

Recombinant Technology

Identification of the antigenic determinants of the American cockroach allergen Per a 1 by error-prone PCR

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Abstract

The group I allergen of cockroach is found in both American and German cockroaches, designated as Per a 1 and Bla g 1, respectively. Members of these allergens so far identified are composed of tandem repeats that may cause the high allergenicity of Per a 1 allergen. In this study, we used monoclonal antibodies HW-8 and HW-19, which can inhibit the binding of patient IgE to Per a 1 allergen, to define the structure of the antigenic determinants in Per a 1.0103 (designated C3), an isoallergen of Per a 1 allergen. Two recognition sites are present, one in the N-terminus (aa 1–208) and the other in the C-terminus (aa 208–395). The N-terminal epitope is not accessible to antibody molecules on the pET-expressed C3 protein. The C-terminal epitope was further localized to the aa 267–354 region (C3E) by colony immunoscreening of the cDNA epitope library. By negative screening of the mutated C3E expression library generated by error-prone PCR (ER-PCR), an approach which has rarely been applied in epitope mapping, the functional epitope was identified to lie in aa 318–337 with aa 323–331 being the core motif. The minimal region of the functional epitope was further delineated, by sequence alignment, to be D-x-[I, L]-A-[I, L]-L-P-V-D-E-[L, I]-x-A-[L, I], where x represents any amino acids. This motif is found in all Per a 1 allergens and may serve as a basis for designing a peptide vaccine for allergen-specific immunotherapy. To our knowledge, this is the first report for (1) detailed mapping of the cockroach allergens and (2) use of error-prone PCR random mutagenesis and negative selection in molecular allergology.

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1. Introduction

Cockroach allergens are one of the major etiologic risk factors for IgE-mediated allergic respiratory illness throughout the world (Kang, 1990; Cuesta et al., 1995; Zhong, 1996; Custovic et al., 1998; Sarpong and Corey, 1998), such as allergic rhinitis and asthma.

Over 3500 cockroach species exist, the most common being German (*Blattella germanica*) and American (*Periplaneta americana*). Three major allergens from *P. americana*, Per a 1 (Cr-PII), Per a 3 (Cr-PI), and Per a 7 (Wu and Lan, 1988; Wu et al., 1996a,b, 1997a,b, 1998), and five from *B. germanica*, Bla g 1, Bla g 2, Bla g 4, Bla g 5, and Bla g 6 (Schou et al., 1990; Pollart et al., 1991; Arruda et al., 1995a,b, 1997; Helm et al., 1996; Asturias et al., 1999), have been identified. Per a 1 and Bla g 1, comprising a family of isoallergens with unknown function, are a shared

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interspecies allergen (Schou et al., 1990; Wu et al., 1997b, 1998). To date, the near-complete nucleotide sequence has been determined for five Per a 1 (Per a 1.0101, Per a 1.0102, Per a 1.0103, Per a 1.0104, and Per a 1.02) cDNAs (Wu et al., 1998; Melen et al., 1999; Wang et al., 1999; Yang et al., 2000) and three Bla g 1 (Bla g 1.01, Bla g 1.02, and Bla g Bd90K) cDNAs (Helm et al., 1996; Pomes et al., 1998). The complete sequence is only available for Per a 1.0103 that encodes a 395 amino acid polypeptide (designated C3), which can be divided into two tandem repeats (positions 22–206 and 211–395) with 74.2% identity (Yang et al., 2000). A common structural feature revealed from these sequences is that they are composed of internal repeats of ~ 100 amino acid residues (Pomes et al., 1998; Yang et al., 2000), implying the possibility of multiple binding sites for an antibody molecule on these proteins. This structure may explain the high reactivity of Per a 1 with IgE from patients sensitive to cockroach extract (Wu and Lan, 1988).

The key event leading to the type I hypersensitivity is the cross linking of FcεRI on the mast cells via cell-bound IgE by allergen. It has been shown that binding of the recombinant epitope of Phl p 1 to IgE antibodies does not induce histamine release from basophils of most pollen allergen patients (Ball et al., 1994). Accordingly, blocking the binding of allergen to the IgE with synthetic B-cell epitopes or induction of blocking IgG with peptide mimotopes may be an ideal therapeutic agent for type I allergic reaction (Valenta et al., 1998, 1999; Ball et al., 1999b). Although several cDNAs coding for cockroach allergens (Arruda et al., 1995a,b, 1997; Helm et al., 1996; Wu et al., 1996b, 1997a; Pomes et al., 1998; Asturias et al., 1999; Melen et al., 1999; Yang et al., 2000) have been sequenced, providing information useful for epitope mapping, the study of B-cell epitopes on cockroach allergens is still in its infancy compared to those on other allergens, e.g. allergens from dust mites (Chapman et al., 1987; van't Hof et al., 1991; Collins et al., 1996; Smith and Chapman, 1997; Takai et al., 1997; Hakkaart et al., 1998; Furmonaviciene et al., 1999), grass pollens (Schenk et al., 1995, 1996; Muller et al., 1998; Ball et al., 1999a; Burton et al., 1999; Flicker et al., 2000; Schramm et al., 2001), and eggs (Elsayed and Stavsen, 1994; Honma et al., 1996). So far, there is only one report describing the

boundaries of the antigenic site on cockroach allergen, in which a potential IgE-reactive region was located within the internal repeat on the C-terminus of Per a 1.02 by gene fragmentation (Wang et al., 1999).

Approaches for studying B-cell epitopes include analyzing the antigenicity of overlapping synthetic peptides (van't Hof et al., 1991; Elsayed and Stavsen, 1994) and allergen fragments produced by chemical degradation (Kahlert et al., 1992), enzymatic digestion (Liebers et al., 1993), or gene fragmentation (Ball et al., 1999a). In addition, antigenic determinants can also be defined using a gene-targeted phage display random epitope library (Wang et al., 1995) or phage display random peptide library (Jespersen et al., 1997; Furmonaviciene et al., 1999).

Error-prone PCR (ER-PCR), in conjunction with functional selection or screening, is a powerful approach in the structure–function analysis of proteins and has been widely applied to generate proteins with desired functions. Antibodies and enzymes with desired properties, such as improved affinity, stability, or altered specificity, have been successfully generated by *in vitro* evolution using ER-PCR as the method of random mutagenesis (Gram et al., 1992; Casson and Manser, 1995; Miyazaki et al., 1999; Riegert et al., 2001; Song and Rhee, 2001). However, this approach has rarely been applied in epitope mapping although it has been demonstrated to be useful in mapping of a conformational epitope (Jespersen et al., 1997). In this study, we employed gene fragmentation and ER-PCR mutagenesis to map the antigenic determinant on Per a 1.0103 allergen using monoclonal antibodies (mAbs) HW-19 and HW-8 capable of inhibiting the binding of human IgE to Per a 1 (Wu et al., 1996a). The results indicated that two recognition sites are present in Per a 1.0103, and only the C terminal one is accessible to antibody molecules on the recombinant C3 protein.

2. Materials and methods

2.1. Construction of expression plasmids

The plasmids constructed in this study are depicted in Fig. 1a, and the C3 protein subfragments are shown in Fig. 1b. Plasmid pET30-C3 was a derivative of pET30a (Novagen, Madison, WI, USA) carrying the

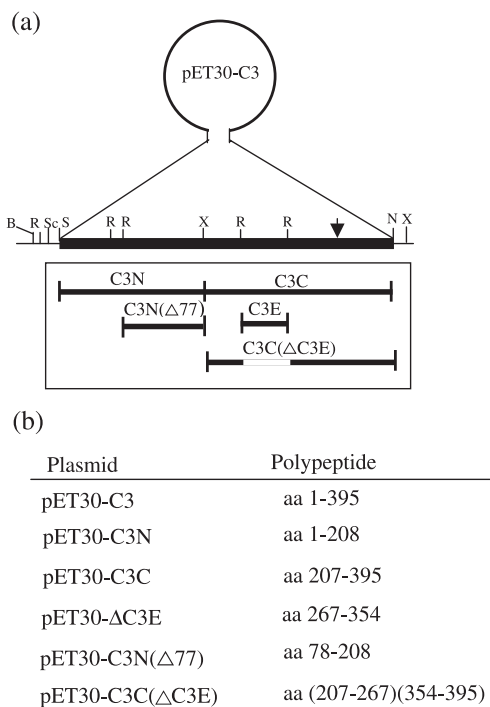


Fig. 1. Schematic diagrams of the pET-expression plasmids (a) constructed in this study and the polypeptide encoded by the inserted DNA (b). Abbreviations for restriction enzymes: B, *Bam*HI; N, *Not*I; R, *Eco*RI; Sc, *Sac*I; S, *Sal*I; X, *Xho*I. The relative position of the stop codon is indicated by an arrow. The plasmid is not drawn to scale.

1.5-kb *Sal*I–*Not*I fragment, with the complete C3 coding region, from the Per a 1.0103 cDNA (Yang et al., 2000). There are two *Xho*I sites in plasmid pET30-C3, one within the C3 DNA (nt 642–647) and the other downstream of the 3' *Not*I site in the vector. Digestion of pET30-C3 with *Xho*I generated two fragments, 6- and 0.8-kb long. Self-ligation of the 6-kb fragment resulted in plasmid pET30-C3N whose insert contained the N-terminal 208 amino acids of C3. To generate plasmid pET30-C3C whose insert contained the C-terminal 189 amino acids of C3, the ~ 0.8-kb DNA fragment was ligated with *Sal*I/*Xho*I double-digested pET30a fragment. Two types of plasmid were present in the ligation products because *Sal*I and *Xho*I are isoschizomers; thus, the 0.8-kb *Xho*I DNA fragment can ligate with the backbone DNA in either direction. These two plasmids can be distinguished by digestions with *Bam*HI and *Not*I. Two DNA bands, ~ 6 and ~ 0.8 kb, would be visualized

when the ~ 0.8-kb *Xho*I DNA fragment was in the desired orientation; otherwise, only one 6.8-kb band would be seen in the gel. Plasmid pET30-C3E was obtained from the expression library (see below). Plasmid pET30-C3C(ΔC3E) was obtained by deleting the *Eco*RI fragment within the C3C. Due to the presence of an *Eco*RI site in the vector DNA of plasmid pET30-C3C, the C3C DNA fragment was first released from pET30-C3C by *Sac*I and *Xho*I digestion and cloned into the corresponding sites of pBlueScript II KS (Stratagene, La Jolla, CA, USA). The resultant plasmid pBS-C3C (~ 3.5 kb) was subjected to *Eco*RI digestion followed by preparative agarose gel electrophoresis and recovering the ~ 3.2-kb *Eco*RI fragment. Plasmid pBS-C3C(ΔC3E) was obtained by self-ligation of the ~ 3.2-kb *Eco*RI fragment. Finally, the C3C(ΔC3E) coding region included in the ~ 0.5-kb *Sac*I–*Xho*I DNA fragment was cloned into the corresponding sites of pET30a to generate plasmid pET30-C3C(ΔC3E). Plasmid pET30-C3N-C was obtained by digestion of plasmid pET30-C3N with *Eco*RI, followed by self-ligation of the large fragment (~ 5.7 kb).

2.2. Construction of libraries

To construct a C3 random fragment pET library, the 1.5-kb *Sal*I–*Not*I DNA fragment from pET30-C3 was subjected to ligation followed by sonication (XL 2020 Sonicator at maximum power for 1 min followed by cooling for 30 s between each burst). The degree of shearing was monitored by agarose gel electrophoresis. The DNA fragments were blunt-ended by treatment with T4 DNA polymerase (New England Biolabs, Beverly, MA, USA), and ligated with an excessive amount of phosphorylated *Eco*RI linkers (New England Biolabs). DNA fragments of 100–300 bp were recovered and ligated to the *Eco*RI-digested pET30a, pET30b, and pET30c vectors. The ligation products were electroporated into electrocompetent *Escherichia coli* BL21(DE3) prepared as described in Sambrook and Maniatis (1989). Electroporation was done using a Bio-Rad Gene Pulse that was set at 25 μF, 2.5 KV, and 200 Ω.

For construction of a library of random C3E variants, error-prone PCR mutagenesis was carried out as described (Gram et al., 1992) with some modifications. PCR (30 cycles, 1 min at 94 °C, 2

min at 50 °C, 1 min at 72 °C) was performed with pET30a-C3E as the template, using Pro Taq DNA polymerase (Protech Technology Enterprise, Taipei, Taiwan). The primers used were the forward 5' pET *Bam*HI primer (5'-GCCATGGCTGATATCGGATCCGAATTC-3') and the backward primer (5'-GCTAGT-TATTGCTCAGCGGTGGC-3') corresponding to the region containing the *Nco*I to *Eco*RI sites and complementary to the upstream and the first 5 nt of the T7 terminator in pET30 vector, respectively. The reaction mixture contained 1 ng of pET30a-C3E, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM MgCl₂, 0.1% (w/v) gelatin, 1% Triton X-100, 0.5 mM MnCl₂, 1 mM each of dCTP, dGTP, and dTTP, 0.2 mM dATP, 0.1 mM dITP, and 0.5 µM each of the primers. The DNA products were digested with *Bam*HI and *Hind*III, gel-purified and ligated into the corresponding restriction enzyme sites of pET30a. The ligation products were electroporated into *E. coli* BL21(DE3) and the transformants were spread on LB plates containing 50 µg/ml ampicillin (amp).

2.3. Colony immunoscreening

Screening of libraries was performed by colony immunoscreening (Sambrook and Maniatis, 1989). To obtain an expression library, sterilized IPTG-impregnated nitrocellulose filters were laid on the plates containing arrayed colonies. The filter, with the bacterial colony contacted side up, was transferred to a fresh LB/amp plate and incubated for 6–8 h at 30 °C. Filters were removed and processed as described (Sambrook and Maniatis, 1989). Briefly, the bacterial colonies on the filter were exposed to chloroform vapor for 15 min, then lysed by submerging the filter to lysis buffer containing 100 mM Tris-HCl (pH 7.8), 150 mM NaCl, 5 mM MgCl₂, 1.5% BSA, 1 µg/ml DNase I, and 40 µg/ml lysozyme at room temperature (RT) with agitation overnight. The filter was washed twice with 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.05% Tween 20 before immunoscreening. After blocking with 5% nonfat milk in PBS, the filter was incubated with the culture supernatant containing mAbs followed by probing with alkaline phosphatase (AP)-conjugated anti-mouse antibodies. Positive clones were visualized by developing the blot with BCIP/NBT.

2.4. Expression and purification of C3 fusion protein and its derivatives

E. coli BL21(DE3) harboring pET30 derivatives were grown in LB containing 50 µg/ml ampicillin to OD₅₅₀ = 0.5–0.8 at 37 °C. Target protein expression was induced by adding 1/1000 vol. of IPTG (200 mg/ml) to the culture and incubating at 28 °C for 8 h. Bacterial cells were collected by centrifugation. Recombinant fusion proteins were purified by using HisBind Purification Kits (Novagen). The concentration of recombinant proteins was determined by using the Bio-Rad Protein Assay Kit (Bio-Rad, CA, USA) and the purity was monitored by SDS-PAGE.

2.5. SDS-PAGE and Western blot analysis

Proteins were separated in 12.5% SDS-polyacrylamide gel following the method of Laemmli (1970). Western blot analysis was carried out essentially as described in Sambrook and Maniatis (1989). The blots were probed with culture supernatants collected from hybridoma HW-8 or HW-19 (Wu et al., 1996a) or rabbit anti-cockroach extract (CRA) (Wu and Lan, 1988) and detected with AP-conjugated goat anti-mouse or anti-rabbit antibodies.

2.6. ELISA

Microtiter plates were coated with 100 µl of purified C3 fusion protein at a concentration of 10 µg/ml in PBS and incubated at 4 °C overnight. The plates were washed three times with 0.05% Tween 20 in saline. Uncoated sites on the plastic were blocked with 5% nonfat milk in PBS for at least 2 h at room temperature (RT). The wells were rinsed three times with Tween 20 saline solution. To determine the optimal concentration of antibody for inhibition ELISA, serial dilutions of hybridoma supernatants (100 µl per well) were added, and the plates were incubated at 4 °C overnight. After rinsing with the Tween 20 saline solution, 100 µl of a 1:5000 dilution of alkaline phosphatase-conjugated goat anti-mouse IgG (Fc specific) (Sigma) was added followed by incubation at RT for 2 h. After rinsing with Tween 20 saline solution, *p*-nitrophenyl phosphate substrate (Sigma) in diethanolamine buffer (pH 9.8) was added. The reaction was stopped by adding 20 µl of 5 N

NaOH, after incubation for 1 h in the dark. The absorbance at 405 was read in a Bio-Kinetic Reader EL 312e (Bio-Tek Instrument Winooski, VT, USA). The supernatant dilution that gave ~ 50% binding was used for inhibition ELISA in which varied amounts of inhibitor were included.

3. Results

3.1. At least two binding sites for mAb HW-19 and HW-8 are present in the C3 allergen

As the first step to map the antigenic determinants of Per a 1, the full-length C3 cDNA was cleaved into two subfragments with *Xho*I endonuclease, which were then separately inserted in correct reading frames into the pET30 expression vector and expressed as fusion proteins in *E. coli*. The resultant fusion proteins containing the N-terminal half (Met1–Arg208) and the C-terminal half (Thr207–Pro395) of the C3 poly-

peptide were designated as C3N and C3C (Fig. 1), respectively. In Western blot analysis, both Ni^{2+} -resin-purified polypeptides were able to react with rabbit anti-CRa and mAb HW-19 with similar specific activities, as determined by comparing the intensities of the immunoreactive bands relative to the corresponding Coomassie blue-stained bands (Fig. 2). Similar intensities were also obtained using mAb HW-8 (data not shown), whose epitope has been proposed to be the same as or overlapping with that of HW-19 (Wu et al., 1996a). The results suggest that the epitope for mAbs HW-19 and HW-8 may not be conformational, and at least two recognition sites, one residing in C3N and the other in C3C, are present in the C3 protein.

3.2. The C-terminal epitope for HW-8 and HW-19 is located within aa 267–354 fragment

To further delimit the C3 antigenic determinants, a random cDNA fragment expression library was con-

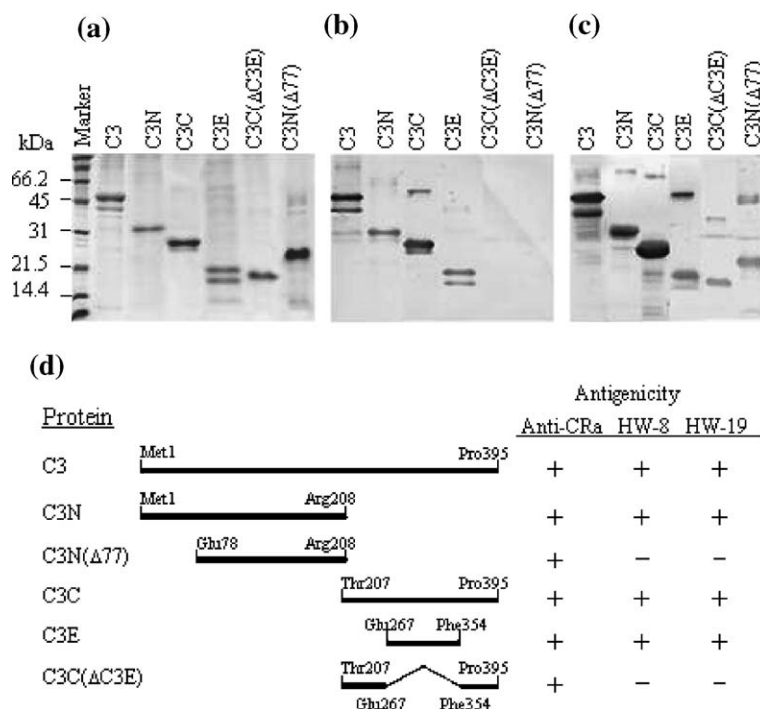


Fig. 2. SDS-PAGE analysis (a), immunoblots (b and c), and the antigenicity of recombinant C3 (Per a 1.003) allergen and its truncated derivatives (d). Ni^{2+} -resin adsorbed proteins were separated in a 12.5% gel under reducing condition and stained with Coomassie Blue (a). The protein bands were transferred to a nitrocellulose membrane and probed with monoclonal antibody HW-19 (b) or rabbit anti-CRa serum (c).

structed. Five hundred clones were randomly picked from the resultant transformants (~5000 colonies) and probed with the mixture of mAbs HW-19 and HW-8 in a colony immunoblotting analysis. Seven reactive clones were obtained. The plasmids were isolated from these seven clones and subjected to nucleotide sequence determination. All plasmids carry the same insert spanning nt 821–1084 encoding Glu267–Phe354, designated as C3E. These results indicate that this region contains the functional epitope for mAbs HW-19 and HW-8. Notably, this region is highly homologous (96% identity) to aa 358–446 of Per a 1.02 which contains an IgE-reactive region (Wang et al., 1999).

To gain further support for this notion, the C3E fragment was deleted from C3C and the resultant fragment, designated as C3CΔ(C3E), as well as the C3E fragment were subjected to Western blot analysis (Fig. 2). Immunoreactive bands corresponding to the fusion C3E (~18 kDa) and fusion C3CΔ(C3E) (~17 kDa) were present in the blot when rabbit anti-CRa antiserum (Fig. 2c) was used as the primary antibody. However, immunoreactive bands were only observed on the lane of fusion C3E protein (lane 4) but not that of C3CΔ(C3E) (lane 5) in the blots probed with HW19 (Fig. 2b) or HW-8 (data not shown). These data confirmed the observation that the Glu267–Phe354 region contains the epitope for mAbs HW-19 and HW-8.

3.3. The HW-19/HW-8 binding site(s) in C3N is contained within the N-terminal 77 amino acid residues

Sequence comparison revealed that the C3E-homologous region in C3N starts from aa78 (Yang et al., 2000). In an attempt to localize the HW-19/HW-8-binding site in the C3N polypeptide, a C3N derivative without the N-terminal 77 amino acid residues, designated as C3N(Δ77), was produced and subjected to Western blotting analysis (Fig. 2b and c, lane 6). The integrity of the resultant polypeptide as a C3 derivative was confirmed by its immunoreactivity with the rabbit anti-CRa antiserum (Fig. 2c). To our surprise, no band was visualized when probed with either HW-19 or HW-8, indicating that the N-terminal 77 amino acid residues are necessary for the integrity of HW-19/HW-8 epitope. The antigenicity of frag-

mented recombinant C3 allergens is summarized in Fig. 2d.

3.4. Although two binding sites for HW-19 and HW-8 are present on C3 protein, only the C-terminal one is accessible to the mAbs

To gain information about the accessibility of the epitope(s) on the non-denatured protein, the relative binding activity of each of the fragmented C3 proteins to HW-8 and HW-19 was evaluated by inhibition ELISA (Fig. 3). The binding of HW-19 and HW-8 to plate-bound C3 was inhibited by soluble C3, C3C, C3E, and C3N, but not C3CΔ(C3E). The inhibition curves for C3 and C3C are almost superimposable to each other, indicating that they have similar affinity to

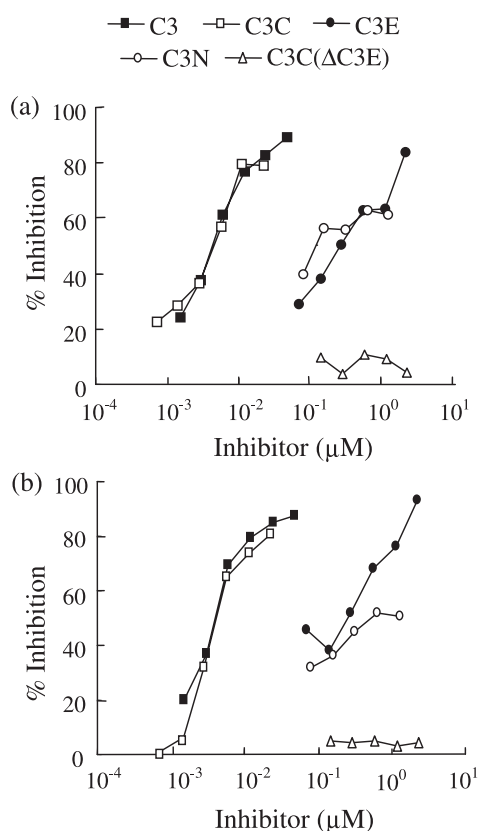


Fig. 3. Inhibition of binding of monoclonal antibodies HW-8 (a) and HW-19 (b) to plate-bound fusion C3 by the soluble fusion C3 and its derivatives.

the test monoclonal antibodies. Approximately 60 times more C3E was required to give a similar inhibition effect as that by C3 or C3C. Although specific inhibition was observed with high concentrations of C3N, the maximal inhibition only reached ~ 65% and ~ 50% for HW-8 (Fig. 3a) and HW-19 (Fig. 3b), respectively. The concentrations of soluble C3, C3C, C3N, and C3E required to give 50% inhibition were approximately 5, 5, 120, and 280 nM for HW-8 and 4, 4, 510, and 260 nM for HW-19, respectively (Fig. 3). Based on these results, we conclude that (i) although two binding sites for HW-19/HW-8 are present in C3 protein, only the one in the C-terminal domain is accessible to the B cell (the primary epitope); (ii) the epitope structure in C3C is similar to that in C3 protein; (iii) the epitope structure in C3E is less accessible than that in C3C and C3; and (iv) the antibody combining sites of HW-8 and HW-19 are slightly different, albeit similar binding activities were observed with the denatured C3 proteins, as judged by the concentrations of C3E and C3N required to give a 50% inhibition effect.

3.5. The primary epitope is within aa 318–317

To define the minimal requirement for forming the functional epitope, error-prone PCR (ER-PCR) was performed using the C3E DNA fragment as the template. The ER-PCR products were cloned in-frame into the pET30 expression vector to generate a mutated C3E library containing ~ 10⁵ transformants.

After three cycles of colony immunoscreening using mAb HW-19 as the probe, 11 negative clones were randomly picked for Western blot analysis. All C3E mutants were reactive with rabbit anti-CRa antiserum; however, no detectable band was visualized in 10 of the clones, C3E-1 to C3E-10, when HW-19 or HW-8 were used as the probes, and a very faint band was observed in clone C3E-11 (data not shown). These results indicated that the epitope for HW19/HW8 was destroyed in clones C3E-1 to C3E-10 and partially preserved in clone C3E-11.

The mutation(s) in each of the eleven clones were determined by nucleotide sequencing of the corresponding plasmid DNA. Twenty-one base substitutions were found in a total of 2904 nucleotides determined. The predominant type of mutation was A → G (9/21), followed by T → C (7/21). Translation mutations occurred rarely with C → A (two cases), and only each for C → T, G → C, and A → T. The rate and types of mutations obtained under our experimental condition are similar to those of previous studies that used an antibody gene as the target (Gram et al., 1992). These 21 nucleotide exchanges resulted in 19 amino acid substitutions distributed between aa 276 to 337 and clustered in the aa 315–337 region (Fig. 4). Of the 10 HW-19- and HW-8-negative clones, C3E-1, C3E-2, C3E-3, and C3E-4 had a single amino acid substitution occurring at aa 323 (Leu → Pro), 327 (Glu → Gly), 328 (Leu → Pro), and 331 (Leu → Pro), respectively. All clones with multiple mutations contained at least one change at these four positions,

	270	280	290	300	310	320	330	340	350
C3E	EFANFYNFL	<u>K</u> GHGLDVADYINEIHS	<u>S</u> IIGLPPEFVPPSRRHARR	GVGING	<u>L</u> IDDVIA	<u>I</u> LPVDELKAL	<u>F</u> QEKLE	TSPDFKALYDAIRSP	EF
C3E1							<u>P</u>		
C3E2								<u>G</u>	
C3E3								<u>P</u>	
C3E4								<u>P</u>	
C3E5						<u>P</u>	<u>P</u>		
C3E6		<u>R</u>						<u>V</u>	
C3E7					<u>G</u>		<u>G</u>		
C3E8						<u>V</u>		<u>P</u>	<u>E</u>
C3E9				<u>L</u>			<u>T</u>	<u>R</u>	<u>P</u>
C3E10			<u>H</u>				<u>S</u> L <u>G</u>		
C3E11						<u>G</u>	<u>T</u>		<u>G</u>

Fig. 4. Comparison of the deduced amino acid sequence of C3E mutants with that of C3E (top line). The single-letter amino acid code is used. The changed amino acid residues are underlined and shaded.

except for C3E-10 that had three consecutive mutations at aa 324–325 and a mutation at aa 291. These results suggested that aa 323 to 331 are critical and may constitute the core motif of the antigenic determinant for HW-8/HW-19. C3E-11 with attenuated reactivity to HW-19 and HW-8 contained mutations at positions 318 (Asp → Gly), 322 (Ile → Thr), and 337 (Glu → Gly). None of these are within the core region: positions 318 and 337 are four and five residues away from the core region (aa 323–331), whereas I322T is adjacent to it. Based on the above observations, peptides corresponding to aa 321–331 and aa 318–337 were synthesized and their binding ability to the monoclonal antibodies was evaluated by dot immunoblottings. Only weak binding activity was observed in aa 318–337 (data not shown), indicating that amino acid residues in the core region are essential but not sufficient to form a functional epitope for HW-19/HW-8; therefore, the minimal structure is included in aa 318–337. This result also confirmed the previous postulate based on results of inhibition ELISA (Wu et al., 1996a) that HW-19 and HW-8 bind to the same or overlapping region of Per a 1 allergen.

3.6. The minimal structure of a functional epitope for HW-19/HW-8 contains LPVDE as the central region

To localize the epitope on C3N, aa 318–337 was aligned with C3N using LALIGN program. Three regions, aa 7–25, aa 30–48, and aa 130–149 with 31.6%, 47.4%, and 85% identities, respectively, were found (Fig. 5). However, only aa 30–48 and 130–149 contain segments with significant homology to the core region aa 323–331 identified by ER-PCR; they are aa 34–43 and aa 140–149. The aa 34–43 segment differs from the aa 323–331 segment at positions 1, 7, 8, and 10. The substitutions at positions 1, 7, and 10 are conserved (Ile ↔ Leu), while a change from Lys to Thr at position 8 is dramatic. The aa 140–149 differs from the core region only at positions 4 and 6, Val to Ile and Glu to Asp, respectively, which have similar properties. Based on the sequence comparison, it is more reasonable to propose that aa 134–143 contributes more than aa 30–43 does in C3N antigenicity. However, epitope mapping with the gene fragmentation approach has demonstrated above that the antigenicity is completely abolished by deleting the N-

85.0% identity in 20 aa overlap			
318	DVIAILPVDELKALFQEKLE		337
			
130	DIIAILPIDDLKALFQEKLE		149
47.4% identity in 19 aa overlap			
318	DVIAILPVDELKALFQEKL		336
	: : . . .		
30	DFLALLPVDEITAI		48
31.6% identity in 19 aa overlap			
319	VIAILPVDELKALFQEKLE		335
	 :		
7	ILAVLGVAFGKSLPNRN		25

Fig. 5. Segments in C3N that aligned with aa 318–337 region of C3. The minimal functional epitopes are boxed. The central region is underlined.

terminal 77 amino acid residues of C3N (Fig. 2). This result attributes the antigenicity of C3N for mAbs HW-19/HW-8 to the region containing aa 34–43, thus implying that the size of the amino acid residue at positions 4 and 6 of the core region is critical in forming the epitope for HW-19/HW-8. The minimal structure of the epitope may consist of at least the first 14 amino acid residues with the LPVDE as the central region delineated from the sequence alignment (Fig. 5) and can be depicted as D-x-[I, L]-A-[I, L]-L-P-V-D-E-[I, L]-x-A-[I, L], where x represents any amino acids.

4. Discussion

In this study, we have systematically elaborated the antigenic determinants of Per a 1 allergen using monoclonal antibodies as the tool. Per a 1.0103 (C3) was used as the representative Per a 1 allergen because it is the only Per a 1 member whose cDNA has been fully sequenced (Yang et al., 2000). It contains two “duplexes”, each comprising of two tandem repeats of ~ 100 amino acid residues. The “duplex”, as a common structural feature for Bla g 1 and Per a 1 allergens, has been proposed to be the basic allergenic unit of Bla g 1 (Pomes et al., 1998) and Per a 1 (Yang et al., 2000). Our results, showing that both C3N and C3C fragments contain a single

duplex which were recognized by mAbs HW-19 (Fig. 2) and HW-8 (data not shown), provide strong support for this proposal. However, our results of inhibition ELISA (Fig. 3) indicated that only the C3C-epitope is accessible to the monoclonal antibodies.

The epitope-containing region of C3C was further narrowed down to aa 267–354 (C3E) by immunoscreening of the cDNA epitope expression library derived from the full-length C3 cDNA. The fact that we could not obtain a clone carrying C3N-epitope from the cDNA epitope expression library was puzzling because the library contained excessive colonies to cover all possible clones. In these experiments, approximately 5000 transformants were obtained from the ligation products. As the insert DNA can ligate with pET30 a-, b-, and c-vector DNA in either direction with all possible reading frames, 50% (~ 2500) of the transformants would have been able to express C3-derived peptides. Therefore, one of the possible explanations is that the C3N-epitope is not functional due to an incompetent structure or is inaccessible under the conditions of colony immunoscreening in which proteins were denatured with chloroform and in a mixture instead of the SDS-, heat-denatured and separated proteins in the Western blots when probed with antibodies.

The functional epitope in C3C was fine-mapped by ER-PCR to aa 318–327 segment in which aa 323–331 was determined to be essential because mutation at any of these residues abolishes the antigenicity completely. Despite the unambiguous result of the mutagenesis analysis, a synthetic 20-residue peptide corresponding to aa 318–327 only weakly bound to HW-19 and HW-8 antibodies in a direct binding assay; antibody blocking was not observed in an inhibition ELISA nor Western blot analysis. For several reasons, synthetic peptides frequently are poor antigen mimics (Benjamin et al., 1984) including that the structure of a free peptide is different from that of a peptide segment within a polypeptide (Stanfield et al., 1990). Taking this into consideration, ER-PCR mutagenesis may be a more beneficial approach in determination of both sequential and conformational epitopes.

Structural analysis of antigen–antibody complexes revealed that antibodies with a sequential epitope generally have a deep binding pocket complementary to three to eight amino acid residues, while antibodies bound to globular antigens typically make contact

with the antigen across a larger flat face with 15–20 side chains (Goodman, 1989; Davies et al., 1990; Stanfield et al., 1990). The sequential epitopes identified in this study are longer than 10 amino acid residues because a synthetic peptide corresponding to aa 321–331 including the core motif does not bind to the antibodies (data not shown). Based on this observation, we propose that the antibody-combining site for HW-19/HW-8, unlike a typical anti-peptide antibody molecule with a deep cavity or a narrow groove, may have an undulating surface complementary to the surface of the epitope. This is the first example implying that antibodies interact with a sequential epitope in a large complementary interface instead of a deep pocket. This is a reasonable proposition because the antibodies were obtained from a mouse immunized with the native protein (Wu et al., 1996a).

Results from different studies have suggested that a functional epitope is dominated by a few side chains that are often charged or polar (Novotny et al., 1989; Davies et al., 1990; Jin et al., 1992). The ranking of some side chains in the contribution to the binding energy of protein–protein interactions has also been determined for the hGH function epitopes as the following: Arg>Pro>Glu–Asp–Phe–Ile>Leu–Asn–Gln (Ala, Cys, and Trp were not tested) (Jin et al., 1992). Based on the sequence alignment, it appears that the Val and Glu of the central region (LPVDE) may dominate the binding energy because the aa 130–149 region that completely lacks the antigenicity is different from aa 318–336 in only these two residues. The importance of Glu is highlighted by the fact that the antigenicity of C3E is destroyed completely by the single substitution of this residue with Gly or Val (C3E2, C3E5, and C3E6 in Fig. 3). Because we did not have a mutant with only the Val being replaced, the significance of the Val at this specific position remains to be elucidated.

The minimal structure of the functional epitope is present at least once in all members of Per a 1 allergen, whereas four copies with 100% identity were found in Per a 1.02 (Wang et al., 1999; Yang et al., 2000). Although not all epitopes may be accessible to membrane-bound or free antibody in the native proteins, these proteins are most likely present in a denatured form in the environment or after entering the human body. The presence of multiple copies of the epitope in all isoallergens of Per a 1 identified so

far may contribute to its high allergenicity, compared to that exhibited by Per a 3, although its relative abundance is much lower than Per a 3 (Wu and Lan, 1988).

Note that in the Western blots, immunoreactive bands at positions other than the expected size were also observed (Fig. 2). The bands appearing at the higher molecular weight positions might represent aggregated products or cross reactivity of the minor contaminants from *E. coli* proteins. The band at the position below the 45-kDa marker in the sample containing C3 protein has previously been shown to be a nonfusion C3 protein (Yang et al., 2000). A doublet was observed in the Ni^{2+} -absorbed C3E protein when blotting with mAb HW-19 (Fig. 2b); however, only the top band was distinctly reactive to the anti-CRa (Fig. 2c). The smaller band only appeared in the Ni^{2+} -absorbed protein but not in the blots of the *E. coli* (pET30-C3E) crude extract (data not shown). These indicated that this band might be the degraded product generated during the purification process and prolonged storage. The fact that the rabbit anti-CRa antiserum recognizes predominantly the top band of the doublet, while both bands were reactive to mAb HW-19 indicated that the epitopes recognized by these two types of antibodies are not the same. This is not surprising because anti-CRa was obtained by immunizing a rabbit with the crude extract of American cockroach antigen (Wu and Lan, 1988), while mAb HW-19 was prepared from CR-PII (Per a 1) immunized mouse (Wu et al., 1996a). It is likely that the anti-CRa recognized either the N- or C-terminus of the C3E protein that had been deleted in the degraded product.

To our knowledge, this paper is not only the first report on the detailed mapping of the cockroach allergens but also the first report on the use of error-prone PCR random mutagenesis and negative selection in molecular allergology. The structure defined in this study may provide useful information in designing peptide vaccine specific for Per a 1 allergen.

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