient #8 is a 43-year-old female with severe allergic responsiveness to cashew from age 1 with an ImmunoCAP® score of 41.9 KU/L. She is also clinically allergic to other tree nuts (unspecified) and peanuts. Patient #9 is a 35-year-old female with severe allergic responsiveness to cashew from age 2 with a cashew-specific ImmunoCAP® score of 35.1 KU/L. She is also clinically allergic to walnuts, pecans, hazelnuts, and almonds.

2.7. Purification of native Ana o 2 from cashew nut extract

To compare the fusion protein reactivity of the mAbs with that of native Ana o 2, the 11S globulin fraction was purified from cashew nut extract as previously described (Sathe et al., 1997), by using a Superdex 200 column (GE Healthcare, Waukesha, WI, USA) and high performance liquid chromatography (HPLC, Pump 126, UV Detector 168, Beckman Coulter, Fullerton, CA, USA). The protein eluted as a single peak and NDND-PAGE gels of the purified native Ana o 2 indicated >90% purity (data not shown).

2.8. Dot blot screening of proteins with Ana o 2-specific mAbs

Each mAb was screened for reactivity to nitrocellulose (NC) strips (0.2 µm, NC, Schleicher & Schuell, Keene, NH, USA) containing 0.2 µg of dotted fusion proteins, 6.0 µg whole cashew extract, 2.0 µg of purified native Ana o 2, and 0.1 µM of MBP (New England Biolabs, Beverly, MA, USA). Dotted membranes were blocked overnight at 4 °C in 5% non-fat dry milk in TBS-T (20 mM Tris, 137 mM NaCl, 0.2% Tween 20, pH 7.6) and cut into strips containing the desired antigen array. The membranes were incubated for 1 h at room temperature (RT) with mAbs diluted 1:50–1:2500, depending on signal intensity observed in previous assays. Three 15-min washes followed by three 5-min washes with TBS-T were performed. Membranes were then probed with an HRP-labeled goat-anti-mouse-IgG (Jackson ImmunoResearch, West Grove, PA, USA) for 1 hr at RT and washed as described above. Reactive dots were identified after a 5 min incubation in ECL Plus (GE Healthcare), prepared following the manufacturer's instructions, and subsequent exposure to Kodak X-OMAT X-ray film (Kodak, Rochester, NY, USA).

2.9. Assay for conformationally labile epitopes

Linear and conformational epitope-binding mAb candidates were chosen based on our previously reported SPOTs analysis and Western blot screening of the fusion proteins (Wang et al., 2003; Venkatachalam et al., 2008) as described below. Each of 20 mAbs was used to probe native and variously denatured proteins. Denatured proteins were produced by incubation at RT for 10 min in BSB containing either 0.1–10% SDS, 0.25–1.0 M β-mercaptoethanol (ME), or 0.1–6.0 M urea, or by boiling in BSB for 10 min. Samples were dotted onto NC for use in blotting assays as described above. For native proteins, 0.5 µg in 100 µl of whole cashew BSB extract or purified nAna o 2 samples were used per dot as described above. Undenatured, control proteins were maintained at RT in BSB devoid of denaturing agents for a comparable period of time prior to blotting.

2.10. Mouse mAb and patient IgE inhibition ELISAs

The ability of mouse IgG mAbs to inhibit Ana o 2-specific IgE reactivity was assessed by inhibition ELISA. Plates were coated with 20 µg/ml of rAna o 2 in coating buffer (0.1 M NaHCO₃, pH 9.6) for 2 h at 37 °C, blocked in 5% non-fat dry milk in PBS-T (PBS, 0.2% Tween 20) for 1 h at 37 °C and washed three times with PBS-T. 50 µl of each undiluted mAb culture supernatant was then

added per well and incubated overnight at 4 °C. For controls, a non-Ana o 2 binding mAb, 3G1 (walnut 2S albumin specific) and PBS-T were used as inhibitors. After three 5 min washes with PBS-T the plates were incubated with Ana o 2-reactive human sera, diluted 1:20 (v/v) in blocking buffer, overnight at 4 °C. After washing as above, HRP-labeled mouse-anti-human-IgE (Zymed Labs, San Francisco, CA, USA) was diluted 1:1000 (v/v) in blocking buffer and incubated for 1 h at 37 °C. Detection was achieved by incubation with an o-phenylenediamine tablet (Zymed Labs) diluted to a working concentration of 0.4 mg/ml in substrate buffer (0.1 M citrate phosphate buffer, pH 5.0) for 10 min. Plates were read at 495 nm using a µQuant ELISA reader (Bio-Tek Instruments, Highland Park, VT, USA) and accompanying software according to the manufacturer's instructions. All assays were repeated at least in triplicate.

2.11. Dot blot inhibition with human sera of mAb binding

To show inhibition between the mouse mAb IgG and human polyclonal IgE, dot blot inhibition assays were performed wherein dotted proteins were blocked as described above and pre-incubated with patients' sera diluted 1:10 (v/v) in blocking buffer overnight at 4 °C. Membranes were then washed with TBS-T and re-probed with the appropriate mAb culture supernatant diluted 1:10,000 (v/v) in TBS-T. A non-Ana o 2 binding mAb (walnut 2S albumin specific) was used as a negative control inhibitor. Direct human-IgE binding to the dotted proteins was also performed after blocking by blotting with patients' sera, washing with TBS-T, and probing with HRP-labeled mouse-anti-human-IgE Ab (Zymed Labs) diluted 1:5000 (v/v). Reactive dots were identified after a 5-min incubation in ECL Plus chemiluminescent reagent (GE Healthcare) as described above.

3. Results

3.1. Ana o 2 and Gly m 6 11S globulin, chimeric and subunit genes

The 1332 base pair (bp) open reading frame of the Ana o 2 gene, excluding the leader sequence, encoding a 443 aa protein (Wang et al., 2003) was amplified as described above. Similarly, a 771 bp fragment corresponding to the 257 aa large subunit and a 558 bp fragment corresponding to the 186 aa small subunit of Ana o 2 were also amplified. The leader sequence-deleted 1425 bp open reading frame-encoding the 475 aa 11S globulin Gly m 6 (GenBank accession #X15121) (Sims and Goldberg, 1989) (allergen designation Gly m 6) (Holzhauser et al., 2009) was similarly amplified as was the 867 bp fragment corresponding to the 289 aa large subunit and the 558 bp fragment corresponding to the 186 aa small subunit. The deduced aa sequence alignment of the two 11S globulins, shown in (Fig. 2), revealed 45% identity and 63% similarity overall, with 46% identity and 65% similarity between the two large subunits, and 52% identity and 71% similarity between the two small subunits.

To determine the subunit specificity of the Ana o 2-specific mAbs (described below), we created two chimeric molecules each possessing the large subunit from one allergen (Ana o 2 or Gly m 6) associated with the small subunit of the other allergen as depicted in Fig. 1B–E. As controls, we also produced chimeras with inverted subunit order (GSM–ALG and ASM–GLG) (Fig. 1F–I) and free (unassociated) Ana o 2 and Gly m 6 large and small subunits (Fig. 1J–M).

3.2. Expression and purification of MBP-fusion proteins

Fusion proteins were expressed and purified as previously described (Wang et al., 2002). Yields ranged from 5 to 50 mg/L of cultured bacteria. Attempts to enzymatically cleave and remove the MBP fusion partner typically resulted in insoluble protein aggre-

Ana o 2	1	LSVCFLLILFHGCLA-SRQEWQQQDECQIDRLDALEPDNRVEYEAGTVEAWDPNHEQFRCA	59
Gly m 6	6	S+CFL+ C A S +E QQ+ECQI +L+AL+PDNR+E E G +E W+PN++ F+CA	65
Ana o 2	60	FSLCFLLFSGCCFAFSSRQEPQQNECQIQKLNALKPDNRIESEGGLIETWNPNNKPFQCA	119
Gly m 6	66	GVALVRHTIQPNGLLLPQYSNAPQLIYVVQGEGMTGISYPGCPETYQAPQQGRQQGQSGR	125
Ana o 2	120	GVAL R T+ N L P Y+N PQ IY+ QG+G+ G+ YPGCP T++ PQQ +Q+GQS R	179
Gly m 6	126	GVALSRCTLNRRNALRRPSYTNGPQEIIYIQQKGIFGMIYPGCPSTFEFPQQPQQRGQSSR	185
Ana o 2	180	FQDRHQKIRRFRRGDIIAIPAGVAHWCYNEGNSPVVTVTLTLDVSNSQNQLDRTPRKFHLA	224
Gly m 6	186	QDRHQKI FR GD+IA+P GVA W YN ++PVV V+++D ++ +NQLD+ PR+F+LA	245
Ana o 2	225	PQDRHQKIYNFREGDLIAVPTGVAWWMYNNEDTPVVAVSIIDTNSLENQLDQMPRRFYLA	254
Gly m 6	246	GNPKDVFQQQQ-----HQSR-----GRNLFSGFDTELLAEAFQVDERLIKQLKS	304
Ana o 2	255	GN + F + QQ HQS+ G ++ SGF E L AF VD+++ K L+	314
Gly m 6	305	GNQEQLFKYQQEQGGHQSQKGKHQEEENEGGSILSGFTLEFLEHAFSVDKQIAKNLQG	353
Ana o 2	315	ED---NRGGIVKVKDELVRIRP-----SRSQSERGSE	374
Gly m 6	354	E+ ++G IV VK L VI+P Q RGS+	413
Ana o 2	375	ENEGEDKGAIVTVKGG-LSVIKPPTDEQQQRPQEEEEEEDEKPKQCKGKDKHCQRPRGSQ	434
Gly m 6	414	SEEESEDEKRRWGQRDNGIETICTMRLKENINDPARADIYTPVGRLTTLNSLNLPIK	478
Ana o 2	435	S+ R NGI+ETICTMRL+ NI + DIY P+ G +TT SL+ P L	
Gly m 6	474	SKS-----RRNGIDETICTMRLRHNIQTSSPDIYNPQAGSVTTATSLDFPALS	
Ana o 2	315	WLQLSVEKGVLYKNALVLPWNLSHSIIYGCKGKGQVQVDNFGNRFVDGEVREGQMLV	
Gly m 6	354	WL+LS E G L KNA+ +PH+NLN++SIIY G+ +QVV+ G RVFDGE++EG++L+	
Ana o 2	375	WLRLSAEFGSLRKNAMEFVPHYNLANSIIYALNGRALIQVVCNGERVFDGELQEGRVLI	
Gly m 6	414	VPQNFV R++ + FE++SFKTND M LAG S+L +PEEV+ + F + + AR+	
Ana o 2	435	VPQNFVVAARSQSDNFEYVSFKTNDTPMIGTLAGANSLNALPEEVIQHTFNLKSQQARQ	
Gly m 6	474	IKFNN 439	
		IK NN	
Gly m 6	474	IKNNN 478	

Fig. 2. Sequence alignment of Ana o 2 and Gly m 6 11S globulins. Large subunits are colored red and small subunits blue. Identical amino acids are shown, (+) indicates similar residues, and (–) indicates gaps assigned through BLAST alignment. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

gates. Consequently all analyses were carried out on the intact fusion protein.

3.3. Monoclonal antibody (mAb) production and Ana o 2 reactivity

Twenty mAbs were identified as being cashew extract-reactive when screened by ELISA (data not shown). Of these, eight showed varying degrees of reactivity to native and recombinant Ana o 2 in subsequent ELISAs (data not shown). We chose the five most strongly reactive mAbs (2B5, 4C3, 4H9, 1F5, and 5G10) for further characterization.

3.4. Dot blot screening of Ana o 2 and Gly m 6 11S globulin, chimeric constructs, and isolated subunits

Eight fusion proteins were tested for mAb reactivity: full-length rAna o 2 and rGly m 6 11S globulin, independently expressed Ana o 2 and Gly m 6 large (LG) and small (SM) subunits, and the Ana o 2 LG–Gly m 6 SM and Gly m 6 LG–A2 SM chimeras. Each was probed for reactivity with the five selected Ana o 2-reactive mAbs in a dot blot assay (Fig. 3A). Four of the five mAbs bound to all Ana o 2 LG subunit-containing constructs (rAna o 2 full length, rAna o 2 LG, and rAna o 2 LG–Gly m 6 SM chimera) and none bound in a pattern consistent with reactivity to rAna o 2 SM (or Gly m 6 LG or SM). One mAb, 2B5, however, recognized the full length rAna o 2 fusion protein and the rAna o 2 LG–Gly m 6 SM chimeric molecules but not the free rAna o 2 LG subunit. These data indicated that mAb 2B5 binds to an Ana

o 2 large subunit conformational epitope that is not displayed on the free rAna o 2 subunit but which is correctly configured through association with a homologous (soybean Gly m 6) small subunit. None of the mAbs bound to the full-length rGly m 6 11S globulin, or its independently expressed constituent subunits, nor did they bind to the MBP fusion partner. The negative control mAb, 3G1, which is specific for walnut 2S albumin, also did not bind any of the dotted fusion proteins as anticipated. Because mAb 2B5 binds both the mature native Ana o 2 (data not shown) and the recombinant form, which represents the uncleaved precursor protein (contiguous LG and SM subunits), any conformational changes associated with *in vivo* plant tissue protein processing (i.e., posttranslational cleavage into large and small subunits during nut maturation) do not significantly influence expression of the 2B5 epitope.

Together, the above data reveal a large subunit epitope that is conformationally dependent upon association with an 11S globulin small subunit. However, neither simple mixing and co-incubation of the ALG–MBP and ASM–MBP constructs nor premixing the two and co-digesting them with thrombin to free them from their MBP fusion partners resulted in a 2B5 reactive complex (data not shown). As an additional control we reversed the order of the large and small subunits creating a set of recombinant molecules with an N-terminal small subunit and C-terminal large subunit, fully anticipating that these constructs would not react with the 2B5 mAb. The four “tail-head” chimeric proteins were: GSM–GLG, ASM–ALG, GSM–ALG, and ASM–GLG (depicted in Fig. 1F–I). We were surprised when those constructs containing the cashew 11S globulin large subunit (ASM–ALG and GSM–ALG, arrowheads

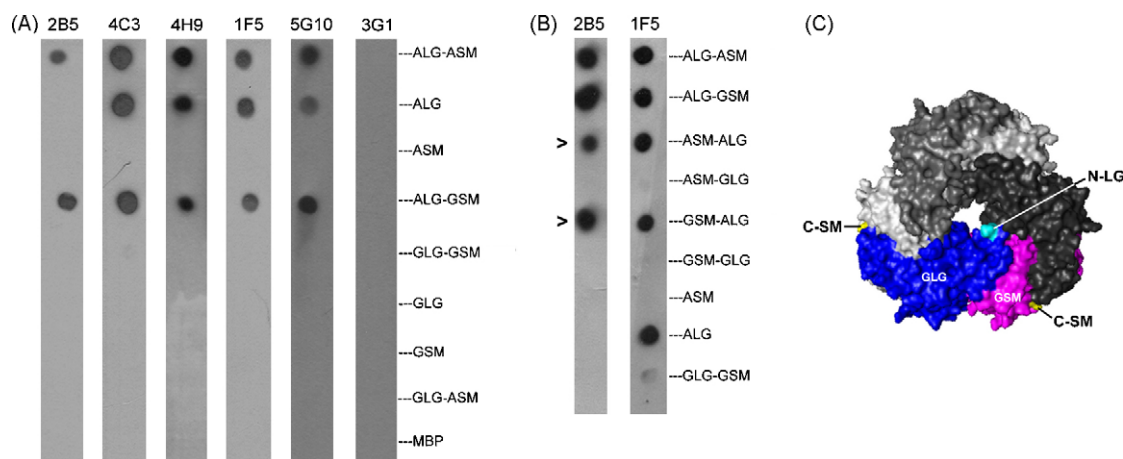


Fig. 3. MAb dot blotting of (A) rAna o 2 full length protein (ALG–ASM) and rGly m 6 full-length protein (GLG–GSM), independently expressed subunits, chimeric fusion proteins, and (B) inverse order (tail–head) proteins. MAbs are identified above each strip and the dotted recombinant proteins to the right. Ana o 2 large (ALG) and small (ASM) and Gly m 6 large (GLG) and small (GSM) subunits in natural order (LG–SM), inverse order (SM–LG) and as free subunits (LG and SM) are indicated. Arrowheads in (B) indicate reverse order constructs containing the cashew 11S globulin large subunit (ASM–ALG and GSM–ALG). MBP in (A) serves as a negative control (all constructs are expressed with associated MBP) as does mAb 3G1 (a mAb walnut 2S albumin). Panel (C) shows the relative locations of the C-terminal aa of the small (yellow, C-SM) and the N-terminal aa of the large (cyan, N-LG) subunits in native Gly m 6. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

in Fig. 3B) demonstrated significant 2B5 reactivity. Because the C-terminal of the SM subunits (C-SM) and the N-terminal of the LG subunits (N-LG) of 11S globulins are distant from each other in the native molecule, as illustrated in Fig. 3C, steric constraints prevent the small and large subunits of each inverted monomeric form from correctly self associating or even from associating with neighboring subunits within a regular trimeric/hexameric configuration. Consequently, our data imply that the large and small subunit of each inverted-order chimera can associate with the small and large subunits, respectively, of other inverted monomers to form structurally complete monomers, though not regular trimers or hexamers, and thus regenerate the 2B5 conformational epitope. Column chromatography size exclusion data indicate molecular configurations in excess of 200 kDa predominate in the expressed inverted chimeric protein preparations, which are consistent with multimerization (data not shown).

3.5. Mouse mAbs recognize epitopes targeted by patient anti-cashew IgE

To determine if the epitope region targeted by 2B5 is also recognized by patient IgE, an inhibition ELISA was performed. A second mAb, 1F5, was chosen as a non-conformational control. 1F5 not only bound to isolated rAna o 2 large subunit but had previously been shown to bind a synthetic 15 amino acid long peptide homologous to the C-terminal 15 aa segment of the large subunit in SPOTs assays (peptide 257EESEDEKRRWGQRDN271), a peptide also reactive with patient IgE (Wang et al., 2003; Venkatachalam et al., 2008). Both the linear epitope-binding (1F5) and conformational epitope-binding (2B5) mAbs could block IgE binding sites when pre-incubated with rAna o 2-coated ELISA plates prior to probing with the patient (#8 and #9) anti-cashew sera (Fig. 4). Modest inhibition (~16–19%) is observed in both patients when the 2B5 mAb was used as an inhibitor and 1F5 inhibited to a greater extent (~37–45%). Significant inhibition was not observed when mAb 3G1 (anti-walnut 2S albumin) was used as a negative control.

3.6. Patients' sera inhibit mAb 2B5 binding

A dot blot inhibition assay was used to corroborate the ELISA data. The results showed that pre-incubation of 2B5-reactive proteins with sera from patient #8 or #9 prior to probing with 2B5, greatly reduced or eliminated 2B5 binding (Fig. 5).

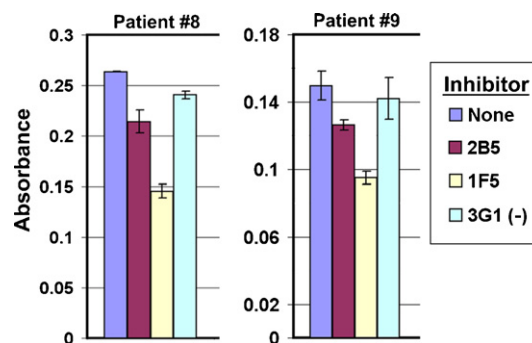


Fig. 4. Inhibition of Ana o 2-specific human IgE with mAbs 2B5 and 1F5. Inhibition of IgE from patient #8 and #9 with mouse mAbs to linear (1F5) and conformational (2B5) epitopes of rAna o 2.

3.7. The conformational epitope targeted by mAb 2B5 is sensitive to denaturation

To further assess the nature of the apparent conformational epitope, mAb 2B5 was tested in dot blot assays for binding to

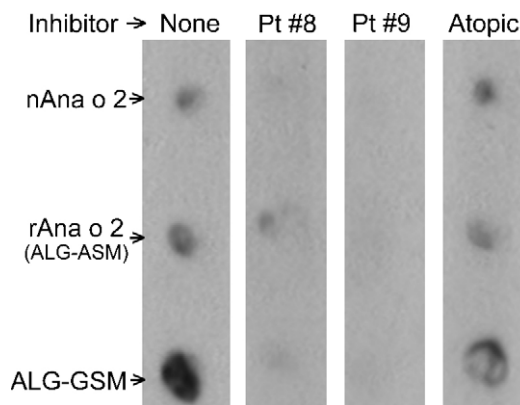


Fig. 5. Inhibition of mAb 2B5 reactivity by sera from two cashew-sensitive Ana o 2-reactive patients. Strip 1 (none), binding of mAb 2B5 to HPLC-purified native (n)Ana o 2, rAna o 2 (ALG–ASM), and ALG–GSM chimera. Similarly dotted strips were preincubated with sera from patients (Pt) #8, #9, and a non-cashew sensitive atopic patient (Atopic) prior to incubation with mAb 2B5.

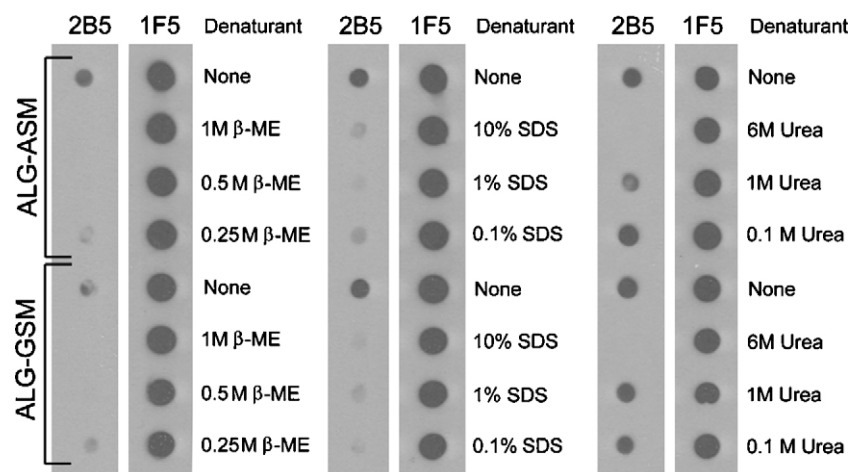


Fig. 6. Dot blot showing the binding of mAbs 2B5 and 1F5 to rAna o 2 (ALG-ASM) and the ALG-GSM chimera under various denaturing conditions.

rAna o 2 and rAna o 2 LG-Gly m 6 SM that had been subjected to various denaturants (Fig. 6). The results showed that the linear epitope-binding mAb 1F5 was not affected by any of the denaturing conditions. Binding of 2B5 to the recombinant allergens, however, was lessened and in some cases completely eliminated when samples were treated at RT with various concentrations of several widely used denaturing agents (0.25, 0.5, and 1.0 M β-ME; 0.1%, 1.0%, and 10% SDS; 1.0 M and 6.0 M urea) prior to blotting. In addition, boiling for 10 min in the absence of chemical denaturants prior to immunoblotting led to a complete loss of 2B5 binding to rAna o 2 and rAna o 2 LG-Gly m 6 SM fusion proteins (data not shown). Boiling did not, however, destroy the mAb 1F5-specific linear epitope (data not shown). Because the recombinant constructs are in the form of proproteins (i.e., single chain LG-SM polypeptides), simple dissociation of the two subunits, as might occur in the native protein, would not explain the loss of the 2B5 epitope.

Binding of 2B5 and 1F5 to native Ana o 2 subjected to similar denaturing conditions gave results comparable to those shown in Fig. 6 for the recombinant forms (data not shown) further demonstrating a conformational similarity between the native and recombinant molecules with respect to these epitopes.

4. Discussion

To fully understand the molecular basis of type I allergic immune responses, identification of conformational IgE-binding epitopes on the surface of allergen molecules is required. Unfortunately, because the technology for identifying linear epitopes is considerably more straightforward than the characterization of conformational epitopes, rather little attention has been paid to the latter (Bredehorst and David, 2001; Robotham et al., 2002). During the last decade, for example, the IgE-binding epitopes of various allergens have been typically identified by assessing reactivity to short (usually 8–15 amino acid) overlapping peptides, a method restricted to the identification of linear IgE-binding epitopes (Gershoni et al., 2007).

Despite this focus, currently available data from a variety of structural studies suggest that most B-cell epitopes on proteins are conformational (Roggen, 2006) and there is no reason to believe that allergen interaction with IgE is an exception. For example, Neudecker et al. (2003) found that a single amino acid mutation which dramatically disrupted the conformation of the cherry pathogenesis-related protein, Pru av 1, could completely eliminate IgE binding (Neudecker et al., 2003). The fact that most food allergens, with the notable exception of many of those involved in pollen-food syndrome allergens, are highly resistant to denatura-

tion and gastric digestion suggests that 3D surface properties rather than cryptic linear peptides should constitute the bulk of the cognate epitopes (Huby et al., 2000; Taylor and Hefle, 2001; Moreno, 2007; Untersmayr and Jensen-Jarolim, 2008).

One approach to studying conformational epitopes involves the production of variously sized protein fragments, generated either through enzymatic digestion or genetic engineering, and their subsequent assessment for IgE binding (Gershoni et al., 2007). Conformational epitopes are often retained on fragments, especially if the fragments encompass discrete structural domains, though inter-domain interaction can also influence conformational epitope structure and would not be retained in such constructs.

A loss of reactivity to an allergen upon fragmentation can also be taken as evidence for the expression of conformational epitopes. For example, Ramachandran et al. (2002) used *Aspergillus fumigatus* allergen Asp f 3-reactive patients' sera to probe cloned and expressed fragments of various lengths and degrees of overlap corresponding to the sequence of this fungal product (Ramachandran et al., 2002). The authors found that sera from certain patients did not recognize any of the smaller overlapping fragments indicating that there are one or more conformational epitopes on the allergen that are exclusively targeted by these patients. A similar approach was used by Schramm et al. (2001), to analyze a grass pollen allergen.

We have used an alternative "gain/loss of function" approach wherein segments of the test allergen, in our case the cashew 11S globulin, Ana o 2, were molecularly substituted for orthologous regions of soybean homologue (Gly m 6, 11S globulin, glycinin) and then tested for antibody reactivity. Similar chimera-based allergen epitope mapping strategies have been previously employed by others (King et al., 2001; Karisola et al., 2002; Alcocer et al., 2004).

Of particular significance to our cashew allergen model is the fact that multiple IgE-reactive linear epitopes of Ana o 2 have already been identified (Wang et al., 2003) and mapped onto a 3D atomic model of the protein (Barre et al., 2007; Robotham et al., 2009), thus allowing us to focus on the more intractable, yet potentially more important, conformational epitopes of this molecule. We first showed that neither the intact soybean rGly m 6 11S globulin, nor its free large (GLG) and small (GSM) subunits were cross-reactive with IgE from the two cashew-allergic patients selected for their reactivity to native and rAna o 2 (ALG-ASM, data not shown). We then screened a panel of five cashew Ana o 2-specific murine mAbs for reactivity to independently expressed rAna o 2 large (ALG) and small (ASM) subunits as well as the ALG-GSM and GLG-ASM chimeric constructs (Fig. 3). Four of the mAbs bound exclusively to the ALG subunit when expressed alone

or in association with the ASM or GSM small subunit. However, mAb 2B5 showed binding to native Ana o 2, ALG–ASM and ALG–GSM but not to the unassociated ALG domain (Figs. 3 and 5). These data imply that the epitope is primarily located on the large subunit but requires association with the small subunit for correct conformation.

Surprisingly, even constructs in which the positions of the large and small subunits are reversed are 2B5-reactive as long as they express the cashew large subunit. As discussed above, due to steric considerations, it seems highly unlikely that the large and small subunits of a given inverse-order monomer can self-associate to create the 2B5 epitope nor can the inverse-order monomers correctly associate into trimers or hexamers. Also, co-incubated free large and small subunits, with or without MBP, do not generate the 2B5 epitope. We hypothesize that an illicit association between LG and SM subunits of different reverse-order monomers occurs during their synthesis to create correctly folded (with respect to the 2B5 epitope) monomeric subunits.

The observation that 2B5 bound both native and rAna o 2 is significant because the *E. coli*-expressed contiguous large and small subunit structure of the recombinant form mimics the native proprotein rather than the native mature protein which predominates in the nut. This proprotein form is largely, though not entirely, enzymatically cleaved into a disulfide bond-linked large and small subunit form in the maturing nut (Shewry et al., 2003), a structural change which could potentially influence the expression of conformational epitopes. The fact that the proprotein to mature protein transition is commensurate with a trimer to dimer of trimer (hexamer) transition is consistent with at least some change in conformation upon maturation (Shewry et al., 2003; Adachi et al., 2003).

Despite the structural differences between forms, native (data not shown) and recombinant (Fig. 6) Ana o 2 reacted similarly to denaturation with respect to 2B5 reactivity. The simplest interpretation of the data is that the conformational epitope defined by mAb 2B5 is located on the large subunit and is conformationally dependent upon association with the small subunit. An alternative possibility is that the actual epitope may directly include residues contributed by the small subunit (either Ana o 2 or the Gly m 6 homologue). This situation could occur if the portion of the 2B5 epitope on the small subunit coincides with structural features that are similar or identical between the cashew and soybean counterparts. Additional studies to distinguish between the alternatives and to more finely map the cognate residues of the 2B5 epitope are underway.

The potential significance of this conformational epitope in terms of patient IgE binding was confirmed by inhibition assays which demonstrated that 2B5 binds at or near an epitope also recognized by IgE from Ana o 2-reactive patient sera (Fig. 4). When the mAb was used to inhibit the binding of patient IgE to rAna o 2 in an ELISA, a moderate reduction in the binding of patient IgE was observed. That a single mAb is only partially inhibitory is not surprising since multiple epitopes are the norm in allergens. However, when patient IgE was first reacted with the allergen, binding by 2B5 was nearly completely inhibited in a dot blot assay, demonstrating patient IgE binding to epitopes overlapping or sterically adjacent to the 2B5 epitopes (Fig. 5). Because allergen-specific IgE is found in low amounts in patients' sera and serum availability is can be limited, murine IgG mAbs are often used as surrogates to study allergen epitopes (Mueller et al., 2001; Midoro-Horiuti et al., 2006; Rolland and O'Hehir, 2008; Pedraza-Escalona et al., 2009). It remains to be seen if the epitope(s) recognized by patient's IgE that are inhibited by 2B5 are also conformational.

To our knowledge, the 2B5 epitope represents the first conformational epitope to be identified on a tree nut allergen. The information provided by these and other epitope mapping studies,

could prove useful in the effort to create specific reagents for tree-nut allergen identification, disease diagnosis, immunotherapy, and vaccine development. Our studies may also help lay the foundation for future work in determining the physical state (conformation) of the allergen during the priming and reactive phases of allergy and might reveal subtle differences in the manner by which allergens are digested and absorbed into the body in allergic and non-allergic subjects.

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