

Antigenic Surface of the Bee Venom Allergen Phospholipase A₂

Structural and Functional Analysis of Human IgG4 Antibodies Reveals Potential Role in Protection¹

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In the human immune response to the main bee venom allergen phospholipase A₂ (PLA₂), Abs of the IgG4 isotype are associated with protection. The antigenic sites of PLA₂ that are recognized by two mAb of this isotype have been analyzed at the molecular level. The interaction of PLA₂ with these two Abs has previously been shown to depend on the conformation of the Ag and to be sensitive to lysine modifications. Therefore, genetically engineered Ag displaying single point mutations of lysine residues were generated. Nine out of twelve surface-exposed lysine residues were substituted by glutamate or glutamine, and the effect of individual mutations on mAb binding was analyzed in the context of the folded Ag. Substitution of lysine at position 25 with either glutamate or glutamine completely abrogates binding to both mAb. All other mutants do not show a difference in binding when compared with wild-type Ag. Probing of sera from bee keepers with lysine 25 mutants reveals that a significant proportion of serum Abs of the IgG4 isotype display specificity for this epitope, suggesting that this represents a major B cell-antigenic site in hyperimmune individuals. We further found that both mAbs inhibit the catalytic activity of PLA₂, a property that may account for the protective role of IgG4 Abs in hyperimmune individuals. *Journal of Immunology*, 1994, 152: 5514.

Abs bind to their cognate Ag by interaction of the paratope, the Ag-binding site of the Ab, with the corresponding epitope, a restricted, mostly surface-exposed area of the Ag (1). Although epitopes (or antigenic sites) can be either continuous, defined by a continuous stretch of amino acids, or discontinuous (2), the majority of Abs elicited by exposure to native protein Ag are believed to recognize conformation-dependent, discontinuous epitopes (1, 3). Furthermore, all epitopes analyzed so far by x-ray diffraction studies of complexes of mAb

F(ab) with their protein Ag are discontinuous (4–8). In this report we describe a molecular approach to study discontinuous epitopes recognized by human Abs elicited by natural exposure to an allergen.

Phospholipase A₂ (PLA₂)³ is the main immunogenic and allergenic component of bee venom (9–11). This Ag is a single-chain polypeptide of 134 amino acids and is glycosylated at a single site, resulting in a molecular mass of 15 to 17 kDa (12). The primary structure of PLA₂ has been determined by cDNA sequencing (13), and its three-dimensional structure has recently been resolved by x-ray crystallography (14). After initial exposure to bee venom, individuals generally respond by producing low affinity anti-PLA₂ IgG1 Abs (15). Some individuals can mount an IgE Ab response to PLA₂ and develop an allergy to bee venom (10, 11). In most subjects, however, repeated exposure results in protective immunity associated with a

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³ Abbreviations used in this paper: PLA₂, phospholipase A₂ from honey bee venom (*apis mellifera*); diC₆thio-PM, racemic 2,3 bis-(hexanoylthio)propyl-1-phosphomethanol lithium salt; DMPM, 1,2 dimyristoyl-*sn*-glycero-3-phosphomethanol lithium salt.

predominant IgG4 Ab response against PLA₂ (15). The protective role of IgG4 Abs has been demonstrated by passive immunization of bee sting allergic individuals with γ -globulins isolated from bee keepers (16, 17). This observation raises the question about the antigenic structure recognized by these Abs as well as their biologic function in protection.

We have previously reported the isolation of two heteromyloma lines derived from mononuclear cells of a bee keeper, which secrete IgG4 Abs specific to discontinuous determinants on PLA₂ (18). Cross-inhibition assays indicated identical or overlapping epitopes for these two Abs. Furthermore, the interaction of PLA₂ with both Abs was shown to be sensitive to lysine-modifying reagents (19). Similar observations were made with polyclonal IgG4 Abs from bee keepers, whereas polyclonal IgE Abs from allergic individuals bound to lysine modified PLA₂ with variable affinities (19).

Because some of the lysine residues of PLA₂ are part of the discontinuous epitope(s) recognized by human Abs, we followed a targeted approach for a molecular definition of these antigenic sites. By using site-directed mutagenesis, single lysine residues were mutated, and the effect of individual substitutions on Ab binding was assessed in the context of the folded Ag variant. In this report we demonstrate that the same lysine (residue 25) is a critical contact residue within the epitope of both mAbs. We also show that this reactivity is shared by a considerable portion of polyclonal IgG4 Abs from bee keepers. In addition, a possible biologic function of these Abs was investigated by enzyme inhibition studies.

Materials and Methods

Enzymes and reagents

Chemicals of the highest available grade were purchased from Fluka (Buchs, Switzerland) or Sigma Chemical Co. (St. Louis, MO). DNA-modifying enzymes were obtained from Boehringer (Mannheim, Germany) and used as recommended. All commercial kits were used according to the manufacturer's instructions. Purified natural PLA₂ was purchased from Boehringer, and recombinant wild-type PLA₂ was prepared as described (20). Concentrations of commercial and recombinant PLA₂ were determined according to the Bradford method by using a commercial kit (Bio-Rad, Richmond, CA) or were calculated from the absorbance by using an $\epsilon_{280}^{1\%}$ of 13.0 (21). DiC₆thio-PM and DMPM were prepared as described (22).

Abs

The isolation and preliminary characterization of two PLA₂-specific human mAbs, designated BVA1 and BVA2, which were derived from a hyperimmune donor, have been described in detail (18). Both Abs were isolated from heteromyloma supernatants and purified by protein A affinity chromatography (8). Mouse anti-PLA₂ mAbs 5D5 and 2F5 (23) produced in ascites were kindly provided by Dr. W.-D. Müller (Friedrich Schiller Universität, Jena, Germany). Mouse mAb RJ 4 specific for human IgG4 was obtained from Oxoid (Oxoid Ltd., Basingstoke, UK). Alkaline phosphatase-labeled goat anti-mouse Ig γ - and λ -chains and alkaline phosphatase-labeled goat anti-human IgG were purchased from TAGO (Burlingame, CA).

Bacterial strains, plasmids, and media

Escherichia coli strain M15 (24) bearing the repressor plasmid pRep4 (25) was used as a host for the expression plasmid and was grown in 2X YT medium supplemented with 100 μ g/ml ampicillin and 25 μ g/ml kanamycin. This strain and the expression plasmid pDS56/RBSII, 6xHis (25) were kindly provided by Dr. D. Stüber (Hoffmann-LaRoche, Basel, Switzerland). Design and construction of a synthetic gene-encoding PLA₂ and its expression in this system have been described previously (20).

rDNA techniques

Plasmid DNA was prepared with anionic exchange columns (Qiagen kit, Qiagen Inc., Düsseldorf, Germany). Restriction fragments and PCR products were purified on agarose gels and recovered with GeneClean (Bio 101 Inc., La Jolla, CA). Ligations were performed with the use of standard protocols (26). Transformations were performed by electroporation with the use of a GenePulser (Bio-Rad) and transformants were selected on LB plates containing the appropriate antibiotics. dsDNA sequencing was performed according to the dideoxy chain-termination method (27) on an Applied Biosystems 373A sequencer with the use of the Taq DyeDeoxy Terminator cycle sequencing kit supplied by the same company. The Type III/Type IV and reverse primers (Qiagen Inc.) were used for sequencing.

Site-directed mutagenesis

The synthetic gene encoding the mature PLA₂ polypeptide (20) in a pBluescript II SK vector (Stratagene, La Jolla, CA) was used as the target for mutagenesis. Site-specific substitutions were introduced into the PLA₂ gene by PCR with the use of mismatch primers. Mismatch primers and T3 or T7 flanking primers (Stratagene) used to generate the mutant gene fragment, as well as the restriction sites used for the corresponding fragment replacements, are shown in Table 1. Gene segments encoding the residue selected for mutagenesis were replaced by PCR amplification products encoding the modified sequence. Whenever possible, additional silent mutations were included in the mutagenic primers to eliminate or generate restriction sites to allow screening of plasmids containing the mutant insertions. Depending on the presence of a unique restriction site in the proximity of the substituted residue, a single or double PCR strategy was applied. PCR was performed by using Vent heat-stable DNA polymerase (New England Biolabs, Schwalbach/Taunus, Germany). Annealing temperatures for the various mismatch primers were calculated by using the program OLIGO (National Biosciences, Plymouth, MN). PCR was performed in 100 μ l of sample containing the appropriate buffer, 200 μ M of each deoxynucleotide triphosphate, 0.5 μ M of each 5'- and 3'-primer, 10 ng of target DNA, and 1 U of Vent DNA polymerase. The samples were subjected to two cycles of amplification consisting of denaturing for 1 min at 95°C, annealing for 1 min at the appropriate temperature, and extending for 1 min at 72°C, followed by 25 cycles under identical conditions except that the annealing was done at 50°C. The PCR product was gel purified and, if the mismatch primer contained a convenient restriction site, treated with the appropriate restriction enzymes for direct subcloning into the wild-type expression construct. If no convenient restriction site was available for subcloning, the primary amplification product was used as a primer in conjunction with the appropriate flanking primer in a second round of PCR to amplify the entire gene carrying the desired mutation. The second PCR reaction was performed as described above except that the first two annealing cycles were omitted. Amplified gene fragments were cloned into appropriate restriction sites of the wild-type expression construct (20) (Fig. 1), and constructs displaying the desired analytical restriction sites were analyzed by DNA sequencing of the entire coding sequence. By applying this strategy, the desired mutations were found in more than 90% of the selected clones.

Production of PLA₂ mutants

All of the PLA₂ variants described in this study were expressed as fusion proteins with six adjacent histidine residues linked to the amino terminal end, allowing single step purification by immobilized metal ion affinity chromatography (28, 29). Protein expression of mutant constructs was assessed in test induction assays (25) by analyzing culture lysates on

Table 1. PCR primers and restriction sites used for mutagenesis

Mutant ^a	Mismatch Primer ^b	Flanking Primer(s) ^c	Restriction Sites
K14E	5'-ACCAGAAGACTCGTTGCCGTG-3'	T3/T7	<i>Sma</i> / <i>Hind</i> III
K25(E/Q)	5'-ACTCGGCCGCTTC (C/G) AACAC-3'	T7	<i>Eag</i> I/ <i>Bst</i> II
K47Q	5'-TGTCTGCAGGTGAATCTCAGCACG-3'	T7	<i>Pst</i> I/ <i>Hind</i> III
K66E/Q	5'-CGTAGAATT (C/G) GTCGTCGCA-3'	K25(E/Q)/T7	<i>Mlu</i> I/ <i>Hind</i> III
K72(E/Q)	5'-ACTGTATCGGCGGAGTTCT (C/G) GAGGCA-3'	K25(E/Q)	<i>Sph</i> I/ <i>Eco</i> RV
K85(E/Q)	5'-TGAAATACATCT (C/G) GCCAACG-3'	K47Q/T7	<i>Mlu</i> I/ <i>Hind</i> III
K97(E/Q)	5'-CCGGTTACGGGTGCTCGAGCT (C/G) GTA-3'	T3	<i>Eag</i> I/ <i>Bst</i> II
K122Q	5'-ACCGTCGACAAATCTCAGCCG-3'	T7	<i>Sal</i> I/ <i>Hind</i> III
K133E	5'-ACCAAGCTTCAGTATTCGCGC-3'	K47Q	<i>Mlu</i> I/ <i>Hind</i> III

^a The designed mutant Ags are referred to by the single letter amino acid code in which numbers designate the position in the mature polypeptide of the lysine residue (K) substituted with glutamate (E) and/or glutamine (Q).

^b The triplet encoding the mutation is depicted in bold, (C/G) is a wobble of C and G at a molar ratio of 1:1.

^c T3 and T7 primers (Stratagene) annealing to vector sequences or appropriate mutagenic primers were used as flanking primers to isolate the gene segment encoding the desired amino acid substitution. When two flanking primers are shown, the mutant gene fragment has been generated by two sequential PCR reactions.

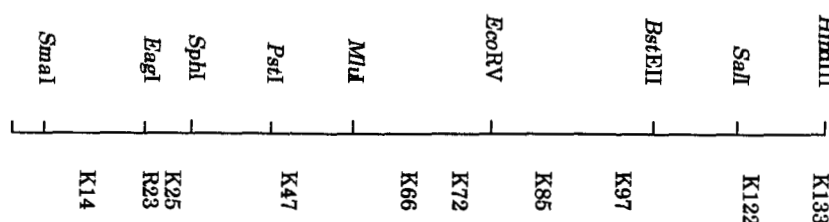


FIGURE 1. Restriction map of the synthetic gene-encoding wild-type PLA₂ (20). Amino acid residues targeted for mutagenesis and unique restriction sites used for subcloning of mutant gene fragments are indicated.

SDS-PAGE (30). Selected clones were grown on a large scale and recombinant protein was produced as described (20). Affinity isolation was done as before (20), except that fresh resin was used for every individual mutant. Renaturation and purification of PLA₂ mutants was performed according to the protocol developed for the wild-type enzyme (20), except that mutant proteins were bound to SP-Sephadex (Pharmacia, Uppsala, Sweden) at pH 7.0 instead of pH 9.0. For all of the studies described in this report, recombinant proteins were used without proteolytic removal of the N-terminal affinity peptide because it has previously been shown that these 15 residues do not affect the catalytic activity or the antigenicity (20).

Enzymatic assays

Enzymatic activities of the PLA₂ mutants were measured by using the spectrophotometric assay with diC₈thio-PM as substrate (31). The method described previously (31) has been adapted to a microtiter plate. Substrate mixture (150 μ l) consisting of 0.1 mM substrate in 25 mM Tris-HCl, 100 mM KCl, 10 mM CaCl₂, and 0.8 mM Ellman's reagent, pH 8.0, in a 96-well polystyrene plate (Nunc, Roskilde, Denmark) was mixed with enzyme (10–20 ng), and the reaction progress was monitored at 405 nm on a kinetic microtiter plate reader (Molecular Devices, Menlo Park, CA). Activity was inferred from individual slopes calculated from at least four data points in the initial velocity region ($r > 0.98$). The sp. act. for each mutant is expressed by equivalence to a commercial PLA₂ standard included in every assay.

The effect of Ab binding on enzyme activity was determined in a pH-stat assay on DMPM vesicles in the presence of Polymyxin B (20, 22). PLA₂ (100 ng) was incubated with Ab at various molar ratios in PBS, pH 7.4, for 1 h at room temperature. Aliquots of these reaction mixtures (75 ng PLA₂) were added to assay solution 30 s after addition of Polymyxin B, and the initial rate of substrate hydrolysis was determined (22).

CD spectra

CD spectra were obtained by using a J-720 spectropolarimeter (JASCO Instruments, Tokyo, Japan). The protein samples were dissolved in 10

mM sodium phosphate buffer, pH 7.0, at a concentration of 10 μ M. Spectra (average of 12–16 scans) were collected over a range of 180 to 300 nm by using a 0.2-mm path length cuvette.

Ab-binding analysis

The binding of Abs to the wild-type and engineered PLA₂ preparations was evaluated by direct solid phase ELISA performed as described (32). Briefly, purified recombinant Ag preparations were coated (0.1 μ M diluted in PBS, pH 8.0; 100 μ l/well) overnight at 4°C onto polystyrene microtiter plates (Maxisorb, Nunc). After blocking the remaining free sites with blocking buffer (PBS, pH 7.4, 1% casein hydrolysate (Oxoid), 5% (w/v) Tween 20) at 37°C for 1 h, wells were incubated for 2 h at 37°C with primary Ab (100 μ l) serially diluted in blocking buffer. Bound Abs were detected by 2 h incubations at 37°C with RJ 4 (diluted 1:4000) followed by phosphatase-labeled goat anti-mouse Abs (diluted 1:6000 in blocking buffer). Assays were developed by addition of 100 μ l of substrate (32) and evaluated on the kinetic microtiter plate reader.

Relative binding affinities were determined by inhibition ELISA. Microtiter plates were coated with wild-type Ag and blocked as described above. Primary Ab or sera at appropriate dilutions were preincubated for 2 h at 37°C in a separate untreated plate with serially diluted wild-type or mutant PLA₂. Thereafter this mixture was transferred to the Ag-coated plate and allowed to react for 2 h at 37°C. Bound Abs were detected as described for the direct solid phase ELISA.

Results

Design of mutants

Previous studies with the use of chemically modified PLA₂ have shown that lysine residues play a prominent role in the association of the PLA₂ Ag with human monoclonal and polyclonal Abs derived from nonallergic individuals (19). Furthermore, these Abs did not cross-react

Table II. Specific catalytic activity of PLA₂ variants

Variant ^a	Sp. Act. ^b	Variant ^a	Sp. Act. ^b
WT	95.2	K66E	87.1
K14E	104	K72E	93.2
R23E	91.8	K85E	85.4
K25E	87.1	K97E	77.9
K25Q	92.4	K122Q	118.0
K47Q	79.2	K133E	102.1

^a Nomenclature of the mutants as in Table I.^b Sp. act. of recombinant PLA₂ preparations were determined from the initial rate of substrate hydrolysis and are given in U/μg enzyme. Activity is expressed by equivalence to purified natural PLA₂ as a standard arbitrarily defined as 100 U/μg protein. The estimated error is ±20%.

with PLA₂-derived synthetic peptides in a pepscan analysis (19). The discontinuous nature of the epitopes and the lack of naturally occurring PLA₂ variants required genetically engineered Ag for a better definition of the antigenic sites recognized by these Abs. Therefore, lysine residues scattered over the surface of PLA₂ were individually mutated. As summarized in Table I, 9 of 12 lysine residues were selected for substitution with either glutamate and/or glutamine. Of the three lysines clustered in the C-terminal loop of the Ag (K120, K122, and K124) only the lysine at position 122 was mutated, as we reasoned that this single mutation would sufficiently affect the local side chain interaction network to influence the binding of Abs exhibiting electrostatic interaction with either K120 or K124. Glutamine and glutamate were chosen as replacements for lysine because, like lysine, each bears a polar side chain and, therefore, is frequently exposed at the molecular surface. We anticipated that these substitutions, when displayed at the molecular surface in the context of the entire folded Ag, would not cause long range alterations of the secondary or tertiary structure. Replacement of the cationic side chain (at physiologic pH) by a negatively charged or neutral residue, however, should substantially reduce the binding energy of Abs containing the corresponding lysine residue in their epitope. The arginine to glutamate mutant at residue 23 (R23E) was designed for another study and was analyzed because of its location next to the critical lysine residue (position 25).

Production and biochemical analysis of mutants

All of the PLA₂ variants were expressed in the pDS expression system, isolated, refolded, and purified as described (20, 25). Yields after affinity isolation ranged from 30 to 50 mg/l of culture for all of the mutants described. Refolding yields (20–30%) were comparable with those reported for the wild-type Ag (20). An exception was the mutant K97E, which consistently yielded only 6 to 8% of active protein. All of the mutants showed >95% purity and displayed an apparent m.w. comparable to wild-type PLA₂ when analyzed on SDS-PAGE (data not shown).

To verify functional folding of the mutant Ags, their sp. act. in enzymatic assays were compared with wild-type PLA₂. All mutant proteins exhibited catalytic activity after refolding, and the sp. act. of purified mutants were comparable to wild-type enzyme in all cases (Table II). In addition, the CD spectra of the mutant PLA₂ are comparable with that of recombinant wild-type or natural protein (data not shown). Although local conformational changes at the molecular surface cannot be excluded, the complex structural requirements for catalytic function inferred from x-ray analysis (14, 33) strongly suggests that mutation of the surface lysine residues would not cause long range alterations of the three-dimensional structure.

Binding of mAb to Ag variants

Purified PLA₂ mutants were assayed by solid phase ELISA for their reactivity with PLA₂-specific human mAb BVA1 and BVA2. The binding of BVA1 and BVA2 to the individual PLA₂ mutants or to wild-type Ag is depicted in Figure 2. To verify that the same amount of each Ag was coated to the plate, murine mAb 2F5 and 5D5 (23) (which are not sensitive to chemical lysine modifications) were included. All of the lysine mutants except those carrying substitutions at residue 25 showed no reduction of reactivity with BVA1 and BVA2. Changing lysine 25 to glutamate (K25E) or glutamine (K25Q) completely abrogated Ab reactivity even though nonstringent conditions (Ag coated at 0.1 μM) were used. The control Ab 2F5 showed comparable binding to all of the PLA₂ mutants tested; similar results were obtained with mAb 5D5 (data not shown). These findings indicate that lysine at position 25 of PLA₂ is crucial for the interaction of both BVA1 and BVA2 with their cognate Ag.

Inhibition ELISA

To rule out the possibility that the observed absence of reactivity of PLA₂ mutants displaying nonconservative substitutions at residue 25 is a result of partial denaturation of solid phase-coated Ag, inhibition ELISA experiments were performed. Binding of Ab to solid phase coated wild-type Ag was inhibited with increasing amounts of mutant protein present in solution. Typical inhibition plots of selected mutants are presented in Figure 3. We find that substitution of lysine 25 with glutamine or glutamate resulted in a loss of detectable binding to BVA1 and BVA2, whereas all the other mutants except R23E showed reactivity comparable with wild-type Ag with both Abs.

The reactivity of the whole panel of mutants with BVA1 and BVA2 is summarized in Table III. Substitution of lysine 25 with either glutamine or glutamate led to a more than 200-fold decrease in the relative affinities to BVA1 and BVA2, whereas all the other lysine mutants display affinities within a factor of 3 of the affinity to wild-type Ag. In addition, no difference in reactivity between BVA1

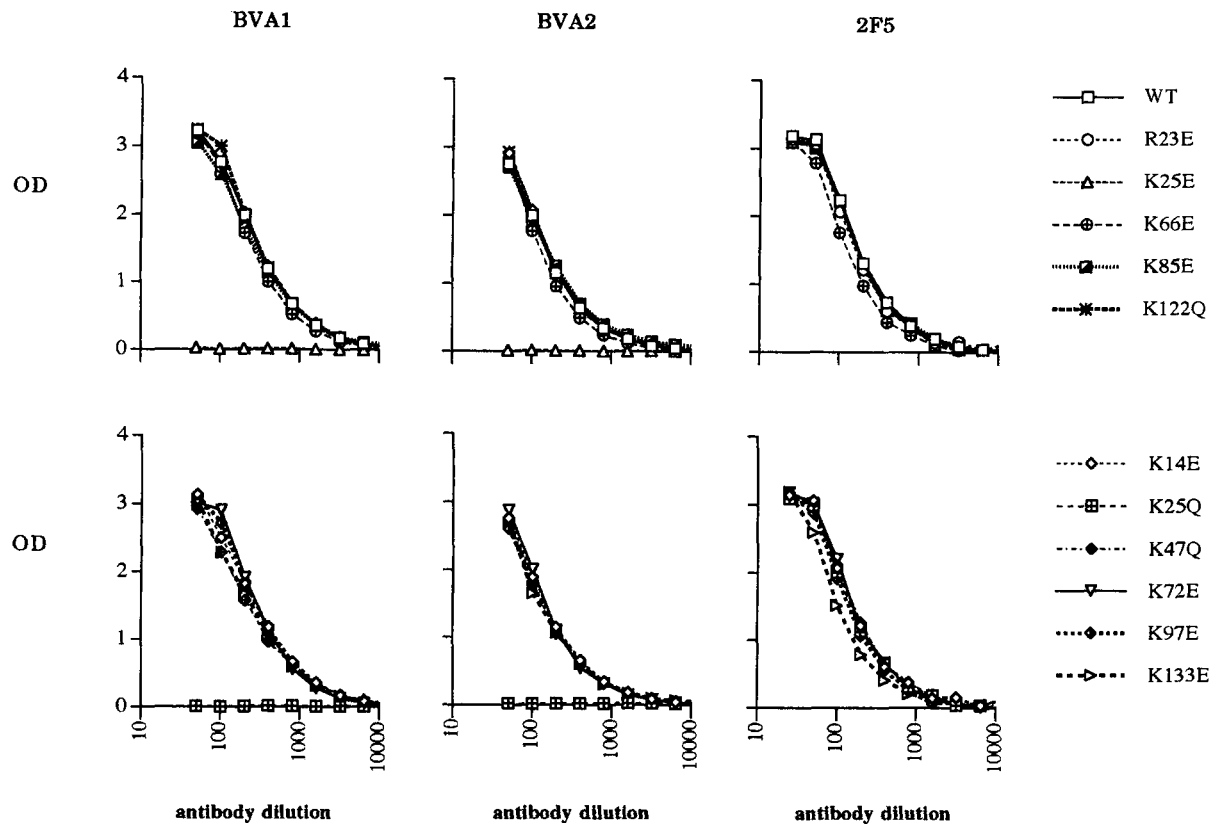


FIGURE 2. Reactivity of BVA1, BVA2, and 2F5 with PLA₂ variants in direct solid phase ELISA. Wild-type and mutant PLA₂ were coated onto microtiter plates and incubated with serially diluted mAb.

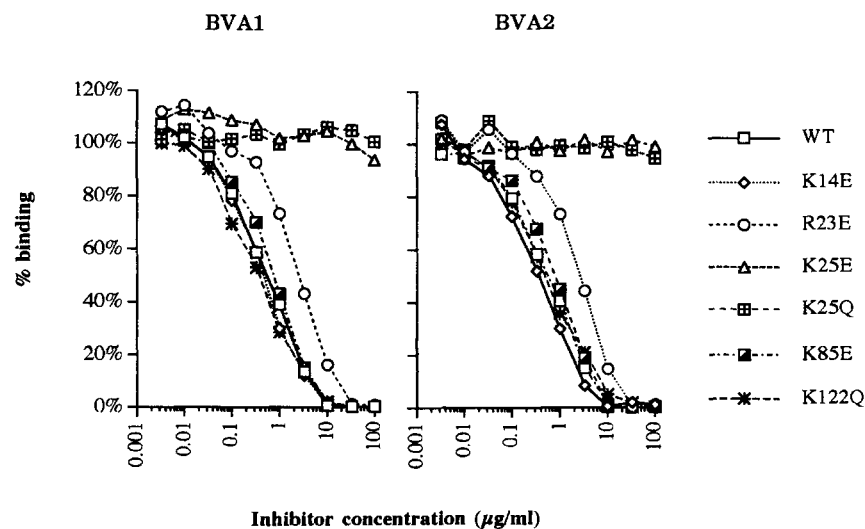


FIGURE 3. Inhibition of BVA1 (left panel) and BVA2 (right panel) binding to solid phase bound wild-type PLA₂ by PLA₂ variants. Varying amounts of wild-type or mutant PLA₂ were preincubated with an amount of mAb that gave 50% of maximal binding in direct solid phase ELISA. Preincubated samples were transferred to wild-type PLA₂-coated plates and mAb binding was subsequently analyzed by direct solid phase ELISA. The extent of binding is expressed as percent of the reactivity in the absence of inhibitor, after subtracting background for each point.

and BVA2 was observed for any of the lysine substitutions tested. These data suggest that both Abs recognize the same antigenic site involving lysine 25 as a critical residue

(hereafter referred to as the K25 epitope). BVA1 and BVA2 exhibited a five- to sixfold decreased affinity to the arginine mutant R23E in comparison with wild-type Ag.

Table III. Relative affinities^a of PLA₂ mutants to BVA1 and BVA2

Mutant ^b	BVA1	BVA2
K14E	1.1	1.2
R23E	0.2	0.15
K25E	<0.005	<0.005
K25Q	<0.005	<0.005
K47Q	0.9	0.7
K66E	3	3
K72E	3	3
K85E	0.7	0.8
K97E	1.4	1.3
K122Q	1.3	1
K133E	2.5	3

^a The affinities of the individual mutants to BVA1 and BVA2 relative to wild-type PLA₂ were inferred from inhibition ELISA experiments. Numbers given above correspond to: IC₅₀ (wild type)/IC₅₀ (mutant) where IC₅₀ corresponds to the inhibitor concentration needed to reach 50% inhibition.

^b Nomenclature of the mutants as in Table I.

This slight reduction of reactivity suggests that R23 contributes to a minor extent to the binding energy of the Ab-Ag interaction.

Reactivity of bee keeper sera

We further investigated whether the K25 epitope recognized by the two human mAbs of the IgG4 isotype also defines the antigenic determinant for a measurable portion of IgG4 Abs in hyperimmune sera. Inhibition ELISA experiments were performed as described above by using bee keeper sera instead of mAb. In these studies, K85E was selected as a control mutant because lysine 85 is located on the opposite side of the molecule from lysine 25 (14). Because of spatial constraints, Abs that react with an antigenic site containing lysine 25 cannot simultaneously interact with lysine 85. Inhibition plots for two typical bee keeper sera are depicted in Figure 4. Clear differences between K25E and K25Q on one hand, and wild-type and K85E on the other, are observed. Although all Ag variants react with IgG4 Abs in a concentration-dependent manner, K25E or K25Q show a marked decrease in reactivity over a broad range of Ag concentration. This decrease varies approximately 10% (serum WF) and reaches up to 40% (serum BR). Similar patterns of IgG4 reactivity were reproducibly found in 11 of 12 bee keeper sera (data compiled in Table IV). The binding of one serum (AS) was not significantly affected by mutations of lysine 25. These results suggest that a significant fraction of polyclonal IgG4 Abs in most bee keepers reacts with the same antigenic site as BVA1 and BVA2, indicating that the mAbs that were isolated are representatives of a prominent portion of the PLA₂-specific IgG4 repertoire conserved in hyperimmune individuals.

Effect of Ab binding on enzymatic activity

Because the biologic function of an Ab directed against an enzyme may rely on its ability to block enzyme activity,

the effect of BVA1 and BVA2 on the catalytic activity of PLA₂ was assessed on DMPM vesicles. In this assay the phospholipid substrate is present in large negatively charged vesicles, a situation that mimics the physiologic substrate in a living cell. We find that both Abs cause a 70 to 80% reduction of the initial rate of substrate hydrolysis at an equimolar ratio of Ab to PLA₂, and this inhibition was not increased further upon addition of excess Ab. Identical inhibition levels were obtained with the mutant K85E, whereas the activity of the epitope mutant K25Q is not affected even by a 20-fold molar excess of mAb, demonstrating that this inhibitory effect is specific and dependent on Ag-Ab interaction.

Discussion

Although the predominance of IgG4 Abs in the immune response to bee venom PLA₂ in hyperimmune individuals such as bee keepers has been recognized for a long time (15), and an anti-anaphylactogenic role for such Abs has been suggested (16, 17), their function and the molecular basis of their Ag recognition remained obscure. To gain more detailed insight into the nature of these presumably protective IgG4 Abs, the epitopes recognized by two mAbs of this isotype derived from PBLs of a bee keeper have been analyzed in detail.

The origin of these Abs is particularly noteworthy because they are elicited by natural exposure to the Ag rather than by artificial immunization with adjuvant. In the latter case Abs are often directed against at least partially denatured Ag and cross-react poorly with the native protein (3). Indeed, the recent characterization of murine mAb specific to PLA₂ revealed that five of seven Abs react poorly with native Ag in solution (23), suggesting that they were originally elicited by denatured Ag. In contrast to these Abs, the mAbs isolated from an individual naturally exposed to bee venom are conformation dependent and exclusively interact with the folded Ag.

Previous studies on Ag-Ab interactions have made successful use of synthetic peptides (34, 35), chemical alterations of amino acid side chains, (36) and naturally occurring genetic variants (37) of the Ag to define antigenic sites. In our study, however, these approaches were of limited success. The conformational nature of the epitopes and the lack of naturally occurring antigenic variants required a genetic approach for introducing targeted substitutions at the molecular surface of PLA₂ and displaying them in the context of the entire folded Ag.

To identify the lysine residue(s) that are crucial for the PLA₂-mAb interaction, nine residues were substituted by glutamine and/or glutamate. We have chosen this particular replacement rather than an alanine scanning strategy applied by other authors (38) because the x-ray crystal structure reveals that all the lysine residues are exposed at the molecular surface (14). Therefore, lysines have been substituted by polar rather than by nonpolar residues.

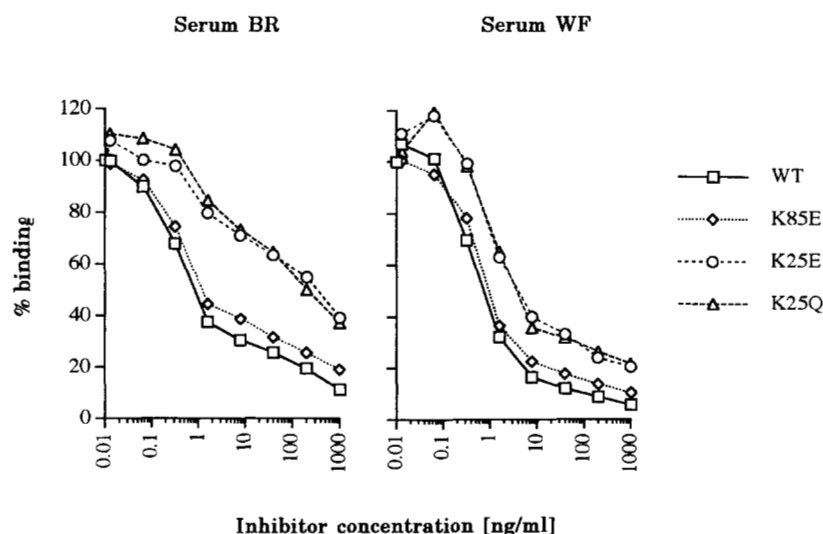


FIGURE 4. Reactivity of IgG4 Abs of bee keeper sera with wild-type PLA₂ and selected mutants. Inhibition ELISA was performed as described in Figure 3.

Table IV. Recognition of K25 epitope by IgG4 Abs of hyperimmune individuals

Serum ^a	% IgG4 Reactivity to K25 Epitope ^b	Serum ^a	% IgG4 Reactivity to K25 Epitope ^b
AG	14 ± 2	AS	1 ± 4
BB	17 ± 5	BR	34 ± 4.5
CH	24 ± 5	GB	22 ± 6
GK	29 ± 3	HB	14 ± 2
HM	25 ± 3	MR	15 ± 4
PZ	13 ± 2	WF	11 ± 1

^a Sera were taken from bee keepers with no clinical history of bee sting anaphylaxis.

^b The percentage of PLA₂-specific IgG4 Abs reactive with the K25 epitope was estimated from the reactivity-difference between wild-type Ag and the mutant K25E in inhibition ELISA experiments (Fig. 4). Data represent the mean ± SD of four data points using inhibitor concentrations of 1 to 1000 ng/ml.

Furthermore, these substitutions do not increase the side-chain volume, a condition that is important inasmuch as replacement of a small noncontact residue by a bulky side chain can significantly affect binding because of steric hindrance (1).

The whole panel of PLA₂-variants was expressed, purified, and analyzed for proper folding. Analysis by CD-spectroscopy shows no significant difference in secondary structure between PLA₂-variants and wild-type Ag. Biochemical analysis, shown in Table II, reveals that all of the mutant Ags display hydrolytic activity, with sp. act. that do not significantly differ from those of the wild-type Ag. This leads to the conclusion that all of the mutant molecules display a native-like 3D structure. The data also suggest that any structural effects resulting from these substitutions are local and do not cause long range structural alterations which may indirectly influence Ab reactivity. This observation is in agreement with previously reported

mutational studies of the antigenic surface of staphylococcal nuclease (39, 40).

Immunochemical analysis of the entire panel of PLA₂ variants reveals that both BVA1 and BVA2 are directed against the K25 epitope. Substitution of this lysine residue by an anionic or a neutral residue leads to a complete loss of detectable Ab reactivity in both solid phase (Fig. 2) and in fluid phase (Fig. 3) assays. Because substitution of K25 results in a strong reduction of binding in the absence of gross structural alterations and without increasing the side chain volume, it can be considered to be a contact residue. The invariable reactivity of both mAb to the entire panel of PLA₂ variants (Table III) suggests that BVA1 and BVA2 share a high degree of similarity in terms of their Ag recognition.

We further investigated a potential *in vivo* dominance of the K25 epitope by assessing the polyclonal IgG4 reactivity by using bee keeper sera as an Ab source. Although the polyclonal IgG4 reactivity was not exclusively confined to this epitope, a significant reduction of reactivity of the contact residue mutants (K25E and K25Q) in comparison to wild-type enzyme or a control mutant (K85E) was observed in 11 of 12 sera (Table IV). As shown for typical examples in Figure 4, this reduction of reactivity of the contact residue mutants is virtually constant over a broad concentration range. This indicates that the proportion of Abs reacting with the K25 epitope completely lose their Ag-binding capacity once this contact residue is modified, whereas Abs exhibiting a different epitope specificity remain unaffected. The preferential polyclonal reactivity indicates an important role of the K25 epitope in the binding of IgG4 Abs and suggests a conserved Ag recognition in hyperimmune individuals.

Analysis of the effect of Ab binding on the catalytic function of PLA₂ reveals a 70 to 80% inhibition of substrate hydrolysis by both BVA1 and BVA2. This effect is dependent on cognate interaction of PLA₂ with Ab, as evidenced by the lack of inhibition with the contact residue mutant K25Q. The mechanism of this inhibition is not understood in detail, but it cannot be caused by a mutually exclusive interaction of the Ab and the phospholipid vesicle with PLA₂. If the latter were true, the catalytic activity of the enzyme would fall to zero as the Ab concentration is increased, rather than reaching a constant level of inhibition as is observed. Ab-mediated inhibition of PLA₂ activity may be of biologic significance, a concept that is underscored by several reports describing cytotoxicity of PLA₂ associated with its enzymatic function (41–43) as well as by studies showing that PLA₂ can degranulate mast cells in the absence of IgE sensitization (44). In view of these observations, it is tempting to speculate that the inhibitory effect exerted by BVA1 and BVA2 (two prototype IgG4 Abs from a hyperimmune individual) may account for the protective role of IgG4 Abs from such individuals in vivo (16, 17). Importantly, we also find that sera from several bee keepers were able to block the enzymatic activity of PLA₂ up to 100%, whereas control sera show no inhibitory effect (data not shown). On the basis of these observations it will be of interest to investigate whether inhibition of enzymatic activity correlates with clinical protection against bee sting allergy. Moreover, because bee keeper IgG4 Abs have been proposed to confer protection by blocking IgE-mediated allergic reactions (16, 17, 45), it will be of considerable importance to localize the antigenic sites recognized by IgE Abs from allergic individuals. With the Ag variants in hand, it will now be possible to address this issue.

In summary, the antigenic sites of PLA₂, a well defined allergen with a known crystal structure, which are recognized by IgG4 Abs from hyperimmune individuals have been analyzed. By using site-directed mutagenesis an identical contact residue for two mAbs has been identified, indicating that they map to the same epitope. Furthermore, a significant portion of polyclonal IgG4 Abs in hyperimmune individuals reacts with this epitope. It is further demonstrated that none of the surface mutations resulted in long range structural alterations, underscoring the validity of this approach for antigenicity studies. On the basis of the ability of these Abs to inhibit the catalytic activity of PLA₂, a biologic role of these Abs in protection is suggested.

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