

Bla g 6: A troponin C allergen from *Blattella germanica* with IgE binding calcium dependence

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Background: The known cockroach allergens do not appear to account for the full repertoire of IgE responses.

Objective: To identify and investigate the importance of other *Blattella germanica* allergens contributing to cockroach allergy.

Methods: A *B. germanica* cDNA library was screened with pooled sera from patients with cockroach allergy. Three isoallergens of troponin C (Bla g 6) were cloned and expressed in *Pichia pastoris*. Homology modeling was performed by using Swiss-Model. IgE responses to purified allergens were simultaneously measured in 104 sera by using a fluorescent multiplex array system. The effect of calcium on IgE binding was investigated by ELISA.

Results: Three isoallergens, Bla g 6.0101, Bla g 6.0201, and Bla g 6.0301, were identified which share homology with insect troponin Cs and vertebrate calmodulins (61% to 78% and 42% to 44% amino acid identity, respectively) and have 2 EF-hand calcium binding domains. Molecular models of Bla g 6 showed 2 structurally homologous lobes connected by a linker that confers flexibility to the allergen. The prevalence of IgE binding to recombinant Bla g 6 was 14%. Calcium depletion by 10 mmol/L ethyleneglycol-bis-(β -aminoethylether)-N,N,N',N'-tetraacetic acid did not significantly affect IgE binding in most cases. Interestingly, addition of 10 mmol/L CaCl₂ after calcium

depletion increased IgE binding by approximately 2-fold, a finding not previously reported for calcium binding allergens. **Conclusion:** Bla g 6 is a troponin allergen with a calcium dependent IgE reactivity that may be involved in muscle contraction.

Clinical implications: Bla g 6 homologous allergens may occur among other insects and cause cosensitization or allergenic cross-reactivity. (J Allergy Clin Immunol 2006;117:1389-95.)

Key words: Asthma, allergy, allergens, cockroach, calcium binding proteins, multiplex array technology

Cockroaches produce potent allergens associated with the development of IgE antibody responses. Early exposure to cockroach allergens in susceptible children may contribute to recurrent wheezing and asthma in early childhood.¹ In the United States, 37% of children with asthma living in inner-city areas have allergy to cockroach, especially to the German cockroach *Blattella germanica*.² Five allergens have been cloned and immunologically characterized from *B. germanica*: Bla g 1, Bla g 2, Bla g 4, Bla g 5, and Bla g 7.³⁻⁸ In a recent study, we reported the relative importance of these allergens for sensitization by measuring IgE antibodies in more than 100 sera from cockroach-sensitized subjects.⁹ The highest prevalence of IgE antibodies was to recombinant (r) Bla g 2 (54%) and rBla g 5 (37%), reaching as high as 71% and 58% among sera with high IgE to cockroach extracts (3.5-100 IU/mL), respectively. Interestingly, the study revealed that 36% of sera did not react to any of the 5 allergens, despite having a positive IgE CAP to *B. germanica*. Most of these sera (75%) had low IgE (0.35-3.49 IU/mL) equivalent to CAP classes 1 and 2. This result suggests that these 5 allergens do not represent the full repertoire of cockroach allergens, and that other allergens remain to be identified.

Knowledge of the molecular structure and biochemical function of an allergen contributes to the understanding of its allergenicity. Engineering of recombinant allergens with lower IgE binding capacity that retain immunogenicity can be used to improve the design of immunotherapy strategies.¹⁰ *B. germanica* allergens differ in structure and function. The primary structure of Bla g 1 contains tandem repeats of ~100 amino acids, with mitochondrial energy transfer motifs.⁴ Bla g 1 is secreted only in the midgut of the cockroach. Adult females produce and excrete significantly more Bla g 1 in their feces than males and nymphs, most likely because of their higher food processing rate.¹¹ Bla g 1 is thought to be involved in digestion,

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The names Bla g 6.0101, Bla g 6.0201, Bla g 6.0301, and Per a 6 were accepted by the World Health Organization/International Union of Immunological Societies Subcommittee of Allergen Nomenclature. The GenBank accession numbers for the nucleotide sequence encoding for these allergens are DQ279092, DQ279093, DQ279094, and AY792950, respectively.

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Abbreviations used

EGTA: Ethyleneglycol-bis-(β -aminoethylether)-N,N,N',N'-tetraacetic acid
r: Recombinant

although the specific function remains unknown.¹¹ Bla g 2 shares amino acid homology and structure with aspartic proteases, but is enzymatically inactive and has a putative ligand binding function.^{5,12-14} Bla g 4 is a lipocalin with structural homology to mammalian allergens (Bos d 2, Can f 1, Equ c 1, Mus m 1, Rat n 1, Fel d 4).^{6,15-17} Bla g 4 has a male reproductive function, is secreted with other seminal substances during sexual activity, and is transferred to the female within the spermatophore during copulation.¹⁸ Bla g 5 is a glutathione S-transferase that may have a role in the detoxification of foods and other substances ingested by cockroach.⁷ Bla g 7 is a tropomyosin believed to be involved in muscle contraction.^{8,19,20}

Here we present the identification, cloning, and expression of a new group of *B germanica* allergens. The use of a fluorescent multiplex array system allows simultaneous assessment of IgE reactivity to different isoallergens, as well as the incorporation of new allergens from the *B germanica* allergen repertoire.⁹ In addition, structure analysis revealed that Bla g 6 is a very flexible molecule, with calcium-dependent IgE reactivity.

METHODS**Sera from patients allergic to cockroach**

A pool was prepared with sera from patients allergic to *B germanica* obtained from previous studies.⁹ The pool contained sera from 5 patients with either high RAST values against *B germanica* extract (1080-2370 U/mL; average, 1556 U/mL equivalent to ~150 ng/mL IgE²¹) or high radioimmunoassay values against one of the known cockroach allergens (760 U/mL against Bla g 1; quantitated by using a serum with high levels of IgE antibodies to Bla g 1 to construct the control curve, and with an arbitrarily assigned value of 1000 U/mL IgE antibodies⁵). The pool was absorbed with 1 mg/mL *Escherichia coli* phage lysate (Stratagene, La Jolla, Calif) and diluted 1:2 in blocking solution (1% nonfat dried milk, 0.2% BSA, 0.4% goat serum, 0.03% gelatin).

cDNA cloning of cockroach allergens

A *B germanica* cDNA expression library on the basis of the UniZAP-XR phagemid expression vector system (Stratagene) was screened by plaque immunoassay using the pooled IgE antibodies.⁶ The phage library was plated by using the *E coli* strain XL1-Blue, and expression of cDNA inserts was induced by laying 10 mmol/L isopropyl β -D-1-thiogalactopyranoside-soaked nitrocellulose membranes on the plates. Expressed proteins were transferred to nitrocellulose membranes, and plaques were screened for expression of cockroach allergens using IgE antibodies. Bound IgE antibodies were detected by using alkaline phosphatase-labeled goat antihuman IgE as described.⁶ The cDNA inserts were sequenced with an Applied Biosystems 377 Prism DNA Sequencer (Biomolecular Research Facility, University of Virginia, Charlottesville, Va). The Per a 6 clone was obtained previously.²² Sequences were analyzed by using

ALIGN (available from www.ebi.ac.uk/emboss/align), ClustalW (www.ebi.ac.uk), Transeq (www.ebi.ac.uk), BLAST (www.ncbi.nlm.nih.gov), Translate (www.expasy.org), and ProtParam (www.expasy.org).

Allergen expression in *Pichia pastoris* and purification

PCR-amplified inserts were subcloned into pGAPZ α constitutive expression vectors (Invitrogen, Carlsbad, Calif) using Not I and Xho I restriction sites. Plasmids encoding the allergens (~10 μ g) were linearized with Avr II, transformed by electroporation into the *P pastoris* strains GS115 and KM71, and selected for zeocin resistance.²³

Each allergen was purified by ion exchange chromatography followed by size exclusion chromatography using the Äkta Explorer HPLC system (GE Healthcare, Piscataway, NJ). Concentrated cultured media, dialyzed against 20 mmol/L Tris-HCl, were applied to a HiTrap Q-XL anion exchange column (GE Healthcare) with a 0% to 70% 1 mol/L NaCl gradient. Fractions eluted at 45% to 65% NaCl were further purified over a HiTrap 16/60 Sephacryl S-100 gel filtration column (GE Healthcare) with 50 mmol/L phosphate buffer containing 0.5 mol/L NaCl pH 7.2. Protein content and purity were analyzed by using nonreducing SDS-PAGE followed by silver staining.

IgE binding to recombinant allergens

Initially allergens were screened by plaque immunoassay with 18 sera from patients allergic to cockroach and 2 noncockroach-allergic sera as controls. In subsequent experiments, the purified allergens Bla g 6.01, Bla g 6.02, and Bla g 6.03 were simultaneously analyzed for IgE binding in 104 sera from patients allergic to cockroach using a fluorescent multiplex array system (Bio-Plex; BioRad, Hercules, Calif). Each allergen (10 μ g) was coupled to a different set of fluorescent microspheres (2.5×10^6) following the protocol supplied by the manufacturer (BioRad).⁹

Assays were performed in Multiscreen BV plates (Fisher, Houston, Tex) as previously described.⁹ Briefly, a mixture of allergen-coupled beads was dispensed at 5000 beads per bead-type per well. Diluted sera (50 μ L, 1:4) were incubated with allergen-coupled beads for 30 minutes. Biotinylated goat antihuman IgE (Biosource International, Camarillo, Calif; 2 μ g/mL) was then added to the wells (30 minutes) followed by addition of phycoerythrin-coupled streptavidin (Molecular Probes, Carlsbad, Calif; 50 μ L of 4 μ g/mL for 10 minutes). Fluorescence was detected by using a Bio-Plex System. Fluorescence values were interpolated from a standard curve established by incubating Pharmacia human IgE standard (100 IU/mL) with beads coupled with sheep antihuman IgE antibody (Biosource International). This total IgE curve was standardized against the Pharmacia CAP standard for IgE antibodies.^{24,25} The limit of detection of each assay was obtained by using a value corresponding to the mean plus 2 SDs for 6 blank wells.

Molecular modeling of Bla g 6

Bla g 6 isoallergens were modeled by homology using Swiss-Model, accessible via the Expasy web proteomics server of the Swiss Institute of Bioinformatics.²⁶⁻²⁸ Although the crystal structures with highest homology to Bla g 6 are calmodulins (~45%), the best quality models were based on x-ray crystal structures of troponin C. Templates were manually selected from the 46 troponin C structures available in the Research Collaboratory for Structural Bioinformatics Protein Data Bank (<http://www.rcsb.org/pdb/>), on the basis of the highest degree of homology to the specific Bla g 6 isoform (~35% to 40%). The model quality was assessed by What-Check (through Swiss-Model, and also available at <http://swift.cmbi.ru.nl/gv/whatcheck/>).²⁹

Calcium binding assay

Calcium dependent IgE binding was assessed by ELISA. Multiwell plates were coated with 1 to 2 µg/well of purified allergens or, for control purposes, with natural Der p 1 or rBla g 1. The plates were incubated with cockroach-allergic sera known to contain specific IgE against the allergen coating the wells. Additional negative controls were performed with sera from an atopic patient without cockroach allergy in Bla g 6-coated wells. Sera were diluted 1:2 with PBS and 0.5% BSA alone or in the presence of either 10 mmol/L CaCl₂ or 10 mmol/L ethyleneglycol-bis-(β-aminoethyl-ether)-N,N,N',N'-tetraacetic acid (EGTA). Sera (100 µL/well) were added to allergen coated wells and incubated for 3 hours at room temperature. Separate wells were incubated with serum in the presence of EGTA for 2 hours followed by serum in the presence of CaCl₂ for 1 hour. Bound IgE antibodies were detected by using biotinylated goat antihuman IgE antibodies (KPL, Gaithersburg, Md), diluted 1:4000, followed by streptavidin peroxidase at 0.25 µg/mL.⁵

RESULTS

Molecular cloning of three Bla g 6 isoallergens

Screening of the cDNA expression library with an IgE serum pool resulted in the identification of 3 cDNA clones encoding for troponin sequences, designated as isoallergens Bla g 6.0101, Bla g 6.0201, and Bla g 6.0301. Bla g 6.02 showed >99% amino acid identity to a previously identified sequence encoding for an allergen, Bla g 6, published in a US patent, that had not been immunologically characterized but showed sequence homology to troponin C.^{30,31} Bla g 6.01, chosen as the reference isoallergen for nomenclature, showed 93% amino acid identity to Bla g 6.02 and 68% identity to Bla g 6.03 (Table I). The open reading frames for Bla g 6.01, Bla g 6.02, and Bla g 6.03 cDNA were predicted to be 151 to 154 amino acids long, encoding allergens with predicted molecular weights of 17,095 to 17,750 d and isoelectric points of 3.9 to 4.1, respectively. Although no N-glycosylation signals were found, O-β-GlyNAc glycosylation sites were predicted in the three Bla g 6 sequences by YinOYang, a Web-based server for eukaryotic proteins (<http://www.cbs.dtu.dk/services/YinOYang/>).³² Bla g 6.01, Bla g 6.02, and Bla g 6.03 showed 91%, 87%, and 69% amino acid identity, respectively, to Per a 6 from the American cockroach (*Periplaneta americana*; Table I).

Using BLAST, the three Bla g 6 sequences were shown to contain 2 EF-hand calcium binding regions and to have high homology to 2 calcium binding proteins: troponin C (61% to 78%), involved in muscle contraction, and vertebrate calmodulin (42% to 44%), a regulatory protein (Table I; Fig 1). An EF-hand motif consists of a 12-residue loop, flanked on both sites by α-helices and with 6 residues involved in calcium binding in positions 1, 3, 5, 7, 9, and 12 (denoted by X, Y, Z, -Y, -X and -Z in Fig 1).

Expression and purification of rBla g 6

Three recombinant Bla g 6 isoallergens were successfully expressed in the *Pichia* strain GS115. One out of three Bla g 6.01 clones expressed a protein with the

TABLE I. Amino acid identity (%) between Bla g 6 and homologous proteins (by ALIGN)*

	Bla g 6.01			
Bla g 6.01	100	Bla g 6.02		
Bla g 6.02	92.7	100	Bla g 6.03	
Bla g 6.03	67.7	65.8	100	Per a 6
Per a 6	90.7	86.8	69	100
<i>Plutella xylostella</i>	78.4	75.2	63.1	75.2
<i>Drosophila melanogaster</i>	75.8	74.5	64.1	78.4
<i>Apis mellifera</i>	75.2	75.2	65	75.2
<i>Solenopsis invicta</i>	74.5	74.5	63.7	73.9
Mouse calmodulin	42.8	42.1	43.9	43.4

*Species names represent troponin C from that species.

expected molecular weight, similar to Bla g 6.02, by SDS-PAGE, and resulted in IgE binding. A first purification step by ion exchange HPLC resulted in several fractions eluted at 45% to 65% NaCl containing 2 to 5 distinct bands (including rBla g 6) by SDS-PAGE. These fractions were pooled, and Bla g 6 was further purified by gel filtration HPLC (Fig 2).

Prevalence of IgE binding to the three Bla g 6 isoallergens

IgE binding to the three Bla g 6 clones was initially assessed by plaque immunoassay. Color intensity was visually assessed from 0 to 4 versus negative controls. Considering values >2 as positive, the percentage of sera from patients with cockroach allergy that reacted with the three Bla g 6 isoallergens was 16.7%. Using a multiplex array assay, the prevalence of IgE antibodies against recombinant allergens rBla g 6.02 or rBla g 6.03 was 14% (n = 104). There was an excellent correlation between the IgE levels from the 15 sera that were positive with both or either isoallergen ($r^2 = 0.94$; Fig 3). The mean IgE antibody titers for these 15 sera were 15.9 IU/mL and 24.2 IU/mL to rBla g 6.02 and rBla g 6.03, respectively, and 29.0 IU/mL (range, 0.5-88.3 IU/mL) to *B. germanica* extract. Most of these sera had a high CAP to cockroach extracts (8/15; >17.5 IU/mL, CAP classes 4-6) and had IgE specific to at least 1 of the known cockroach allergens (12/14, 1 not tested). Six out of 7 selected sera that reacted with Bla g 6.02 also recognized Bla g 6.01.

Bla g 6 structural models

Several molecular models for Bla g 6 were constructed on the basis of troponin C structures. The best models, with positive structure z scores by What-Check, were based on calcium saturated troponin C, not bound to troponin I.^{33,34} They are composed of 2 structurally homologous lobes, mainly constituted by α-helices and loops, connected by a long exposed ~9-turn helical linker that spans the residues F65-Y95 in Bla g 6.01, F65-D96 in Bla g 6.02, and F69-D100 in Bla g 6.03 (Fig 4, left). The N-terminal lobe contains 3 α-helices, except in Bla g 6.03, which has 4 extra residues with an additional short α-helix, involving residues A2 to Q7. Short β-sheets are

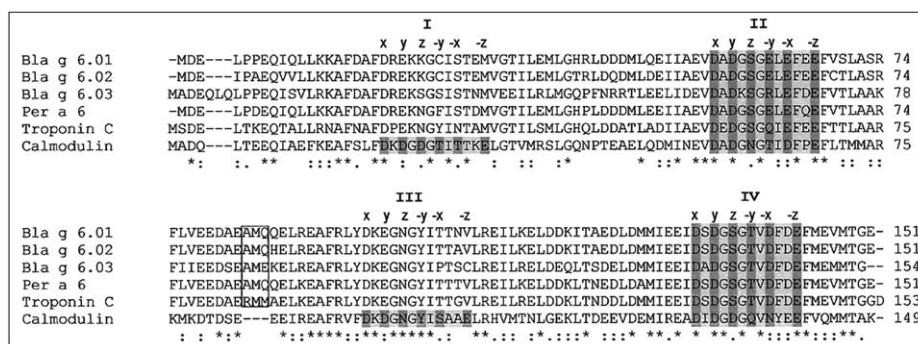


FIG 1. Sequence alignment of Bla g 6 isoallergens with Per a 6, troponin C from *Drosophila melanogaster*, and calmodulin from mouse. EF-hand regions are numbered I, II, III, and IV. Those predicted to bind Ca^{2+} are highlighted in grey. Residues at calcium coordinating positions are denoted by x, y, z, -y, -x, and -z and shaded in dark grey. The boxed area shows the 3 extra residues present in troponin C central α helix.

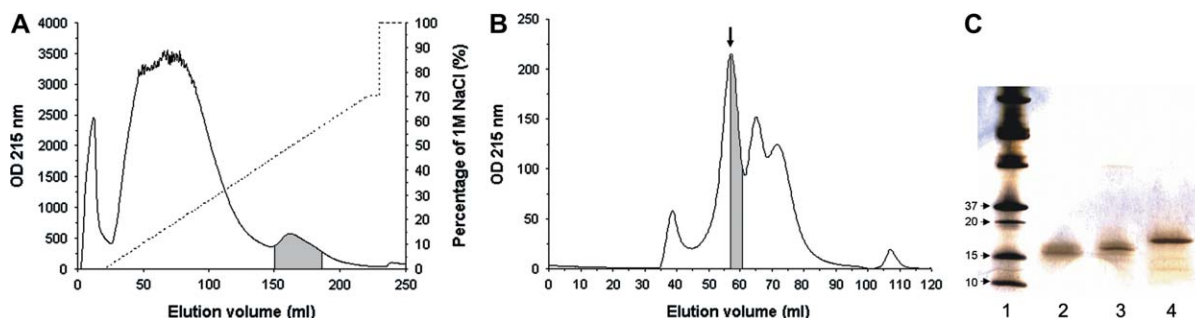


FIG 2. Bla g 6 purification. Elution profiles for rBla g 6.03 purification by ion exchange (A) and gel filtration (B) chromatography. The buffers were 20 mmol/L Tris-HCl pH 8.0, with a 0% to 70% gradient of NaCl for elution (A), and 0.05 mol/L phosphate, 0.2 mol/L NaCl, pH 7.2 (B). (C) Silver stained SDS-PAGE of purified rBla g 6 isoallergens. Lanes: (1) protein marker (molecular weight in kd), (2) Bla g 6.01, (3) Bla g 6.02, and (4) Bla g 6.03. Shaded areas, selected fractions. Arrow, peak containing purified rBla g 6.03.

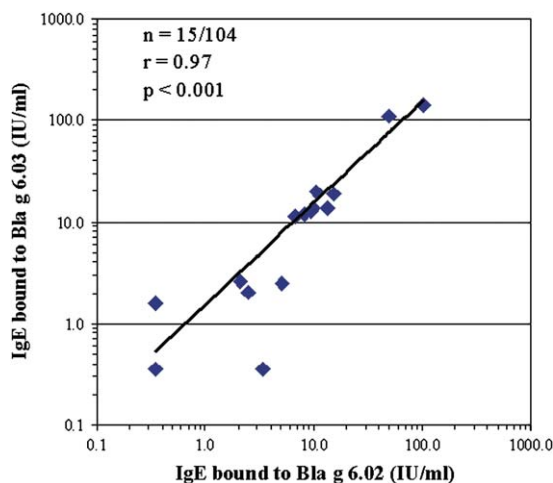


FIG 3. Correlation of IgE binding to Bla g 6.02 and Bla g 6.03 by multiplex assay.

formed between the strands that follow the α -helices 1 and 3 in Bla g 6.01 and Bla g 6.02, and the structurally equivalent α -helices 2 and 4 in Bla g 6.03. Three α -helices are present in the C-terminal lobe. An EF-hand calcium binding motif is present in each of the 2 lobes: after the last

α -helix of the N-terminal lobe, and after the second α -helix of the C-terminal lobe (Fig 4). Identical residues in the 3 isoallergens are indicated in Fig 5.

Three models were also generated on the basis of the crystal structure of troponin C in complex with a fragment of troponin I from rabbit skeletal muscle³⁵ (Fig 4, right). On troponin I binding, the center of the long helical linker unwinds and bends 90°. The 2 lobes shift much closer, and troponin C adopts a more globular shape (Fig 4, right). There are 3 extra residues in Bla g 6 (AMQ/E), not present in calmodulin, that contribute to the longer α -helix of troponin C compared with calmodulin (Fig 1). The first 2 residues (AM) correspond to the end of the unwound helix. Given the similar overall folding of the 3 isoallergens, only the Bla g 6.03 model, with a longer N-terminus, was selected to illustrate the molecular structure of calcium-bound Bla g 6 (Fig 4).

Bla g 6 as a calcium binding allergen

The effect of calcium on IgE binding to the three rBla g 6 isoallergens was assessed by ELISA using sera of 3 patients allergic to cockroach, containing specific IgE for Bla g 6, and a pool of 3 sera from which 2 had IgE specific for Bla g 6. In most cases, allergens with added calcium or EGTA calcium depleted did not show significant

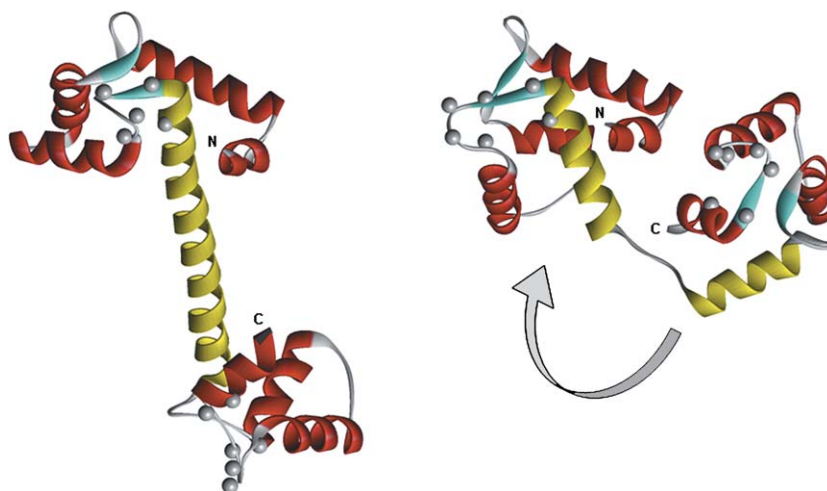


FIG 4. Molecular models of Bla g 6.03, based on the crystal structure of calcium-bound troponin Cs from rabbit skeletal muscle alone (1tcf.pdb) (*left*) and in complex with a fragment of troponin I (1a2x.pdb) (*right*). The α carbons from the 6 residues expected to bind calcium are indicated as grey spheres.

differences in IgE binding. Interestingly, the IgE response to allergens incubated with serum containing EGTA followed by serum with CaCl_2 increased an average of 2-fold compared with allergens incubated with serum only (Fig 6). Natural Der p 1 and rBla g 1, used as positive controls, displayed no significant changes in IgE responses in the 4 conditions tested (Fig 6, *bottom*).

DISCUSSION

We report the identification of 3 cockroach isoallergens, Bla g 6.01, Bla g 6.02, and Bla g 6.03, with sequence homology to troponin C, a protein essential in calcium regulation of skeletal and cardiac muscle contraction. Troponin C is part of a complex of 3 subunits: troponin C binds Ca^{2+} , troponin T binds tropomyosin, and troponin I is the inhibitory subunit.³⁶ Troponin and tropomyosin are located on the actin filament regulating the interaction of actin with myosin. The existence of at least 3 different Bla g 6 isoallergens could be related to the reported presence of several genes encoding for troponin Cs in insects.³⁷⁻³⁹ A correlation between IgE binding to Per a 6 and Bla g 6 suggests cross-reactivity between both allergens (F. T. Chew, Unpublished data, February 2006). Other insects may also induce sensitization by inhalation to Bla g 6 and Per a 6-homologous allergens, which are relatively conserved proteins, given the ubiquitous presence of troponin C in muscles from insects.

The rationale for the screening of a *B germanica* expression library was to identify new cockroach allergens. A previous publication suggests that Bla g 1, 2, 4, 5, and 7 do not account for the full IgE binding repertoire.⁹ Most of the sera reacting to Bla g 6 had IgE specific to at least 1 of the known cockroach allergens. Therefore, Bla g 6 does not account for the reactivity to cockroach extracts in patients who do not have specific IgE to any of the

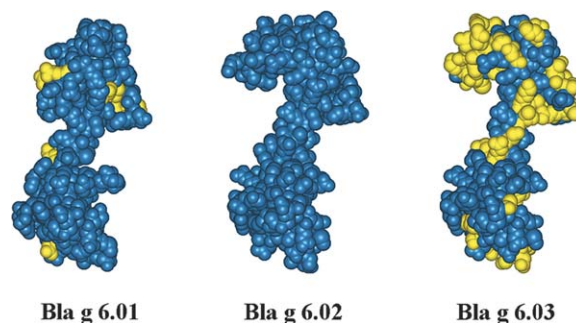


FIG 5. Bla g 6 models based on troponin C from turkey skeletal muscle (5tnc.pdb) for Bla g 6.01 (39.5% amino acid identity), and rabbit skeletal muscle (1tcf.pdb) for Bla g 6.02 and Bla g 6.03 (37.4% and 35.3% identity, respectively). Blue spheres represent atoms from identical amino acids in the 3 isoallergens. Yellow spheres represent the atoms from the amino acids that differ from Bla g 6.02.

known 5 allergens. Bla g 6 is a minor allergen with a prevalence of sensitization of 14% among patients with cockroach allergy, measured by a fluorescent multiplex array assay. The prevalence was similar for the 3 isoallergens by plaque immunoassay. The excellent correlation of IgE binding to Bla g 6.02 and Bla g 6.03, and the fact that individual patients generally reacted to both isoallergens, indicate that the majority of the IgE binding epitopes are most likely located in homologous parts of both proteins.

Bla g 6 (troponin C), together with Bla g 7 (tropomyosin) and Bla g 8 (myosin light chain, another calcium binding allergen identified by the screening of the library; data not shown), are homologous to proteins involved in muscle contraction. Other allergens involved in muscle contraction, different from troponin C and myosin light chain, have been identified from species other than cockroach. Examples are tropomyosin in crustaceans

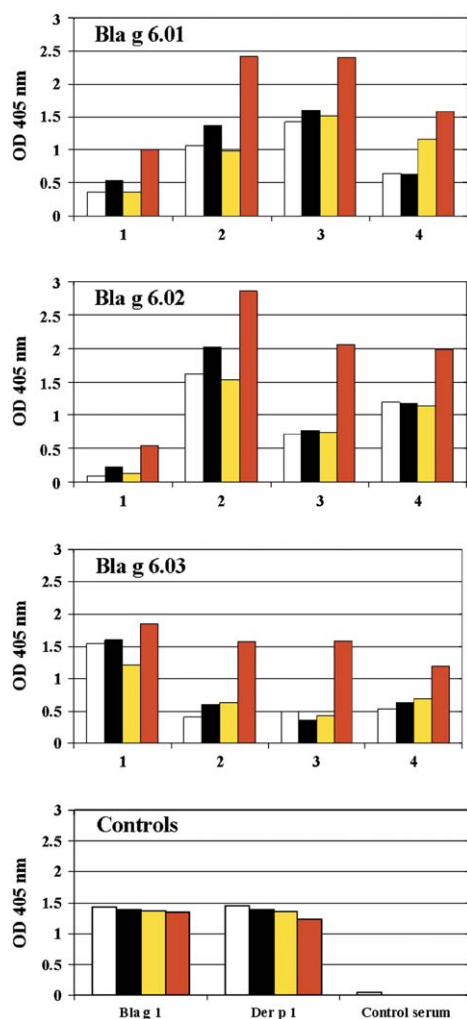


FIG 6. Influence of calcium on IgE binding to Bla g 6 by ELISA. Individual (1-3) or pooled (4) sera known to contain Bla g 6 specific IgE (white) with 10 mmol/L CaCl_2 (black), 10 mmol/L EGTA (yellow), or 10 mmol/L EGTA followed by 10 mmol/L CaCl_2 (red) were used. Controls: sera from an atopic patient without cockroach allergy, and wells coated with nDer p1 and rBla g 1 incubated with sera of patients allergic to mite or cockroach, respectively.

and dust mite, and paramyosin in dust mites and nematodes. Troponin C and calmodulin are Ca^{2+} -sensor regulatory proteins with a very flexible molecular structure that extends from a closed conformation in the calcium-free (apo-) form to a dumbbell-shaped open molecule in the calcium-bound form. This open form has 2 terminal lobes and exposes hydrophobic surfaces to bind peptides or other proteins. Troponin C, upon binding to troponin I, adopts a closed conformation by bending 90° the unwound center of the central α -helix, and regulates muscle contraction by relieving the inhibition of actomyosin by troponin I.³⁵ The flexibility of *B. germanica* troponin C was illustrated with 2 sets of Bla g 6 models generated on the basis of the crystal structure of Ca^{2+} -bound troponin C alone or in complex with a troponin I fragment. Whereas calmodulin functions as an isolated protein,

with both lobes involved in signal transduction, troponin C is integrated into the troponin complex through its structural C-lobe, and only the N-lobe has a regulatory function.³⁵ Two complete EF-hand motifs were found in the second and fourth domains of the N-terminus and C-terminus of Bla g 6, respectively, as expected from troponin C in arthropods (such as cockroaches) and mollusks, which binds 2 Ca^{2+} . However, troponin C binds 3 and 4 Ca^{2+} in vertebrate cardiac and skeletal muscle, respectively, and calmodulin mostly has 4 EF-hand motifs binding Ca^{2+} . Binding of magnesium to the third domain is also likely because Bla g 6.02 and Bla g 6.03, for example, have the same EF-hand motif sequences as the potentially magnesium binding EF-hands in TpnCIIIa from *Apis mellifera* and in TpnC3 from *Lethocerus indicus*, respectively.³⁹

Calcium-dependent modulation of IgE binding has been demonstrated for several calcium binding allergens present mostly in plants (Bet v 3, Cyn d 7, Jun o 2, Bet v 4, Ole e 3, Ole e 8, Aln g 4, Phl p 7, Cyp c 1, Hom s 4).^{40,41} With 1 exception, these are qualitative studies of calcium-bound depletion on IgE binding by using plaque immunoassay or allergen blotted/dotted on nitrocellulose membranes. Here we quantified this effect by ELISA. For most sera, a substantial IgE binding could still be detected after calcium depletion, indicating the presence of most of the IgE epitopes in the apo-form. Interestingly, IgE binding was always increased about 2-fold by addition of calcium to sera previously calcium-depleted with EGTA. This last observation had not previously been reported for calcium binding allergens. It could be related to different conformational changes associated with the nonphysiological process of release of troponin C from the degraded muscle after cockroach death followed by exposure to the patient's calcium. This process is different from the physiological release of allergens associated with pollen. Therefore, the measurement of IgE reactivity in an assay could differ depending on the conditions of allergen preparation and analysis (such as composition of expression and purification buffers, sera dilution) that determine the calcium bound to the allergen. On the basis of these observations, it is important to consider which calcium-dependent changes are associated with IgE recognition when assessing sensitization to calcium-bound allergens such as Bla g 6.

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