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Immunologic characterization of a recombinant American cockroach (*Periplaneta americana*) Per a 1 (Cr-PII) allergen

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Key words: American cockroach; cross-reactive allergen; cDNA
cloning; IgE-binding epitope; Per a 1 (Cr-PII) allergen.

Background: Previously, we have identified several Per a 1 (Cr-PII) allergens from a λ gt22A cDNA library of *Periplaneta americana*. This study aimed to sequence clone C42 and determine its molecular and antigenic properties.

Methods: The cDNA of C42 was sequenced and ligated into a bacteria expression vector, pET21. The recombinant proteins were purified by ion-exchange and affinity chromatographies. Their antigenicities were analyzed by immunoblotting, ELISA, and binding inhibition with human IgE.

Results: The nucleotide of the cDNA has been sequenced and the deduced amino acid which encodes a 446-amino-acid protein (50 kDa) determined. The recombinant C42 protein can bind both anti-Per a 1 monoclonal antibodies and human IgE and showed a 54.4% (12/22) skin reactivity in atopic patients. Sequence homology searches revealed a high degree of identity to two other members of the Per a 1 family, C17 and C6, and the German cockroach (*Blattella germanica*) Bla g Bd90K allergen. Interestingly, these allergens all contain internal repeats, and the crude *B. germanica* extract, Per a 1, and recombinant allergens share similar antigenic determinant(s) as defined by ELISA and IgE-binding inhibition studies. In IgE-binding epitope studies, an immunopositive C42 fragment was first identified from partial protease digestion. Overlapping peptides were then generated by expression of restriction enzyme fragments in *E. coli*. The shortest peptide, C42-P560, identified by monoclonal antibodies and human specific IgE, can inhibit IgE binding to C42.

Conclusions: An additional Per a 1 allergen has been defined at the molecular level and characterized and preliminary results showed that a potential IgE-reactive region is located within amino-acid residue 358–446 of C42, which is an internal repeat. The results defined the boundaries of the antigenic site and will facilitate further epitope-mapping studies.

Date:

Accepted for publication 28 August 1998

To cite this article:

Wang N.M., Lee M.F., Wu C.H. Immunologic
characterization of a recombinant American cockroach
(*Periplaneta americana*) Per a 1 (Cr-PII) allergen.
Allergy 1999, **54**, 119–127.

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ISSN 0105-4538

Cockroaches have been considered major causes of inhalant allergy and asthma (1–14). American cockroach, one of the principal species, is important in producing environmental allergens that provoke immediate hypersensitivity reactions in man. Two major allergenic fractions, Per a 3 (Cr-PI) and Per a 1 (Cr-P II), were previously isolated, identified, and characterized from the crude *Periplaneta americana* extract (CRA-A) (15–17). Per a 3, comprising a family of isoallergens, is a species-specific allergen which has been cloned and found to be related to insect hemolymph proteins (arylphorin) (18–21).

Recently, the construction of a λ gt22A cDNA library and the subsequent identification of several clones recognized by anti-Per a 1 monoclonal antibodies (mAbs) and human IgE have been reported. Among them, clones C6 and C17, which encode a 25.8- and a 31.14-kDa protein, respectively, share 94% identity between them, and no sequence homology was found with Per a 3 (22). Both molecules contain internal repeat sequences and showed 59% and 77.3% skin reactivities, respectively. They were found to have 82.7–85.1% identity to the *Blattella germanica* Bla g Bd90K allergen (23), and 28.9–31.8% identity to ANG12, an African malaria mosquito (*Anopheles gambiae*) protein (GenBank database accession number Z22925, unpublished data). ANG12 is a probable secretory protein with unknown function that is induced in the midgut of female *A. gambiae* after a meal. Cr-P II and Bla g Bd90K were recently renamed Per a 1 and Bla g 1, respectively (22). In this study, we report the sequence, characterization, and identification of a major IgE-binding region of an additional Per a 1 allergen.

Material and methods

DNA sequencing

The C42 cDNA insert in λ gt22A was released by *Not* I and *Sal* I double digestion and then subcloned into pSPORT1 (Gibco BRL/Life Technologies, Grand Island, NY, USA). A set of nested deletions was created with the “Erase-a-base” Exo III/S1 nuclease system (Promega, Madison, WI, USA). Double-strand DNA was sequenced by the dideoxynucleotide chain termination (24) with the T7 Sequencing Kit 2.0 (Pharmacia Biotech, Piscataway, NJ, USA) and ^{35} S-labeled-dATP following protocols provided by the supplier. ^{35} S-labeled products were analyzed on 6% polyacrylamide gels and autoradiographed.

Computer analysis

Sequence data were analyzed with the PC/GENE software (IntelliGenetics, Inc., Mountain View, CA, USA), and sequence homology searches were performed at NCBI with the BLAST network service. The version used was BLAST 1.49 MP (26 March 1996).

Expression and purification of recombinant C42 protein

C42 in pET21 was overexpressed and purified from *E. coli* BL21(DE3). A volume of 20 ml of an overnight culture was transferred into a 200-ml Lauria Bertani containing 50 μ g/ml ampicillin. The culture was grown for 1 h at 37°C and then isopropyl-b-D-thiogalactopyranoside was added to 0.4 mM, and the mixture was incubated overnight. The culture was harvested, and the cells were washed with sonication buffer (50 mM Tris-HCl, pH 8, 200 mM NaCl, 0.1 mM EDTA, and 5% glycerol). The pellets were resuspended in sonication buffer containing 2 mM phenylmethylsulfonyl fluoride, and the cells were sonicated. The lysate was centrifuged (10 000 g), and then the supernatant was mixed with 10 volumes of ice-cold buffer A (50 mM sodium phosphate, pH 7.4, and 1 mM EDTA). The resulting mixture was loaded onto a hydroxyapatite column equilibrated with the same buffer. The column was washed with buffer A, and the proteins were eluted by a 50–500 mM sodium phosphate gradient. Fractions containing the C42 protein, which migrates at 50 kDa on both SDS-PAGE gels and immunoblots, were pooled, concentrated, and then dialyzed against buffer B (20 mM Tris-HCl, pH 7.4, 50 mM NaCl, and 0.1 mM EDTA). The pooled fraction was then loaded onto a DE52 column equilibrated with buffer B. After washing two column volumes with the same buffer, proteins were eluted with a 50 mM to 2 M NaCl gradient. Fractions were analyzed on SDS-PAGE gels and ELISA, and fractions that contained C42 protein were pooled (yield typically 10 mg/l of culture), dialyzed against PBS, concentrated, and frozen at –70°C.

Cockroach extracts and preparation of Per a 3 and Per a 1

Commercial cockroach skin test reagents and crude *B. germanica* extract (CRA-G) were obtained from Allergy Laboratories, Inc. (Oklahoma City, OK, USA), Berkeley Biologicals (Berkeley, CA, USA), Center Laboratories (Port Washington, NY, USA), Greer Laboratories (Lenoir, NC, USA), and Hollister-Stier Laboratories (Spokane, WA, USA). CRA-A was prepared by extracting the whole body of insects with Coca's solution in our laboratory (15). Per a 3 and Per a

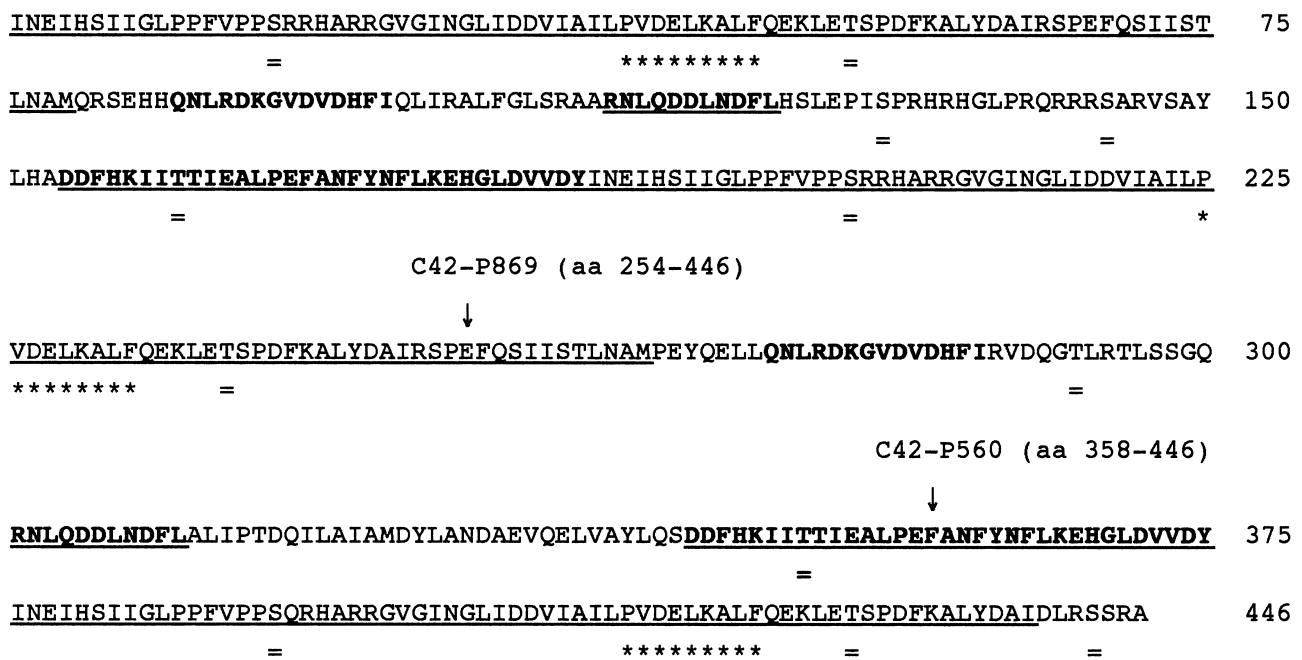


Figure 1. Deduced amino-acid sequence of C42. Single-letter amino-acid code is used, and internal repeat sequences are shown visually by underline and/or boldface. Potential mitochondrial energy transfer peptides and phosphorylation sites are denoted by (*) regions and (=), respectively. Arrow indicates beginning of C42-P869 and C42-P560 peptides.

1 were purified from the CRa-A by gel filtration (15) and preparative isoelectric focusing, respectively (17).

Skin test and IgE antibodies to CRa-A

Commercial American cockroach extracts (1:20 w/v, Greer Laboratories), Per a 1, and recombinant proteins at a concentration of 100 µg/ml in PBS containing 50% glycerol were used for skin testing. Allergens were applied to the test heads with Dip 'N Touch dispenser (Center Laboratories, Port Washington, NY, USA), and the epicutaneous skin test was performed with the sterile disposable Multi-Test applicator of eight test heads (Lincoln Diagnostics, Decatur, IL, USA). PBS containing 50% (v/v) glycerol and 1.0 mg/ml histamine were included as negative and positive control, respectively. The reaction was read at 15 min, and the largest and smallest diameter of the wheal and erythema were measured. The mean diameters of the wheal and erythema were graded against the controls. A reaction comparable to negative control was considered negative. A wheal 2.0 mm larger or erythema 5.0 mm greater than negative control, and a wheal 4.0 mm larger or erythema 10 mm greater than negative control were graded as 1+ and 2+, respectively. A reaction to the positive control that usually had a wheal of 7–10 mm and erythema greater than 20 mm were graded as 3+. A reaction greater than the positive control that was sometimes associated with

pseudopodia and itching was graded as 4+. Sera of cockroach-sensitive atopics were assayed for CRa-A specific IgE antibodies by the fluoroallergosorbent test (FAST), and a class-scoring system based on specific IgE concentration was assigned according to the manufacturer's recommendation, as previously described (20).

Immunodetection of Per a 1 allergen by ELISA using anti-C42 antibody

Antibody to C42 recombinant protein was generated from immunized BALB/c mice using purified protein by the method of Mahana & Paraf (25). ELISA was performed as described previously (20). Wells were coated with either 1.0 µg of CRa-A, 0.5 µg of Per a 3, Per a 1, and purified C42, C17, and C6 recombinant proteins; or 100 ml of a 1:20 dilution of commercial skin test extracts in triplicate. The anti-C42 antibody (500 ng) and peroxidase-labeled anti-mouse immunoglobulin (1:8000, Sigma) were used as primary and secondary antibodies, respectively. The optical density was determined at 415 nm on an SLT SPECTRA (SLT-Labinstruments, Salzburg, Austria).

Protease digestion

Protein was incubated with endoproteinase Arg-C at a protease-to-protein molar ratio of 1:1.8 in reaction buffer (10

Cr4H	-----RNLDLQDFLKL	13
C6	SIISTLNAMPEYQDLLQNLKRDGVDVDHYIELIRALFGLTRAA***Q***N*****	56
C17	SIVQTLNAMPEYQDLLQNLKRDGVDVDHYIELIRALFGLTREAA***Q***N***A*	102
C42	SIISTLNAMQR-SEHHQNLKRDGVDVDHFIQLIRALFGLSRAA***Q***N***HS	125
Cr4H	VPVDQIIAIAITDYIANDAE-VQAAVAYLQSEFETIVTVDALPELQNFNLETN	68
C6	I*T***L***M***L*****-**EL*****D*HK*IT*IEG***FA**Y***KEH	111
C17	I*T***L***M***L*****-**EL*****D*HK*IN*IE***FA**Y***KGH	157
C42	L--EP*SPRRHGLPRQRRRSARVS***HA*D*HK*IT*IE*** <u>FA**Y***KEH</u>	179
Cr4H	GLNAIDFLNGIHDLLGIPHIPVSGRKYHIRRGVGITGLIDDVLAIL PIDDLKALFN	124
C6	**DVA***E**SII*L*PFVPPS*R-*A*****N*****I*** V*E*****D	166
C17	**DVANYI*E**SII*L*PFVPPS*R-*A*****N*****I*** V*E***T**Q	212
C42	**DVV*YI*E**SII*L*PFVPPS*R-*A*****N*****I*** V*E*****Q	234
Cr4H	EKLETSPDFLALYNAIKSPEFQSIVQTLNDMPEYQNLQKLREKGVVDVKIIEI	178
C6	****T***K***D**R*****IS**A*****D***N**D*****HF***	220
C17	****T***K***D**R*****IS**A*****E***N**D*****HF***	266
C42	****T***K***D**R*****IS**A*****E***N**D*****HF*RV	288

Figure 2. Comparison of deduced amino-acid sequences of C6, C17, and C42 with “repeat 1” sequence of Bla g Bd90K (Cr4H). Single-letter amino-acid code is used, and gaps (–) are introduced to optimize alignment. Asterisk (*) represents homology with Cr4H. Potential phosphorylation sites are in boldface, and mitochondrial energy transfer peptides are in bold face and italics. Internal repeat of C42-P560 in C42 is underlined. Only region of homology is represented.

Table 1. Skin tests with recombinant allergen and serum IgE antibodies to CRa-A on atopic patients

Patient no.	Diagnosis*	Skin test/FAST class		
		CRa-A	Per a 1	C42
1	AS	3+/I	1+	–
2	AS	2+/II	1+	1+
3	AS	3+/II	3+	3+
4	AS/AR/AC	3+/I	2+	1+
5	AC/AR/UR	2+/I	2+	2+
6	AC	3+/III	3+	–
7	AS	2+/I	2+	–
8	AS/AR/AC	3+/I	1+	–
9	AS/AR	2+/II	4+	2+
10	AS	2+/II	4+	1+
11	AR	3+/III	2+	3+
12	AS/AR/AC	1+/I	1+	3+
13	AS	1+/II	1+	3+
14	AS/AR	2+/II	1+	–
15	AS/AR/AC	2+/I	1+	–
16	AS	4+/III	3+	4+
17	AS/AR	3+/III	3+	1+
18	AS/AR/AC	1+/I	1+	–
19	AS/AR/AC/UR	1+/I	1+	1+
20	AR	3+/II	3+	–
21	AS/AR/AC	2+/I	2+	–
22	AR	3+/II	2+	–

* AS, asthma; AR, allergic rhinitis; AC, allergic conjunctivitis; UR, urticaria.

mM MOPS, pH 7.5, 200 mM NaCl, 2 mM MgCl₂, 1 mM dithiothreitol] for 24 h at room temperature. After digestion, the reaction was terminated by addition of EDTA to a final concentration of 3 mM and incubated for 5 min.

Restriction enzyme fragmentation

The cDNA was digested with *Eco*RI and *Not*I (26). After digestion, the fragments and the linearized plasmid, pET 30, DNA were ligated together, and the resulting plasmids, C42-P869 and C42-P560, were transformed into *E. coli* BL21(DE3) (20, 27).

C42-P560 and C42-P869 peptides

The growth and the induction of *E. coli* cells containing C42-P560 or C42-P869 are the same as for the C42 already described. After harvesting, the cells were lysed in 5 ml buffer C (8 M urea, 20 mM Tris, 100 mM sodium phosphate, pH 8.0). Insoluble proteins were removed by centrifugation at 10 000 g for 10 min at 4°C. The crude extract was coupled with 1 ml of 50% Ni²⁺-NTA resin (Qiagen, Valencia, CA, USA) for 45 min at 25°C. Unbound proteins were removed by washing the resin three times with 10 ml of buffer C and then 10 times with 10 ml of buffer D (8 M urea, 20 mM Tris, 100 mM sodium phosphate, pH 6.3). The bound histidine-tag protein was eluted with 1 ml of buffer D containing 100 mM EDTA. Proteins were frozen and stored at –70°C. Protein concentrations were determined by the Bio-Rad Bradford

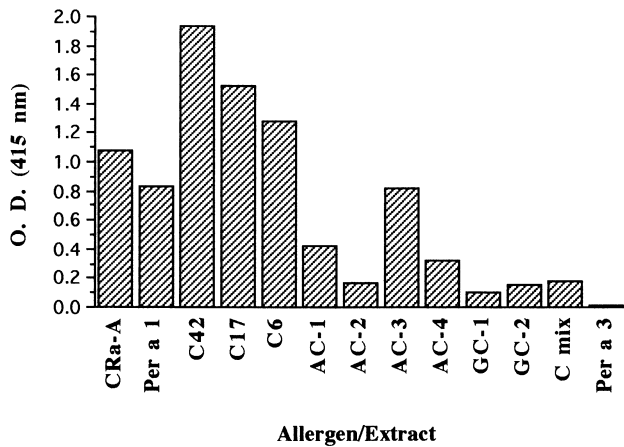


Figure 3. Detection of cockroach allergens by anti-C42 antibody with ELISA. Different commercial *P. americana*, *B. germanica*, and cockroach mix extracts, denoted by AC1-AC-4, GC1-GC2, and C-mix, respectively, were coated onto wells. Anti-C42 was used as primary antibody, and results are average of experiment performed in triplicate, as described.

assay (Bio-Rad, Hercules, CA, USA) with either bovine serum albumin (BSA) or bovine gamma globulin as standard.

SDS-PAGE and immunoblotting

Protein samples were loaded on a 5% polyacrylamide stacking gel above a 12% separating gel, and the gel was run with discontinuous buffer by Laemmli's method (27). After electrophoresis, gels were fixed and stained with 0.2%

Coomassie brilliant blue R-250. For immunoblotting, gels were transferred electrophoretically to polyvinyl difluoride membranes (Millipore Corp., Marlborough, MA, USA) (28). After the transfer, the membranes were blocked in TBST (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20) containing 4% BSA for 2 h at room temperature. Twelve C42-skin-test-positive atopic sera and 10 nonatopic sera were constituted as patient serum pool and negative control pool, respectively. Blots were incubated with a 1:100 dilution of anti-Per a 1 mAbs or 1:10 dilution of patient serum pool or negative control pool in TBST containing 5% nonfat dry milk for 2 h at room temperature. The blots were washed for three 20-min periods in TBST and then incubated with a 1:64 000 dilution of an anti-mouse IgG alkaline phosphatase conjugate (Sigma, St Louis, MO, USA) or 1:100 dilution of antihuman IgE alkaline phosphatase conjugate (PharMingen, San Diego, CA, USA) in TBST containing 5% milk for 2 h at room temperature. The reaction was detected by adding 1.5% of 5-bromo-4-chloro-3-indoyl phosphate and 3% of nitro blue tetrazolium in 0.1 M sodium bicarbonate, pH 8.1.

Direct binding and inhibition ELISA

Purified C42 (1.0 µg/well) or C42-P560 (1.0 µg/well) in coating buffer (0.1 M sodium carbonate, pH 9.6) was coated onto each well and incubated at 37°C for 2 h. After

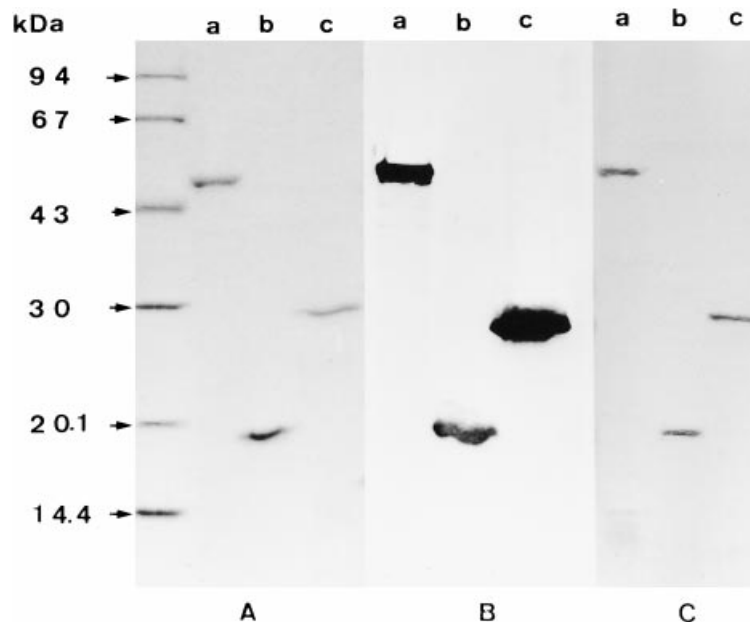


Figure 4. SDS-PAGE analysis (A) and immunoblots (B and C) of recombinant allergens. Purified C42, C42-P560, and C42-P869 recombinant allergens were stained with Coomassie brilliant blue (A), or probed with anti-Per a 1 mAbs (B) or IgE of human allergic serum pool (C). Lane a) C42, lane b) C42-P560, lane c) C42-P869. Numbers at left indicate sizes of protein markers.

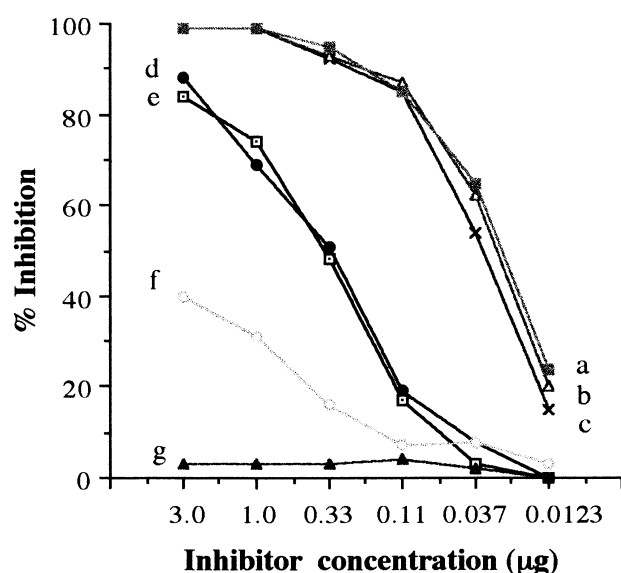


Figure 5. Binding inhibition of IgE to C42 by C42-P560 (a), C42-P869 (b), C42 (c), Per a 1 (d), CRa-A (e), CRa-G (f), and Per a 3 (g). Microtiter plates were coated with purified C42 recombinant protein, and results are average of experiment performed in triplicate, as described.

incubation, wells were washed five times with PBS-0.01% Tween (PBST) and then filled with 1% normal goat serum and incubated for 2 h at room temperature. Wells were washed and incubated overnight at 4°C either with 1:10 dilution of patient or nonatopic serum for direct binding or with different concentrations of CRa-G, CRa-A, Per a 3, Per a 1, C42, C42-P869, and C42-P560 which had been preincubated for 2 h with patient serum pool (1:10) at room temperature for inhibition binding. All sera were diluted in PBST containing 1% BSA. After washing, peroxidase-labeled goat antihuman IgE (1:100, Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA) in PBST containing 1% normal goat serum was added, and the mixture was incubated for 2 h at room temperature. The excess antibodies were washed, and the reactions were developed with 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) with 0.03% H₂O₂ and terminated 45 min after incubation with 5% SDS. The results were read at 415 nm by an EAR 400AT Easy Reader (SLT Labinstruments, Salzburg, Austria).

Results

Sequence analyses of C42

Clone C42, isolated from a λ gt22A cDNA library derived from *P. americana* mRNA, contains a 1.6-kb insert which was subcloned in a pSPORT 1 vector, and the cDNA was

sequenced. The nucleotide sequence (1630 bp) of C42 was deposited in the GenBank under the accession number U69260. The 446-amino-acid sequence derived from the longest open reading frame (ORF) is presented in Fig. 1. The C42 cDNA starts with a 1-bp 5'-proximal end followed by a 1338-bp ORF, which begins with an isoleucine and ends with the TAA stop codon proceed with a 288-bp 3'-untranslated region. The predicted molecular mass is 50.1 kDa with an isoelectric point of 5.16. The C42 gene has several unique features. It comprises several identical repeats (at amino-acid residues 1-79, 154-265, and 343-439) separated from each other by "spacer" sequences (86-99 and 273-286; and 113-123 and 301-311). Three potential mitochondrial energy transit peptides (at amino-acid residues 39-47, 225-233, and 414-422) and several possible cAMP- and cGMP-dependent protein kinase phosphorylation sites were found in each repeating domain. There is no cysteine nor any potential glycosylation site in the sequence.

Amino-acid sequence homologies

Alignment of the C42 protein with two other members of the Per a 1 family, C17 and C6, and the *B. germanica* Bla g Bd90K amino-acid sequence revealed a high level of homology (77.4% identity with C17, 78.1% identity with C6, and 51.6% identity with Bla g Bd90K) among them (Fig. 2). A search of the protein sequence databases showed C42 also to have a 27% identity to *A. gambiae* ANG12 (data not shown).

Allergy skin test

Allergic patients (16-57 years old) and 15 nonallergic adults were skin tested with the commercial American cockroach extracts and Per a 1 allergens. The skin test was further performed on 22 CRa-A- and Per a 1-positive patients with the purified C42, and 54.5% (12/22) of subjects showed a positive skin reaction (Table 1). CRa-A-specific IgE was also determined on these cockroach-sensitive patients using FAST, and all patients were found to carry CRa-A-specific IgE. On the other hand, one of 15 (6.7%) of the nonatopic control group gave a positive reaction to CRa-A and showed no cockroach-specific IgE.

Detection of Per a 1 by anti-C42 antibody

Polyclonal antibody was produced against purified C42, and capacity and specificity were examined by ELISA (Fig. 3).

The response of Per a 1 to anti-C42 antibody in the ELISA was dose dependent, and no Per a 1 allergen was detected with preimmunized mouse serum (data not shown). Polyclonal anti-C42 antibody was able to bind the CRa-A, Per a 1, C42, C17, and C6. All five commercial American cockroach extracts tested also contain detectable Per a 1 allergen, although the reactivities were different among the quantity of allergens in the extracts. Interestingly, anti-C42 antibody was also able to recognize two commercial German cockroach extracts. No detectable allergen was found in Per a 3 allergens using anti-C42 antibody.

Reactivity of murine mAbs and human IgE with recombinant Per a 1

C42 was overexpressed in *E. coli*, and the recombinant protein with an apparent molecular mass of 50 kDa was recognized by both anti-Per a 1 mAbs and human IgE (data not shown). No other protein from *E. coli* BL21 (DE3) was recognized by either probe. C42 was purified by hydroxyapatite and ion-exchange chromatography, and the purification was analyzed on SDS-PAGE gels and immunoblot (Fig. 4). For the IgE-binding epitope studies, the protein was first cleaved enzymatically with endoproteinase Arg-C to generate various internal peptides. After digestion, the proteolytic fragments were separated on SDS-PAGE gels and probed with either anti-Per a 1 mAbs or human IgE (data not shown). The cDNA encoding an immunopositive fragment of C42 was then digested with *EcoRI* and *NotI*. Two overlapping gene fragments (C42-P869, 761–1339 bp; C42-P560, 1073–1339 bp) were then subcloned into a pET30 vector, expressed in *E. coli*, and purified by Ni²⁺-NTA resin. Immunoblotting revealed that both C42-P560 and C42-P869 were able to recognize anti-Per a 1 mAbs and human IgE (Fig. 4B and C). Recombinant proteins were not recognized by the negative control serum pool (data not shown).

IgE-binding and inhibition studies

The specific human IgE antibodies against C42-P560 were demonstrated by ELISA with 12 C42-skin-test-positive and 10 nonatopic sera. All atopic sera contained specific IgE antibodies to C42-P560. The optical densities for the atopic group and nonatopic ranged from 1.001 to 2.002 and 0.098 to 0.531, respectively. The mean absorbances and standard deviation of atopic and nonatopic were 1.266 ± 0.276 and 0.315 ± 0.167 , respectively, and a statistically significant difference ($P < 0.01$) was found between them with the *t*-test. These sera were used for the inhibition of IgE binding to C42

and different cockroach allergens used as competitors (Fig. 5). CRa-G, CRa-A, Per a 1, C42, C42-P869, and C42-P560 inhibited 31%, 74%, 69%, 99%, 99%, and 99% of IgE binding to C42 at concentration of 1.0 µg, respectively. No significant inhibition by Per a 3 was observed, as expected.

Discussion

A new member of the Per a 1 family, C42, was identified and characterized. We have shown the DNA sequence of the C42 gene, the purification of the recombinant C42 protein from *E. coli*, and its immunologic reactivity to anti-Per a 1 mAbs. More importantly, this recombinant allergen elicits 54.5% skin reactivity in CRa-A-positive patients. Sequence searches showed a high degree of homology among C42, *B. germanica* Bla g Bd90K, C6, and C17 of Per a 1 and *A. gambiae* ANG12. In ELISA, anti-C42 antibody was able to recognize the CRa-A, Per a 1, C6, C17, five commercial American cockroach, and two German cockroach skin-test extracts. C42, like C6 and C17, consists of internal repeat sequences, which are highly conserved in Per a 1, and share a high degree of identity with Bla g Bd90K (22). Bla g Bd90K binds 77% of cockroach-allergic IgE antibody, and carries a 4125 bp nucleotide containing seven 576-bp-long tandem repeats (around 90% homology among them) (23). Its deduced amino-acid sequence was untranslatable from all seven repeats except "repeat 1" of the gene. This "repeat 1" (192-amino-acid peptide) shares a 51.6% identity with the internal repeat of C42 at the protein level. In agreement with this, the crude *B. germanica* extract was able to inhibit the binding of IgE antibodies to C42, as with C6 and C17 (22). In addition, the predicted 4.0-kb mRNA of Bla g Bd90K was detected in *B. germanica* by the C42 cDNA probe, like the C6 and C17 probes (22), and similar Northern blot results were obtained in *P. americana* with C42, C6, and C17 cDNA probes (data not shown).

Besides Bla g Bd90K (90 kDa), three other species-specific *B. germanica* allergens, Bla g 2 (36 kDa; an aspartic protease), Bla g 4 (21 kDa; a calycin), and Bla g 5 (23 kDa; a glutathione transferase), have also been cloned (29–31). *P. americana* and *B. germanica* are two major cockroaches occurring commonly in households worldwide. Immunologic cross-reactive allergens between these two species have been reported (32–37), including *B. germanica* Bla g 1 and *P. americana* Per a 1 allergens (33, 36). It is known that most of the population who are allergic to cockroach usually show positive reaction to the extracts of both *P. americana* and *B. germanica* (37, 38). Recently, a Per a 1

and three Bla g 1 cDNA clones were isolated and identified with mAbs and human IgE (22). These clones also have internal repeat sequences and exhibit high homology with Bla g Bd90K, and *P. americana* C6, C17, and C42 clones (unpublished data). It has been suggested that *P. americana* Cr-P11 and *B. germanica* Bla g Bd90K allergens be redesignated Per a 1 and Bla g 1, respectively. Taken together, these results and present studies show unequivocally that cross-reactive allergens exist between *P. americana* and *B. germanica*.

To determine the major sites on the C42 protein that can elicit an antibody response, we attempted to localize the antigenic sites of the allergen. The preliminary IgE-binding epitope studies were done with a combination of proteolytic fragmentation and cloning specific predetermined fragments, which were generated with existing restriction enzyme sites in the cDNA. Immunoblotting revealed that the overlapping C42-P869 and C42-P560 peptides were recognized by both anti-Per a 1 mAbs and IgE from C42-skin-test-positive subjects. In addition, the specific IgE antibodies to C42-P560 were detected in all 12 C42-skin-test-positive subjects by ELISA. Moreover, CRa-A, Per a 1, C42, C42-P869, and C42-P560 were all able to compete in binding of IgE antibodies to C42 with various degrees of inhibition. Lower degrees of inhibitions found in CRa-A and Per a 1 may have been due to the mixtures of different components in the inhibitors. Similar inhibition curves of IgE were obtained among C42, C42-P869, and C42-P560. These results suggest that the shortest fragment C42-P560, an 89-amino-acid-long peptide (amino-acid residues at 358–446), contains a major antigenic site.

It has become apparent that many allergens demonstrate a range of biochemical activities suggesting that these proteins may also contribute to immunogenicity (39). In

addition, repeated motifs are thought to arise through intragenic duplication, and survive if they improve protein function (40, 41). Interestingly, the allergenic C42-P560 fragment is located inside the repeating sequence. The importance of this repeated domain is not yet known. However, this repeat “Per a 1” motif contains the following significant characteristics:

- 1) the B-cell-binding site(s)
- 2) homology to *B. germanica* allergens
- 3) a mitochondrial transit sequence – a potential energy transfer protein
- 4) several kinase phosphorylation sites – a phosphoryl protein.

This “Per a 1” motif is duplicated several times throughout the C42, C6, and C17. This suggests that the “Per a 1” motif exists as a tandem repeat essential for their biologic function.

The results of this study have defined the boundaries of the antigenic site, and we are now in the process of systematically narrowing down the epitope sites. Additional information on the relationship between the multiple T- and B-cell epitopes on other major allergens of *P. americana* will be studied. These characterized allergens make it possible to analyze the antigenic structure of the allergens, and will aid in developing effective immunotherapy and elucidating the mechanism of IgE-mediated reactions.

Acknowledgments This work was supported by grants from the National Science Council (NSC86-2314-B075A-007 and NSC87-2314-B075A-008) and the Taichung Veterans General Hospital (TCVGH 867302C) of the Republic of China. We thank Dr Shue-Fen Luo for help with the skin test experiments.

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