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Recombinant American Cockroach Component, Per a 1, Reactive to IgE of Allergic Thai Patients

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SUMMARY **Twelve similar recombinant Per a 1 clones were produced from an American cockroach (CR) cDNA library. The nucleotide sequence of a representative cline, i.e. clone A6, contained 579 base pairs (bp) and a 372 bp open reading frame (2-373) encoding 124 amino acids. A stop codon was found at position 374-376 followed by a 3' end untranslated region with an AATAAA polyadenylation signal and a poly (A) tail. The estimated molecular mass of the 24 amino acid residue protein was 13.8 kDa, with a predicted isoelectric point value of 4.74. Cysteine or N-linked glycosylation was not found. The deduced amino acid sequence of the A6 revealed 84.68-95.97% identity to other previously reported Per a 1 clones and 65.87-69.60% homology to the previously reported Bla g 1 clones. However, while previously reported Per a 1 clones showed homology to ANG12, a precursor protein in the midgut of the female *Anopheles gambiae* secreted after the blood meal, the A6 DNA sequence was found to have homology (37.1%) to DNA of G2, a putative protein in the midgut of *Aedes aegypti* (AY 050565). The deduced amino acid sequence of A6 contained a mitochondrial energy transfer protein signature, phosphorylation sites for the cAMP-and cGMP-dependent protein kinase C and casein kinase II. Hydrophobic and hydrophilic characteristics of the A6 deduced peptide indicated that it was a transmembrane protein. This is the first report that Per a 1 is a transmembrane protein. The deduced amino acid sequence of the A6, which contained the sequence LIRSLFGLP, differed in one amino acid from two previously reported epitopes, i.e. LIRALFGL and IRSWFGLP, of Per a 1.0104 which bound 80% and 100%, respectively, to IgE of the allergic patients tested. The A6 DNA sequence was deposited in the GenBank (Accession number AY 259514) and has been designated Per a 1.0105. The A6 expressed protein bound to monoclonal antibodies (MAb 3C2) specific to American cockroach and also bound to IgE of all (100%) of the 20 allergic Thai patients**

The cockroach is a domestic pest commonly found in urban dwellings worldwide. During infestation, cockroaches produce a variety of substances containing allergen that can trigger an asthma attack.[1-3] The sources of relevant cockroach allergens include fecal material, secretions, egg castings, dead bodies and body washes.[4-7] American cockroach (*Periplaneta Americana*) allergens involved in immediate hypersensitivity reactions have been identified by various immunochemical methods, such as immunoblotting, crossed-radioimmunoelectrophoresis, and RAST inhibition. The major allergens have been found to range in molecular weights from 6 to 120 kDa.[3,8-11] In 1988, Wu and Lan[11] identified two major allergenic fractions after Sephadex G-50 SF resin filtration of crude American cockroach extract. The high molecular weight protein fraction (> 50 kDa), i.e. the Cr-PI fraction, was found to contain two major allergens of 78 and 72 kDa, which elicited skin reactivity in 73% of cockroach-sensitive patients and also proved to induce T-cell proliferation.[12] Cr-PIII (fraction of proteins at MW of 12 to 45 kDa) contained highly reactive allergens of 45, 40, 38, 32 and 28 kDa that elicited a skin reaction in 93% of patients who were sensitive to the crude cockroach extract.[3,8,9,13] Cr-PI and Cr-PII were cloned and designated as Per a 3 and Per a 1 in accordance with the WHO/IUIS nomenclature system, respectively.[14,15] In addition,

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P. americana tropomyosin was also purified from whole body extract. The 37 kDa protein bound to 41% of IgE of CR allergic patients tested. It was cloned and designated as Per a 7.[16]

The incidence of hypersensitivity to the American cockroach, the most abundant cockroach species in Thailand,[17] among the allergic rhinitis and asthmatic Thai population was 44-61%.[18-20] However, the diagnosis and monitoring of the patients were mostly based on skin testing using commercial cockroach extracts which vary in their allergenic activities. The analysis of specific allergenic levels of 14 commercial American cockroach extracts have shown to vary between 10-250 BAU/ml and only 6 extracts reached the estimated therapeutic dose.[21]

The use of genetically engineered recombinants can overcome this problem. With the access to recombinant allergens, a standardization of cockroach allergens will eventually become possible, which should lead to an improvement of the diagnosis and therapy of patients. We report here the production of a recombinant Per a 1 which reacted to IgE in serum samples of CR allergic Thai patients.

MATERIALS AND METHODS

Patient sera

Serum samples were obtained from 5 non-allergic individuals and 20 patients allergic to cockroach attending the Allergy Clinic of the Department of Pediatrics, Faculty of Medicine Siriraj Hospital, Mahidol University in Bangkok. Equal volumes of sera from 5 patients and 5 non-allergic individuals were separately pooled to constitute an atopic serum pool and a non-atopic serum control, respectively.

Monoclonal antibodies

Monoclonal antibodies (MAbs), namely 38G6 and 3C2 were raised against crude American cockroach extract.[22] The MAb of clone 38G6 reacted to components located between >207 to 72 kDa components of the cockroach antigen, whereas the MAb of clone 3C2 bound to components between 45 to 40 kDa. Both of them showed specificity to American cockroach antigen and did not cross-react to other heterologous antigens including other cockroach species and inhabitant commensal bacteria such as *E. coli*. These two MAbs were shown to be directed against American cockroach allergens by using them as capture antibodies. The affinity-purified antigens of these MAbs were shown to give a positive reaction in the Prausnitz-Kustner (PK) test. The two MAbs were IgG1 isotypes and their indirect ELISA titers against the crude American cockroach antigens were 1:128 (MAb38G6) and 1:64 (MAb3C2).

Genomic DNA extraction

Live adult American cockroaches were trapped, identified, quick frozen, lyophilized and stored at –70°C. Genomic DNA was extracted from the frozen whole bodies of mixed sexes of American cockroaches according to the methods of Sambrook *et al.*[23] and used as a template for DNA probe preparation.

Group 1 cockroach allergen DNA probe

Six Group 1 cockroach allergen mRNA sequences, including 5 American cockroach allergens (accession number AF072222, U69260, U69261, U69957 and U78970) and 1 German cockroach allergen (accession number L47595) submitted to the GenBank were used to align and search for conserved sequences by using the DNAMAN software Version 4.15 (Lynnon BioSoft, Australia). Group 1 cockroach allergen DNA primers were then designed from the conserved sequences by using the OLIGO 4.1 primer analysis software. The primers were: GR1F, 5′-AACGCAAGCATACTCGCA-3′ (sense); and GR1R, 5′-TGGGACAAATGGTGGCAA-3′ (antisense). Heat-denatured cockroach genomic DNA (100 ng) was mixed with the described Group 1 cockroach allergen DNA primers (50 nmol each) in a standard PCR mixture plus 0.5 nmol of DIG-11-dUTP (Roche Applied Science, Germany) to the final 25 µl reaction volume. The mixture was subjected to amplification with *Taq* DNA polymerase (Finnzymes, Espoo, Finland) under the following conditions: initial denaturation at 94°C for 5 minutes, then 35 cycles at 94°C for 1 minute, 55 °C for 1 minute, and 72°C for 1 minute and a final incubation at 72 °C for 7 minutes. After agarose gel electrophoresis, a single band of 560 bp was isolated from the 0.8% agarose gel (Sigma-Aldrich Co., St. Louis, MO) and used as DNA probe.

Cloning of American cockroach cDNAs and screening

Total RNA was extracted from the frozen whole bodies of both sexes of American cockroaches with guanidine isothiocyanate in the presence of β-mercaptoethanol. Polyadenylated mRNA was isolated using

a mRNA isolation kit (Stratagene, La Jolla, CA) according to the method recommended by the manufacturer. An American cockroach cDNA library was prepared from 5 μg of mRNA in the Uni-ZAP XR phagemid expression vector (Stratagene, La Jolla, CA) with an estimated amplified titer of 7×10^9 pfu/ml. The library was size-fractionated to carry inserts greater than 400 bp. Recombinant plaques were grown on NZY agar, and screened with Group 1 cockroach allergen DNA probes. Positive plaques were identified using a 1:10,000 dilution of alkaline phosphatase-labeled anti-DIG (Roche Applied Science, Mannheim, Germany) and 5-bromo-4-chloro-3-indolyl phosphate/nitro-blue tetrazolium substrate (KPL, Gaithersburg, MD). Positive cDNA clones were further screened by plaque immunoassay with a 1:2 dilution of pooled human anti-cockroach serum using biotin-mouse anti-human IgE (ε-specific: Zymed Laboratory, South San Francisco, CA) and streptavidin-alkaline phosphatase as detection systems.

In vivo excision and sequencing

The pBluescript phagemid containing the Per a 1 cDNA inserts, were excised from the LambdaZap II vector by co-infecting XL1-Blue MRF′ cells with a portion of cockroach clone stocks and ExAssist helper phage (Stratagene, La Jolla, CA). The mixture was incubated at 37°C for 15 minutes after which 3 ml of Luria-Bertani (LB) broth was added to the preparation. After incubation at 37°C for 3 hours, the culture was heated at 70°C for 20 minutes, and the cells pelleted by centrifugation at 5,000 × *g* for 15 minutes. The supernatant containing the packaged excised phagemid was then used to infect SOLR cells (Stratagene, La Jolla, CA). SOLR cells containing the Per a 1 inserts were selected on LB-ampicillin plates. Plasmids were isolated from these colonies by standard methods.[23] Double stranded sequencing of the cDNA clone (A6) was carried out by dideoxynucleotide chain termination using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Norwalk, CT)

Sequence analysis

Nucleotide sequences data were analyzed by using the Sequencher software (Gene Codes Corporation, Ann Arbor, MI) and the DNAMAN software Version 4.15 (Lynnon BioSoft, Australia). Deduced amino acid sequences were compared to GenBank databases with the BLAST network service. The version of BLAST used was BLASTX 2.2.4 (26 Aug 2002).

Recombinant DNA subcloning and expression

Clone A6 cDNA was digested with *Bam*H I and *Xho* I restriction enzymes (New England Biolabs, Beverly, MA). The enzyme-digested DNA insert was purified and ligated into the *Bam*H I and *Xho* I sites of the pTrcHis2A expression vector (Invitrogen, Carlsbad, CA). The recombinant DNA was transformed by electroporation into the DH5α *E. coli*. The clones incorporating A6 DNA were identified by PCR. The transformed *E. coli* DH5α was grown in LB medium containing ampicillin at 37°C overnight. Cells of the stationary culture were inoculated into fresh LB medium and incubated at 37°C until the optical density at 600 nm reached 0.6. Then isopropyl-1-thio-β-D-galactopyranoside (Sigma-Aldrich Co., St. Louis, MO) was added to a final concentration of 1 mM and incubated at 37°C with shaking. Cells were harvested at 4 hours after induction and resuspended in SDS sample buffer and boiled for 5 minutes before being subjected to a 12% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

Recombinant allergen purification

Recombinant proteins were over-expressed in *E. coli* DH5α as described. The culture was harvested and the cells were lysed with guanidinium lysis buffer (6 M guanidine hydrochloride, 20 mM sodium phosphate, 500 mM sodium chloride, pH 8.0). The lysates were purified using a Nickel column under denaturing conditions as described by Invitrogen (Probond purification system: Invitrogen, Carlsbad, CA). The eluates were desalted using HiTrap Desalting column (Amersham Pharmacia Biotech, Piscataway, NJ), concentrated using Centriprep particle separators (MW cut-off 10,000: Millipore, Billerica, MA) and assessed for purity by SDS-PAGE and Coomassie Brilliant Blue R-250 staining (Sigma-Aldrich Co., St. Louis, MO). Purified recombinant proteins were used for immunoblotting and inhibition ELISA.

IgE immunoblotting

Purified recombinant proteins were separated on SDS-PAGE gels under denatured conditions with a discontinuous buffer according to the method of Laemmli.[24] After electrophoresis, proteins were transblotted

onto a 0.22 μm nitrocellulose membrane (Bio-Rad, Hercules, CA) by the method of Towbin.[25] The blots were incubated with a 1:2 dilution of 20 individual human anti-cockroach sera and one non-allergic serum pool derived from 5 non-allergic subjects diluted in dilution buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% bovine serum albumin) at 4°C overnight. Biotin-mouse anti-human IgE (ε-specific: Zymed Laboratory, South San Francisco, CA), streptavidin-alkaline phosphatase and 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium substrate (KPL, Gaithersburg, MD) were used as a detection system.

Detection of recombinant Per a 1 by anti-cockroach monoclonal antibodies

The recombinant protein blots, prepared as described, were incubated with 40 μg/ml of monoclonal antibodies secreted from clone 38G6 and clone 3C2 diluted in dilution buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% bovine serum albumin) for 1 hour at room temperature. An alkaline phosphatase-conjugated rabbit anti-mouse antibody (Dakopatts, Glostrup, Denmark) was used as secondary antibody and 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium were used as substrate (KPL, Gaithersburg, MD) for the detection system.

Crude American cockroach extracts

Live adult cockroaches were ground into a powder in liquid nitrogen and then immediately suspended in (1:10 w/v) extraction buffer (10 mM of ethylenediamine tetraacetic acid, 0.1 mM polymethylsulfonyl fluoride and 1 μM E64 in PBS pH 7.4). The cockroach suspension was ultrasonicated at 20 kHz, three times for 3 minutes. The cell debris was clarified by centrifugation, and then dialyzed overnight against distilled water. The protein concentration was determined by Bradford's assay.

Inhibition ELISA

Crude American cockroach extracts (3 μg/well) in 0.1 M bicarbonate buffer, pH 9.6 were coated onto each well of the microtiter ELISA plates (Costar, USA) in triplicates and incubated overnight at 37°C. After washing five times with PBST, the wells were blocked with blocking buffer (1% BSA in PBS) at 37°C for 1 hour. The wells were washed and then incubated with a 1:10 dilution of pooled serum from 5 allergic patients, previously incubated with different concentrations of recombinant protein from clone A6 (3, 2, 1, 0.5, 0.25, 0.125 μg/ml) or 1% BSA for 2 hours at room temperature. The wells were incubated at 4°C overnight, and biotin-mouse anti-human IgE (ε-specific: Zymed Laboratory, South San Francisco, CA), peroxidase conjugated streptavidin solution (Dakopatts, Glostrup, Denmark) and O-phenylenediamine (Zymed Laboratory, South San Francisco, CA) were used as a detection system. Crude American cockroach extract was used as inhibitor for the positive control, and non-allergic sera pre-incubated with the inhibitor used as a negative control.

RESULTS

Isolation of cDNA clones encoding Per a 1

The group 1 allergen DNA probe was used to screen for the Per a 1 isoallergen from the American cockroach cDNA library. Twelve positive clones were isolated after screening with the Group 1 cockroach allergen DNA probe and human anti-cockroach serum pool. After sequencing, they were found to be redundant. Thus, the clone A6 was selected for further study. The nucleotide sequence and the deduced amino acid sequence of the clone A6 are shown in Fig.1.

Nucleotide and deduced amino acid sequences of Per a 1

Clone A6 was found to contain 579 base pairs and a 372 base pair open reading frame (2-373), encoding 124 amino acids. A stop codon (TGA) was found at position 374-376, and followed by a 3′ end untranslated region with an AATAAA polyadenylation signal and a poly(A) tail. The estimated molecular mass of the 124 amino acid residue protein was 13.8 kDa with a predicted isoelectric point value of 4.74. Cysteine or potential N-linked glycosylation sites could not be found. This clone, GenBank Accession Number AY259514, was hereby designated as Per a 1.0105 in accordance with the WHO/IUIS allergen nomenclature.

Hydrophilicity and hydrophobicity

The deduced amino acid sequence of clone A6 was used to predict membrane-spanning domains, potential antigenic sites and regions that were likely to expose on the protein's surface by characterizing its hydrophobic and hydrophilic character (Fig. 2).

Homology between A6 and other sequences

A comparison of deduced

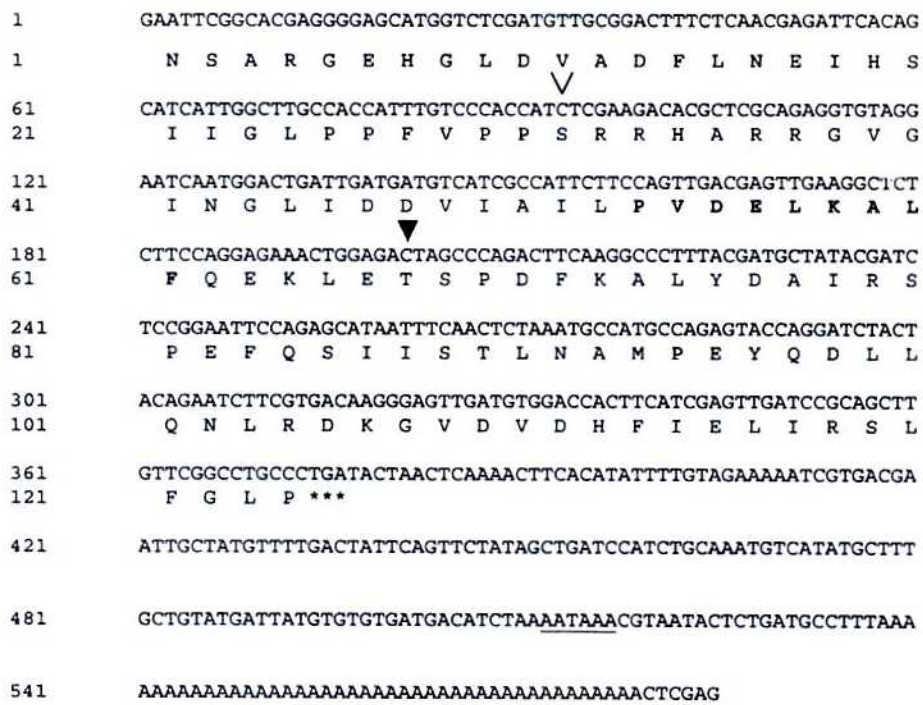

Fig. 1 Nucleotide and deduced amino acid sequences of the *P. americana* cDNA clone A6, encoding *P. americana* Per a 1. The initiation methionine is lacking from the deduced sequence. The mitochondrial energy transfer protein signature is bold, phosphorylation sites for cAMP- and cGMP-dependent protein kinase C (⋁), and casein kinase II (▼) are also indicated. No potential N-linked glycosylation sites were identified. The stop codon TGA is shown (*), and a polyadenylation signal sequence, AATAAA, in the 3′-non-coding region, is underlined. These sequence data are available from the GenBank database under accession number AY259514.

amino acid sequences of clone A6 with the other Per a 1 clones revealed 95.97% (Per a 1.0101, GenBank AF072222), 95.16% (Per a 1.0102, U78970), 91.94% (Per a 1.0103, U69957), 90.32% (Per a 1.0104, U69261), and 84.68% (Per a 1.02, U69260) identity (Fig. 3). When compared to Bla g 1 clones, it was found that A6 shared 67.20% (AF072219), 69.60% (AF072220), and 65.87% (AF072221), identity. Database searches showed that besides the other Group 1 cockroach allergens (Per a 1 and Bla g 1), A6 sequence was found to be homologue to G12, a putative protein in midgut of *Aedes aegypti*, as well as to a microvilli membrane protein of the mosquito. Amino acid sequence alignment of A6 revealed 37.1% homology with both G12 (GenBank AY038041) and the microvilli membrane protein (GenBank AY050565).

Production of recombinant Per a 1

An A6 cDNA insert was isolated and cloned into the *Bam*H I and *Xho* I sites of pTrcHis2A, and the protein was overexpressed in *E. coli* DH5α as a fusion protein. The recombinant protein was purified from the bacterial lysate by Nickel affinity chromatography. The SDS-PAGE analysis of the recombinant Per a 1 revealed that the protein had a molecular mass of 17 kDa (Fig. 4).

Reactivity of human IgE and monoclonal antibodies to recombinant Per a 1

The frequency of IgE binding to recombinant Per a 1 was determined by immunoblotting. Fig. 4 illustrates immunoblot strips, which had been incubated with sera from 20 individual patients and a non-atopic serum pool derived from sera of 5 non-allergic subjects. All patient

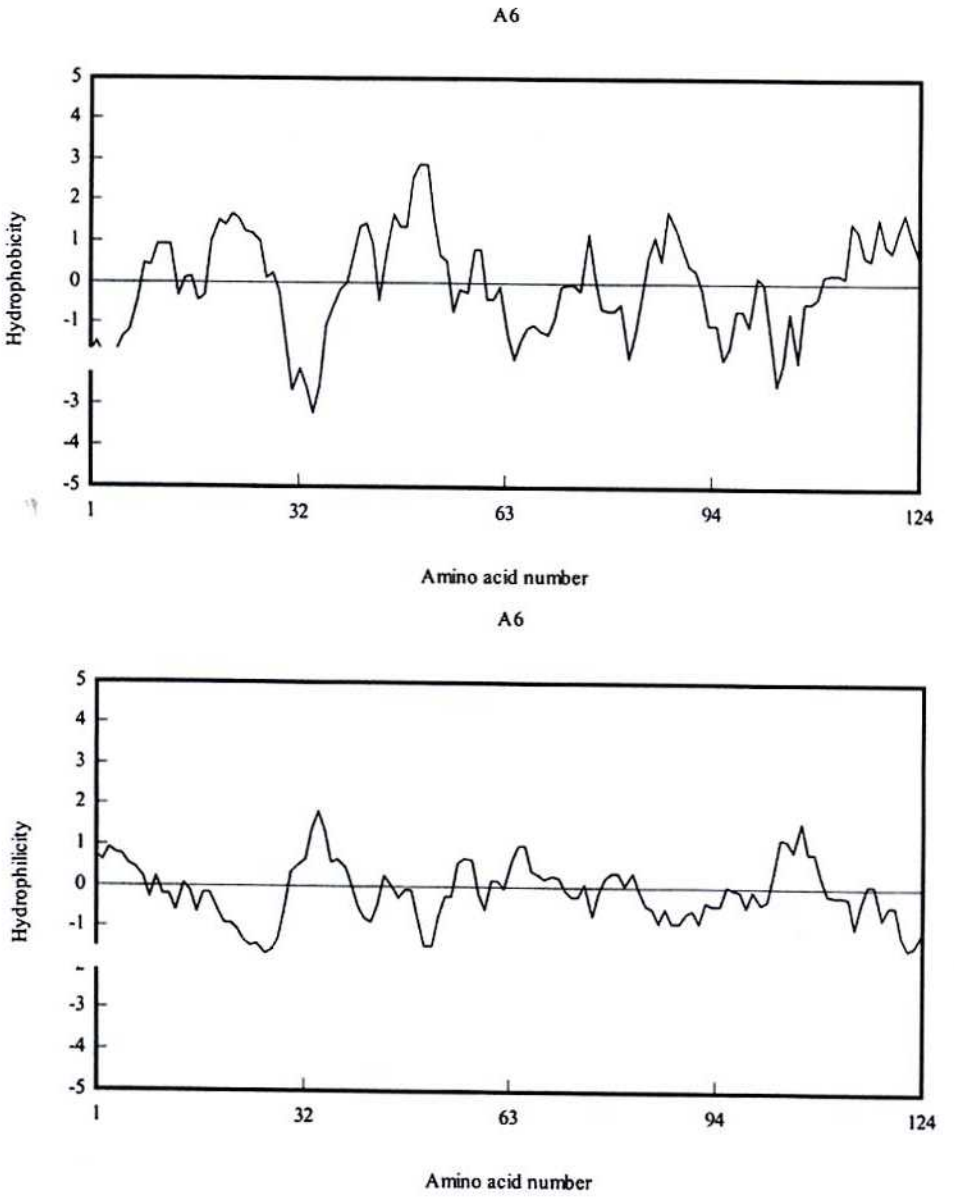

Fig. 2 Hydrophobic and hydrophilic characteristics of clone A6 protein analyzed by DNAMAN software Version 4.15 (Lynnon BioSoft, Australia).

sera tested had IgE that recognized this recombinant cockroach allergen, whereas the non-atopic serum failed to recognize the recombinant protein. These results indicate that the recombinant protein expressed from clone A6 is specific to cockroach-sensitive individuals.

Similarly, anti-cockroach monoclonal antibodies secreted from clone 3C2 bound strongly to recombinant allergen expressed by clone A6, whereas monoclonal antibodies secreted from clone 38G6 gave a negative reaction. These results suggested that the monoclonal antibodies secreted from clone 3C2 were directed against Per a 1 allergen.

IgE binding inhibition of crude cockroach extracts by recombinant Per a 1

The IgE binding specificity of the recombinant cockroach al-

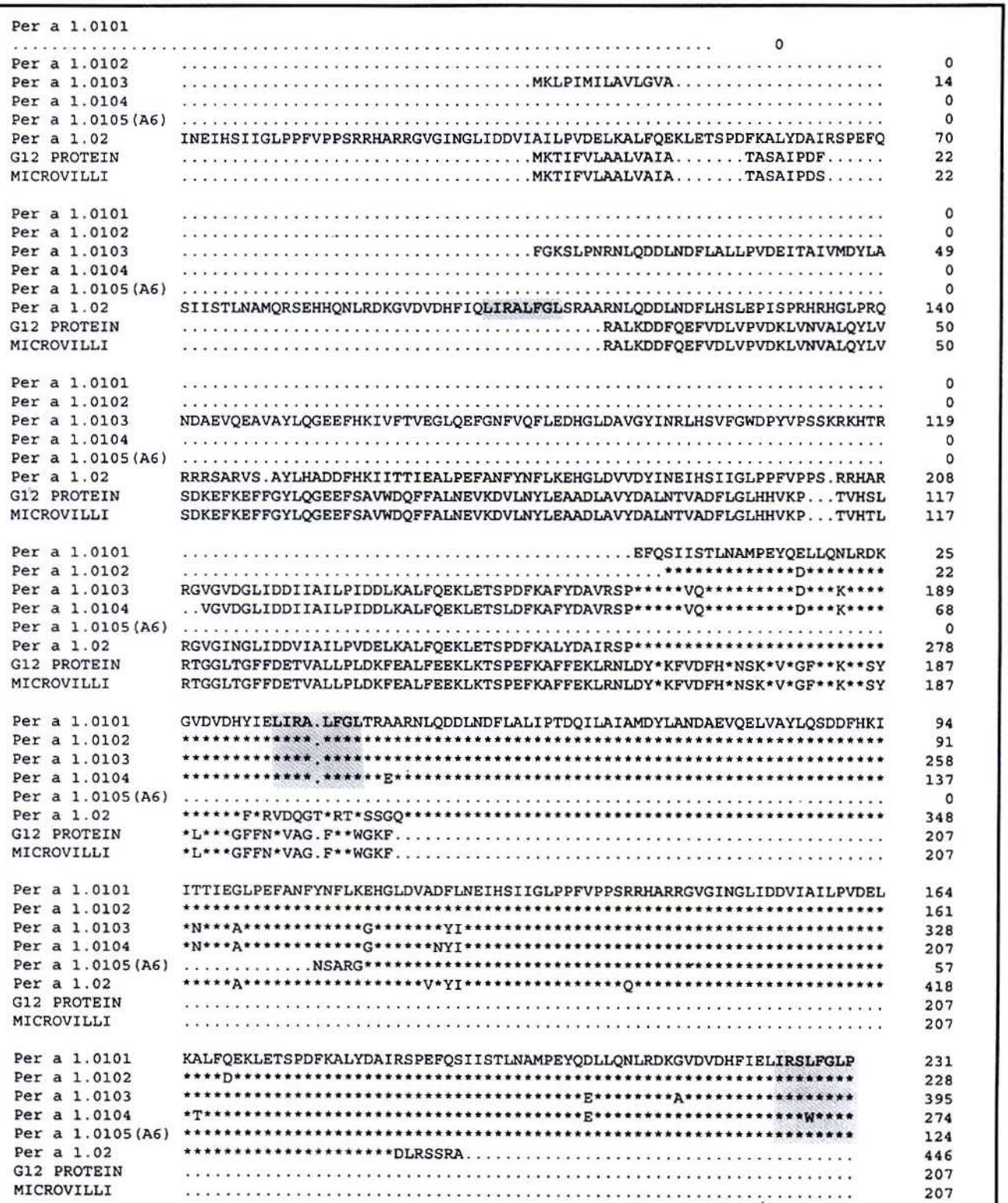

Fig. 3 Comparison of A6 with other Per a 1 clones: Per a 1.0101 (GenBank AF72222), Per a 1.0102 (GenBank U78970), Per a 1.0103 (GenBank U69957), Per a 1.0104 (GenBank U69261), Per a 1.02 (GenBank U69260), mosquito protein G12 (GenBank AY038041), and mosquito microvilli membrane protein (GenBank AY050565). A single-letter amino acid code is used. IgE binding sequences are shaded. The asterisk (*) represents a residue identical to that of Per a 1.0101, and gaps (...) are introduced by the program to optimize alignment.

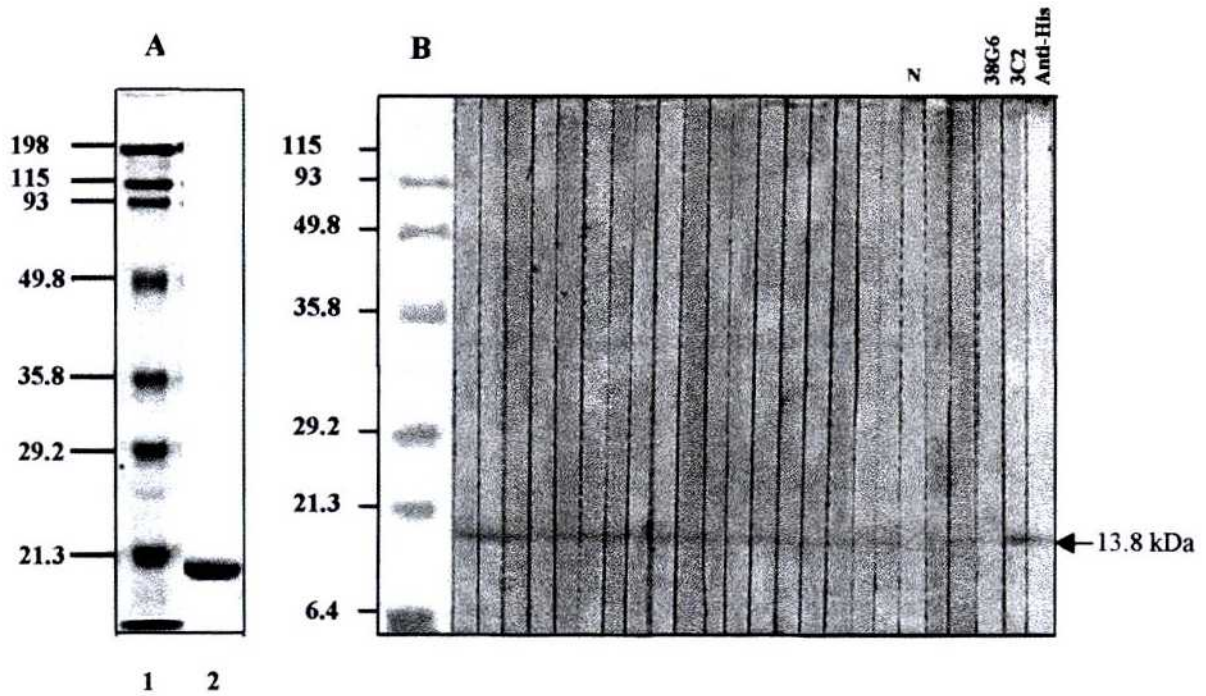

Fig. 4 SDS-PAGE (A) and immunoblotting (B) of recombinant protein expressed from clone A6. The protein was stained with Coomassie brilliant blue (A), or probed with patient sera, a non-allergic serum pool,[37] anti-cockroach monoclonal antibodies (38G6 and 3C2) and anti-Histidine (C-terminal) (B). Numbers on the left indicate the sizes (kDa) of the protein markers.

lergen was assessed by ELISA-inhibition using natural cockroach extract-coated plates and the recombinant Per a 1 as a competitor. Five sera from cockroach allergic patients were pooled and used for the inhibition assay. At a concentration of 3 μg/ml, recombinant Per a 1 was able to inhibit IgE binding to the natural extract up to 62.5% when allergic sera against American cockroach were used, while natural cockroach extracts produced an inhibition of 90.2% (Fig. 5). No significant effect was detected when BSA was used as inhibitor or when recombinant Per a 1 was preincubated with non-allergic serum pool.

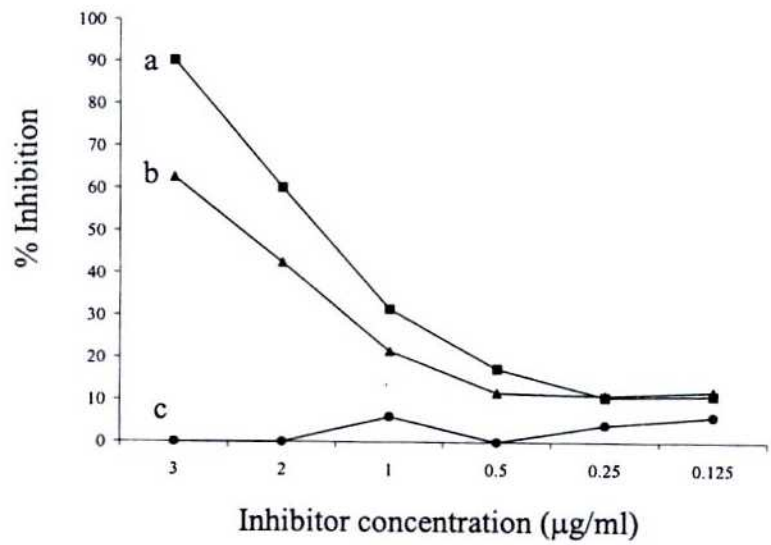

Fig. 5 Inhibition of IgE binding to crude American cockroach extracts by crude American cockroach extracts (a), recombinant Per a 1 (b), and non-allergic serum (c). Microtiter plates were coated with crude American cockroach extract, and results are an average of the experiments performed in triplicates as described in text.

DISCUSSION

The allergenic importance of group 1 cockroach allergens (Per a 1

and Bla g 1) has been documented by several studies.[13,26-28] However, the molecular nature and biologic function of group 1 cockroach allergens have not been clearly defined[14,29-31] and never been tested in cockroach allergic Thai patients.

In the present study, we have produced twelve similar Per a 1 clones from an American cockroach cDNA library. The deduced amino acid sequence of a representative clone, A6, revealed 84.68-95.97% identity to other Per a 1 clones. While all of the previously reported Per a 1 clones showed a homology to ANG12, a precursor protein in the midgut of the female *Anopheles gambiae* secreted after the blood meal, the A6 DNA sequence was found to be homologue to G12 (37.1%), a putative protein in the midgut of *Aedes aegypti* (AY038041) as well as to a microvilli membrane protein of the mosquito (AY050565). The deduced amino acid sequence analysis of A6 contains a mitochondrial energy transfer protein signature, phosphorylation sites for cAMP- and cGMP-dependent protein kinase C and casein kinase II (Fig. 1). Hydrophobic and hydrophilic characteristics of the A6 deduced protein indicated that it was a transmembrane protein (Fig. 2). This finding did not conform with the other studies which reported that Per a 1 and Bla g 1 are not membrane proteins but directed to the outside of the cells through the endoplasmic reticulum secretory pathway or through the mitochondrial matrix.[29,32]

Protein members of the mitochondrial carrier protein family are evolutionarily related membrane proteins with three tandem repeated sequences of approximately 100 amino acid residues, each containing two transmembrane domains.[33] The repeated sequence character has been identified in all members of Per a 1 and Bla g 1[14,28-30,34] but the transmembrane feature was first reported in the A6 protein of this study. Taken together, the information suggests that Per a 1 is a mitochondrial carrier protein localized at the microvilli of the American cockroach digestive system and is involved in the transportation of metabolites across the mitochondrial membrane.

The occurrences of polymorphism in cockroach allergens have been reported and each allergenic isoform showed significantly different skin reactivities among asthmatic patients.[15,28,30,35] Recently, the amino acid sequences LIRALFGL and IRSWFGLP of Per a 1.0104 have been reported to be directly involved in human IgE binding.[31] These two linear epitopes are capable to bind 80% (8/10) and 100% (10/10) of IgE sera tested. The two epitopes were used to analyze the secondary structures (DNAMAN software Version 4.15: Lynnon BioSoft, Australia) and found to be α-helices and α-helices & coils, respectively. Deduced amino acids of clone A6, which contained the LIRSLFGLP sequence, had one amino acid different from both previously reported epitopes and had a α-helices & coil structure. The recombinant protein could be recognized by anti-cockroach monoclonal antibodies (secreted from clone 3C2) as well as by 100% (20/20) of the Thai patient sera tested. This finding has emphasized the importance of the secondary structure of the recombinant proteins.

The treatment of patients by repeated injection with specific allergens has been known to down-regulate the specific IgE and the cellular responses to these allergens. The use of recombinant cockroach allergen containing specific epitopes, rather than crude extract may improve the efficiency of the immunotherapy. In this study, the recombinant Per a 1 was able to inhibit 62.5% of IgE binding to natural cockroach extract, missing about one third. This could be due to the existence of various different cockroach isoallergens involved in the allergenic responses. The use of a panel of IgE haptens, small peptides carrying only one IgE-binding epitope, may be a new immunotherapeutic approach that could reduce anaphylactic side effects.[36]

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