Roles of Ionic Residues of the C1 Domain in Protein Kinase C-α Activation and the Origin of Phosphatidylserine Specificity*

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On the basis of extensive structure-function studies of protein kinase C-α (PKC-α), we have proposed an activation mechanism for conventional PKCs in which the C2 domain and the C1 domain interact sequentially with membranes (Medkova, M., and Cho, W. (1999) J. Biol. Chem. 274, 19852–19861). To further elucidate the interactions between the C1 and C2 domains during PKC activation and the origin of phosphatidylserine specificity, we mutated several charged residues in two C1 domains (C1α and C1β) of PKC-α. We then measured the membrane binding affinities, activities, and monolayer penetration of these mutants. Results indicate that ionic residues of the C1α domain, most notably Arg77, interact nonspecifically with anionic phospholipids prior to the membrane penetration of hydrophobic residues. The mutation of a single aspartate (Asp55) in the C1α domain to Ala or Lys resulted in dramatically reduced phosphatidylserine specificity in vesicle binding, activity, and monolayer penetration. In particular, D55A showed much higher vesicle affinity, activity, and monolayer penetration power than wild type under nonactivating conditions, i.e. with phosphatidyglycerol and in the absence of Ca2+, indicating that Asp55 is involved in the tethering of the C1α domain to another part of PKC-α, which keeps it in an inactive conformation at the resting state. Based on these results, we propose a refined model for the activation of conventional PKC, in which phosphatidylserine specifically disrupts the C1α domain tethering by competing with Asp55, which then leads to membrane penetration and diacylglycerol binding of the C1α domain and PKC activation.

Protein kinases C (PKC) are a family of serine/threonine kinases that play crucial roles in many different signal transduction pathways (1, 2). At least 10 isoforms of mammalian PKCs have been identified to date and they all contain an amino-terminal regulatory domain linked to a COOH-terminal domain (3). The PKC-α gene, respectively, were used for polymerase chain reaction (18). Briefly, four primers, including two complementary oligonucleotides introducing a desired mutation and two appropriate C1 domain mutations were generated by the overlap extension polymerase chain reaction using pVL1392-PKC-α plasmid as a template (18). Brieﬂy, four primers, including two complementary oligonucleotides introducing a desired mutation and two additional oligonucleotides complementary to the 5′-end and 3′-end of the PKC-α gene, respectively, were used for polymerase chain reaction performed in a DNA thermal cycler (PerkinElmer Life Sciences) using three classes; conventional PKC (α, βι, βμ, and γ isoforms), novel PKC (δ, η, and θ isoforms), and atypical PKC (ζ and λ isoforms). Conventional PKCs are activated by the Ca2+-dependent translocation of proteins to the membrane containing anionic phospholipids, preferably phosphatidylserine (PS) and diacylglycerol (DAG). The membrane translocation is mediated by two types of membrane-targeting domains (C1 and C2 domains) in the regulatory region of conventional PKC (3). The C2 domain of conventional PKC is responsible for the Ca2+-dependent binding of protein to anionic membranes (4–7). The conventional PKC also contains a tandem repeat of cysteine-rich, C1 domains (C1α and C1β) that provide a binding site for DAG and phorbol esters (8–13). Based on extensive structure-function studies on PKC-α, we have recently proposed a mechanism for the in vitro membrane binding and activation of PKC-α (14). In this mechanism, PKC-α initially binds to the membrane surface via the Ca2+-dependent membrane binding of the C2 domain. Once membrane-bound, PS specifically induces the insertion of the hydrophobic residues of the C1α domain into the membrane. The membrane penetration allows optimal DAG binding and drives the release of pseudo-substrate region from the active site, hence the PKC activation. Although this mechanism accounts for much of the temporal and spatial sequences of in vitro activation of conventional PKC, questions still remain as to how the C1 and C2 domains of PKC-α interact with each other during PKC activation and how PS specifically induces the membrane penetration of the C1α domain. To address these questions, we performed further structure-function studies on the C1α and C1β domains of PKC-α, with an emphasis on surface ionic residues. Results from these studies provide an important new clue to the understanding of the origin of PS specificity.

EXPERIMENTAL PROCEDURES

Materials—1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho glycerol (POPG), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoserine (POPS), and 1,2-sn-dioleoylglycerol (DOG) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL) and used without further purification. Tritiated POPC ([3H]POPC) was prepared from 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine and [9,10-3H]oleic acid (American Radiochemical Co.) using rat liver microsomes as described (15, 16). Phospholipid concentrations were determined by phosphoate analysis (17). Fatty acid-free bovine serum albumin (BSA) was from Bayer Inc. (Kankakee, IL). γ-[32P]ATP (3 Ci/mmol) was from Amersham Pharmacia Biotech and cold ATP was from Sigma. Triton X-100 was obtained from Pierce Chemical Co. (Rockford, IL). Restriction endonucleases and enzymes for molecular biology were obtained from either Roche Molecular Biochemicals or New England Biolabs (Beverly, MA).

Mutagenesis—Baculovirus transfer vectors encoding the cDNA of PKC-α with appropriate C1 domain mutations were generated by the overlap extension polymerase chain reaction using pVL1392-PKC-α plasmid as a template (18). The abbreviations used are: PKC, protein kinase C; BSA, bovine serum albumin; DAG, 1,2-sn-diacylglycerol; DOG, 1,2-sn-dioleoylglycerol; PG, phosphatidylglycerol; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol; POPS, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoserine; PS, phosphatidylserine; SPR, surface plasmon resonance; CHAPS, 3-[3-cholamidopropyl]dimethylammonionio]-1-propanesulfonate.
Pfu DNA polymerase (Stratagene). Two DNA fragments overlapping at the mutation site were first generated and purified on an agarose gel. These two fragments were then annealed and extended to generate an entire PKC-α gene containing a desired mutation, which was further amplified by polymerase chain reaction. The product was subsequently purified on agarose gel, digested with EcoRI, and cloned into the pVL1392 plasmid digested with the same restriction enzymes. The mutagenesis was verified by DNA sequencing using a Sequenase 2.0 kit (Amersham Pharmacia Biotech).

Expression of PKC-α and Mutants in Baculovirus-infected Sf9 Cells—Wild type PKC-α and mutants were expressed in baculovirus-infected Sf9 cells (Invitrogen, La Jolla, CA) and purified as described previously (5, 18). The transfection of Sf9 cells with mutant pVL1392- PKC-α constructs was performed using a BaculoGold™ transfection kit from Pharmingen (San Diego, CA). The plasmid DNA for transfection was prepared by using an EndoFree Plasmid Maxi kit (Qiagen, Valencia, CA) to avoid potential endotoxin contamination.

Determination of PKC Activity—Activity of PKC was assayed by measuring the initial rate of [32P]Phosphate incorporation from [γ-32P]ATP (50 μM, 0.6 μCi/tube) into the histone III-SS (400 μg/ml) (Sigma). The reaction mixture contained large unilamellar vesicles (0.1 mM), 5 mM MgCl2, 12 mM PKC, and 0.1 mM CaCl2 in 50 mM HEPES, pH 7.0. Protonate sulfate (200 μg/ml) was used to determine the free enzyme concentration in vesicle binding measurements (see below). Protein concentration was determined using a modified method described previously (5, 18). Reactions were initiated by adding MgCl2 to the mixture and quenched by adding 50 μl of 5% aqueous phosphoric acid solution after a given period of incubation (e.g. 10 min for histone at room temperature). Seventy-five microliters of quenched reaction mixtures were spotted on P-81 ion-exchange papers (Whatman) and papers were washed 4 times with 5% aqueous phosphoric acid solution after a given period of incubation. The papers were then transferred into scintillation vials containing 4 ml of phosphoric acid solution and washed once with 95% aqueous ethanol. The papers (Whatman) and papers were washed 4 times with 5% aqueous phosphoric acid solution after a given period of incubation (e.g. 10 min for histone at room temperature). Seventy-five microliters of quenched reaction mixtures were spotted on P-81 ion-exchange papers (Whatman) and papers were washed 4 times with 5% aqueous phosphoric acid solution after a given period of incubation.

Vesicle Binding Measurements—The binding of PKC to phospholipid vesicles was measured by a centrifugation assay using large sucrose-loaded unilamellar vesicles (100 nm diameter) (20). Sucrose-loaded vesicles were prepared as described previously (18). The final concentration of vesicle solution was determined by measuring the ratio of an absorbance at a trace of [H]PPOP (typically 0.1 mol %) included in all phospholipid mixtures. For binding experiments, PKC (∼12 nm) was incubated for 15 min with sucrose-loaded vesicles (0.1 mM, 1 μg BSA, and varying concentrations of Ca2+ in 150 mM of 20 mM Tris-HCl, pH 7.5, containing 0.1 mM KCl. BSA was added to minimize the loss of protein due to nonspecific adsorption to tube walls. Vesicles were pelleted at 100,000 × g for 30 min using Sorvall RC-1210EX Microcentrifuge. Aliquots of supernatants were used for protein determination by PKC activity assay using protamine sulfate as a substrate. The fraction of bound enzyme was plotted against the anionic lipid composition (mol %) of mixed vesicles. Mol % values of PS and PG giving rise to half-maximal binding enzyme was plotted against the anionic lipid composition (mol %) of mixed vesicles. Mol % values of PS and PG giving rise to half-maximal binding and activity ([PS]1/2 and [PG]1/2) were estimated from the experimental data.

RESULTS

Roles of Cationic C1 Domain Residues in PKC Activation—Figs. 1 and 2 illustrate amino acid sequences and model structures of C1a and C1b domains of PKC-α, respectively. In particular, Fig. 2 shows that the C1 domains of PKC-α have a polarized distribution of hydrophobic and ionic residues. The upper part of the molecule, where the DAG/phorbol ester binding pocket is located, contains a few aliphatic and aromatic residues whereas the middle part has a number of cationic residues. We have recently performed an extensive structural-function study on the hydrophobic residues in the C1a and C1b domains of PKC-α, which revealed their distinct roles in PKC activation (4). Hydrophobic residues in the C1a domain are essential for the membrane penetration and DAG-dependent activation of PKC-α, whereas those in the C1b domain are not directly involved in these processes. To assess the role of cationic residues of the C1 domains in the membrane binding and activation of PKC-α, we mutated several cationic residues in the C1a and C1b domains. Specifically, Lys62, Lys76, and Arg77 were replaced with Glu, Asp, and Gln, respectively. The C1 domains were purified on an agarose gel, digested with Pfu DNA polymerase (Stratagene) and amplified by polymerase chain reaction. The product was subsequently purified on agarose gel, digested with EcoRI, and cloned into the pVL1392 plasmid digested with the same restriction enzymes. The mutagenesis was verified by DNA sequencing using a Sequenase 2.0 kit (Amersham Pharmacia Biotech).

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Monolayer Measurements—Surface pressure (π) of solution in a circular Teflon trough (4 cm diameter × 1 cm deep) was measured using a Wilhelmy plate attached to a computer-controlled Cahn microbalance (Model C-32) as described previously (18). Five to ten microliters of phospholipid solution in ethanol/hexane (1:9 (v/v)) or chloroform was spread onto 10 ml of subphase (20 mM Tris-HCl, pH 7.5, containing 0.1 mM KCl and 0.1 mM free Ca2+) to form a monolayer with a given initial surface pressure (π0). The subphase was continuously stirred at 60 rpm with a magnetic stir bar. Once the surface pressure reading of monolayer was stabilized (after about 5 min), the protein solution (typically 40 μl) was injected into the subphase through a small hole drilled at an angle through the wall of the trough and the change in surface pressure (Δπ) was measured as a function of time. Typically, the Δπ value reached a maximum after 30 min. The maximal Δπ value depended on the protein concentration and reached a saturation value (e.g. at [PKC-α] = 1 μg/ml). Protein concentrations in the subphase were maintained above such a concentration to ensure such a saturated Δπ represented a maximal value. The critical surface pressure (πc) was determined by extrapolating the Δπ versus πc plot to the x axis (21).

Surface Plasmon Resonance (SPR) Measurements—400 μg/ml vesicle solutions were prepared in an appropriate flow buffer solution (typically 10 mM HEPES, pH 7.4, containing 0.15 mM NaCl and varying concentra-
in the C1a domain and His\textsuperscript{127}, Lys\textsuperscript{131}, and Lys\textsuperscript{141} in the C1b domain were replaced by alanine (Fig. 1). Since all mutated residues are surface exposed (Fig. 2), these mutations were not expected to cause deleterious conformational changes. Indeed, all six mutants were expressed in baculovirus-infected insect cells as efficiently as wild type, suggesting comparable thermodynamic stability and lack of gross conformational changes.

We systematically analyzed the effects of the above mutations by measuring the anionic phospholipid dependence of vesicle binding and enzyme activity for wild type and mutants. First, we measured the PS dependence of binding to POPC/POPS mixed vesicles containing 1 mol % of DOG. As shown in Fig. 3, two C1a domain mutants, K62A and R77A, required significantly higher mol % of PS for vesicle binding (\([PS]_{1/2} = 20\) and 30 mol %, respectively) whereas another C1a mutant K76A and all C1b mutants behaved essentially the same as wild type (\([PS]_{1/2} = 16\) to 18 mol %). We then measured the kinase activity of wild type and mutants toward histone under the same conditions (i.e. in the presence of POPC/POPS/DOG mixed vesicles). In general, C1a domain mutants exhibited lower activity than did C1b domain mutants at a given PS concentration (Fig. 4). For instance, at 40 mol % PS wild type PKC-\(\alpha\) and C1b domain mutants showed full activity. Under the same conditions, however, K62A and K76A showed only 44 and 55% of the wild type activity, respectively, although they are fully vesicle-bound (see Fig. 3). Most notably, R77A showed no detectable activity with up to 80 mol % PS although the protein should be fully vesicle-bound with 80 mol % PS (see Fig. 3). R77A exhibited full vesicle-binding affinity and enzymatic activity in the presence of 1 mol % of phorbol 12-myristate 13-acetate in the vesicles (data not shown), indicating that the extremely low activity of R77A is not due to deleterious conformational changes.

We have previously shown that the isolated C1 domain (i.e. C1a + C1b) has essentially the same affinity for PS and phosphatidylycerol (PG)-containing vesicles, indicating lack of a specific PS-binding site in the domain (14). This, in turn, suggests that the role of cationic residues in the C1 domains is to interact nonspecifically with anionic phospholipids. If this is the case, the mutations of the cationic residues of the C1 domains of PKC-\(\alpha\) should not affect its PS specificity for vesicle binding affinity and enzyme activity. To test this notion, we measured the PG dependence of the binding of wild type and mutants to POPC/POPS/DOG mixed vesicles and compared it with the PS dependence shown in Fig. 3. As reported previously (18), PKC-\(\alpha\) and all mutants required higher mol % of PG than PS for the same degree of vesicle binding (Fig. 5). As with the PS dependence of vesicle binding, only two C1a domain mutants, K62A and R77A, showed reduced binding affinity for PG-containing vesicles, while other mutants behaved like wild type. We then measured the PG dependence of kinase activity. As shown in Fig. 6, the PG dependence of activity compared...
well with the PS dependence (Fig. 4). In general, wild type and all mutants had much lower kinase activity in the presence of PG vesicles, and displayed significant activity only at high mol \% of PG. Even at high mol \% of PG, however, K62A and R77A exhibited much lower activity than wild type. For instance, R77A exhibited no detectable activity and K62A showed about 50\% of wild type activity at 80 mol \% PG. Thus, the PG dependence was qualitatively similar to corresponding PS dependence, indicating that the mutations of C1 domain residues affect the PS- and PG-dependent vesicle binding and activation of PKC-\alpha to similar extents. Taken together, these results indicate that the cationic residues in the C1a domain, most notably Arg^{77}, make important contributions to the membrane binding and activation of PKC-\alpha by nonspecifically interacting with anionic membrane surfaces.

Roles of C1 Domain Aspartates—Our model structures of C1a and C1b domains of PKC-\alpha reveal the presence of single surface-exposed anionic residues, Asp^{55} (C1a) and Asp^{116} (C1b), located near the cationic patches (Fig. 2). Residues 55 and 116 are not perfectly matched in the sequence alignment but their relative location in the molecule should be similar based on our modeling (see Fig. 1). Conventional and novel PKCs invariably contain an anionic residue, predominantly Asp, in these positions. To determine the role of these unique aspartates, we replaced Asp^{55} in the C1a domain and Asp^{116} in the C1b domain with alanine and lysine, respectively. We then measured the vesicle binding and kinase activity of the mutants as a function of PS composition in POPC/POPG/DAG (99-x:1) mixed vesicles and also as a function of Ca^{2+}. As shown in Fig. 7, D55A showed higher membrane affinity than wild type at a given PS composition in the range of 0 to 20 mol \%. As a result, [PS]_{1/2} (\sim 10 mol \%) for D55A was significantly lower than that of wild type (\sim 17 mol \%). In contrast, D116A behaved similarly to wild type. A similar trend was seen with relative activity. In this case, the maximal activity of D55A was \sim 35\% higher than that of wild type even when both enzymes were fully activated. Fig. 8 shows the calcium dependence of PKC activity of the three proteins in the presence of POPC/POPS/DAG (89:30:1) vesicles. Again, D55A required less Ca^{2+} than wild type and D116A for activation and showed \sim 40\% higher maximal activity. Since our previous studies showed that Ca^{2+} and PS are required for triggering the membrane penetration and DAG binding of the C1a domain (14, 18), lower Ca^{2+} and PS requirements for D55A activation suggest that this mutant might have higher intrinsic activity to penetrate the membrane and bind DAG (see the monolayer penetration data below). This, in turn, implies that Asp^{55} might be involved in the specific tethering of C1a domain, which is relieved upon Ca^{2+}-dependent binding to PS-containing membranes. The observed properties of D55A were not due to stronger nonspecific electrostatic interactions between the C1a domain and the anionic membrane caused by the removal of negative charge on the C1a domain, because D55K behaved essentially the same as D55A. On the basis of simple electrostatic effect, the former would have higher affinity and activity than the latter. To further test the notion that D55A exists in a more or less preactivated conformation, we measured the activity of wild type, D55A, and D116A in the presence of POPC/POPG/DAG

![Fig. 4. Dependence of enzymatic activity of PKC-\alpha and C1 domain mutants on the POPG composition in POPC/POPG/DOG vesicles. Proteins include wild type (■), K62A (▲), K76A (▲), R77A (●), H127A (△), K131A (□), and K141A (○). Total lipid concentration of POPC/POPG/DOG (99-x:1) mixed vesicles and also as a function of Ca^{2+}. As shown in Fig. 7, D55A showed higher membrane affinity than wild type at a given PS composition in the range of 0 to 20 mol \%. As a result, [PS]_{1/2} (\sim 10 mol \%) for D55A was significantly lower than that of wild type (\sim 17 mol \%). In contrast, D116A behaved similarly to wild type. A similar trend was seen with relative activity. In this case, the maximal activity of D55A was \sim 35\% higher than that of wild type even when both enzymes were fully activated. Fig. 8 shows the calcium dependence of PKC activity of the three proteins in the presence of POPC/POPS/DAG (89:30:1) vesicles. Again, D55A required less Ca^{2+} than wild type and D116A for activation and showed \sim 40\% higher maximal activity. Since our previous studies showed that Ca^{2+} and PS are required for triggering the membrane penetration and DAG binding of the C1a domain (14, 18), lower Ca^{2+} and PS requirements for D55A activation suggest that this mutant might have higher intrinsic activity to penetrate the membrane and bind DAG (see the monolayer penetration data below). This, in turn, implies that Asp^{55} might be involved in the specific tethering of C1a domain, which is relieved upon Ca^{2+}-dependent binding to PS-containing membranes. The observed properties of D55A were not due to stronger nonspecific electrostatic interactions between the C1a domain and the anionic membrane caused by the removal of negative charge on the C1a domain, because D55K behaved essentially the same as D55A. On the basis of simple electrostatic effect, the former would have higher affinity and activity than the latter. To further test the notion that D55A exists in a more or less preactivated conformation, we measured the activity of wild type, D55A, and D116A in the presence of POPC/POPG/DAG

![Fig. 6. Dependence of enzymatic activity of PKC-\alpha and C1 domain mutants toward histone on the POPG composition in POPC/POPG/DOG vesicles. Proteins include wild type (■), K62A (▲), K76A (▲), R77A (●), H127A (△), K131A (□), and K141A (○). Experimental conditions are the same as described for Fig. 4. The absolute value of maximal activity is 0.20 \pm 0.04 \mu mol/(mg/min), as described for Fig. 4.
The Origin of PS Specificity—Since PS specifically allows the membrane penetration and DAG binding of C1a domain, we reasoned that PS with a carboxylic group in the head group might be able to release the hypothetical C1a domain tethering by competing with Asp55. If so, D55A (and D55K) should lose PS specificity in vesicle binding and activation due to lack of C1a domain tethering. To explore this possibility, we measured the vesicle binding and kinase activity of wild type and mutants as a function of anionic phospholipid composition in two different mixed vesicles, including POPC/POPS/DOG and POPC/POPG/DOG. As shown in Fig. 10, wild type PKC-\(\alpha\) and D116A showed similar high PS specificity for vesicle binding; i.e. \([\text{PS}]_{1/2} = 17 \text{ mol }\%\) and \([\text{PG}]_{1/2} = 30–35 \text{ mol }\%\). In contrast, D55A showed a lower degree of PS specificity; \([\text{PS}]_{1/2} = 10 \text{ mol }\%\) and \([\text{PG}]_{1/2} = 12 \text{ mol }\%\). Thus, D55A binds PG-containing vesicles, for which conventional PKCs are known to have much lower affinity, as tightly as the wild type PKC-\(\alpha\) binds PS-containing vesicles. This high affinity of D55A for PG vesicles leads to dramatically reduced PS specificity, when compared with wild type. The relative activities of the proteins determined in the presence of different mol \% of POPS and POPG further support our model. All three proteins are essentially fully vesicle-bound when the anionic phospholipid composition of mixed vesicles is above 40 mol \% (see Fig. 10). Thus, the relative activity of the proteins under these conditions should reflect mainly the effects of mutations on PKC activation. As shown in Fig. 11, the three proteins displayed distinctly different degrees of PS specificity. As reported previously, PKC-\(\alpha\) showed high PS specificity, i.e. PS \(\gg\) PG. When compared with wild type, D55A showed a much lower degree of PS specificity at both 40 and 60 mol \% of anionic lipids. At 40 mol \% of anionic lipids, PG was \(\sim 75\%\) as effective as PS in activating D55A. At 60 mol \% of anionic lipids, activities on PS and PG were comparable. Also, D55A was more active than wild type under all assay conditions (up to 140\% of wild type activity). On the other hand, D116A was about 15\% less active than wild type in the presence of PS-containing vesicles but was more active than wild type in the presence of PG-containing vesicles. As a result, D116A showed considerable lower PS specificity than did wild type. Taken together, it is clear that Asp55 in the C1a domain plays an important role in the PS specificity for membrane binding and activation of PKC-\(\alpha\). On the other hand, Asp116 had little effect on the vesicle binding of PKC-\(\alpha\) but modestly lowered the PS dependent activity while enhancing the PG dependent activity. Thus, Asp116 in the C1b domain might also be involved in PS specificity in PKC-\(\alpha\) activation, albeit indirectly (see “Discussion”).
Monolayer Measurements—We have shown that PS specifically induces the penetration of C1a domain into the membrane (18). To corroborate the notion that PS specifically disrupts the tethering and thereby induces the penetration of hydrophobic residues of the C1a domain into the membrane.

SPR Measurements—The SPR analysis of membrane-protein interactions offers an advantage over other methods in that the effects of the mutations of membrane-binding residues on membrane association \( k_d \) and dissociation \( k_i \) rate constants can be directly determined (25, 26). In our recent study on the membrane binding of phospholipase A2, we showed that electrostatic interactions driven by ionic residues mainly affect \( k_d \), whereas hydrophobic interactions resulting from the membrane penetration of hydrophobic residues largely influence \( k_i \). By means of the SPR analysis, we determined the values of \( k_d \) and \( k_i \), for PKC-\( \alpha \), D55A, and D116A at varying surface lipid compositions and calcium concentrations. First, we coated the sensor chip with POPC/POPS/DOG (69:30:1) vesicles and measured the binding in the presence of 0.1 mM Ca\(^{2+} \). As summarized in Table I, little variation of \( k_d \) or \( k_i \) was observed for the mutants when compared with wild type, hence comparable \( K_d \) values. This is consistent with our vesicle binding data (see Fig. 10), in which all three proteins exhibited the maximal binding under these conditions. Since there was a much larger difference in relative binding affinity with POPC/POPS/DOG (69:30:1) vesicles and 0.1 mM Ca\(^{2+} \) (see Fig. 10), we then measured the penetration of PKC-\( \alpha \) and mutants into mixed vesicles as a function of PS and PG composition. Wild type (○), D55A (△), and D116A (□) were incubated with 0.1 mM POPC/POPS/DOG (99:1:1) vesicles (open symbols) or POPC/POPG/DOG (99:1:1) vesicles (closed symbols). Experimental conditions are the same as described for Fig. 3. Each data point represents an average of triplicate measurements.

FIG. 10. Binding of PKC-\( \alpha \) and the C1 domain aspartate mutants to mixed vesicles as a function of PS and PG composition. 

FIG. 11. Enzymatic activity of PKC-\( \alpha \) and C1 domain aspartate mutants toward histone at two different anionic lipid compositions. The kinase activity of wild type, D55A, and D116A was measured in the presence of POPC/POPS/DOG (open bars) and POPC/POPG/DOG (solid bars) mixed vesicles. Total lipid concentration and calcium concentration were 0.1 mM and DOG composition was 1 mol %.

FIG. 12. Effect of the initial surface pressure of monolayers on the penetration of PKC-\( \alpha \) and mutants. Proteins used were wild type (○), D55A (△), and D116A (□) and their concentrations in the subphase were 1.5 \( \mu \)g/ml. Monolayers contained either POPC/POPS (6:4) (open symbols) or POPC/POPG (6:4) mixed monolayers (filled symbols). The subphase contained 20 mM Tris buffer, pH 7.5, containing 0.1 M KCl and 0.1 mM Ca\(^{2+} \). Each data point represents an average of duplicate measurements.

etrinate into the phospholipid monolayer with \( \pi_c \) comparable to or higher than that of biological membranes (i.e. \( \pi_c \geq 31 \) dyn/cm) (24). As reported previously (18), PS showed the unique ability to induce the Ca\(^{2+} \)-dependent penetration of PKC-\( \alpha \) into the monolayer: \( \pi_c \approx 33 \) dyn/cm for the POPC/POPS (6:4) monolayer and 27 dyn/cm for the POPC/POPG (6:4) monolayer (Fig. 12). D116A again displayed a similar property with \( \pi_c \approx 34 \) dyn/cm for the POPC/POPS monolayer and 29 dyn/cm for the POPC/POPG monolayer. In contrast, D55A showed no appreciable selectivity for PS and penetrated equally well into POPC/POPS and POPC/POPG monolayers (i.e. \( \pi_c \approx 34 \) dyn/cm for both PS- and PG-containing monolayers). These results provide strong evidence for the notion that Asp\(^{55} \) is involved in the C1a domain tethering and that PS specifically disrupts the tethering and thereby induces the penetration of the hydrophobic residues of the C1a domain into the membrane.

2 R. V. Stahelin and W. Cho, submitted for publication.
ured the binding of the three proteins with the sensor chip coated with POPC/POPG/DOG (69:30:1) vesicles. In agreement with vesicle binding data, all three proteins showed lower affinity for the POPC-coated chip than for the POPS-coated chip. Again, D116A mutation did not significantly influence \( k_a \) and \( k_d \) under these conditions. However, D55A had 2.2-fold lower \( k_d \) than wild type while having comparable \( k_a \), indicating that the mutation leads to the enhanced penetration into the POPG-containing vesicles. This is also consistent with the monolayer penetration data shown in Fig. 12. We then measured the binding to immobilized POPC/POPS/DOG (69:30:1) vesicles at the lowest possible Ca\(^{2+}\) concentration that gave rise to detectable SPR signal under our experimental conditions (i.e. 7 \( \mu \)M Ca\(^{2+}\)). In accordance with Ca\(^{2+}\) dependence data in Fig. 8, D55A had 15-fold higher affinity (in terms of \( K_d \)) than wild type and D116A. Interestingly, enhanced affinity of D55A derived from both a 3.2-fold increase in \( k_a \) and 4.6-fold decrease in \( k_d \). As was the case with binding to POPC/POPG/DOG (69:30:1) vesicles, the decrease in \( k_d \) should be due to enhanced membrane penetration. On the other hand, the increased \( k_a \) might originate from the contribution from C1a cationic residues that can readily interact with anionic membranes due to the lack of C1a domain tethering. This contribution would become more important when the C2 domain cannot effectively drive the membrane association at low calcium concentrations. Together, these data further supports the notion that Asp\(^{55}\) of PKC-\( \alpha \) is involved in C1a domain tethering, the disruption of which allows the membrane penetration of the C1a domain, which in turn leads to more favorable interactions between C1a cationic residues and anionic membrane surfaces (see Fig. 2 and “Discussion”).

**DISCUSSION**

**Differential Roles of C1a and C1b Domains**—Both conventional and novel PKCs contain a tandem repeat of C1 domains, which serve as a binding site for DAG and phorbol esters (8–13). Irie et al. (27) recently reported that despite high sequence homology, isolated C1 domains of conventional and novel PKCs have different phorbol ester affinity, with dissociation constants ranging from 1 nM to >3 \( \mu \)M. Oncia et al. (28) also reported that isolated C1a and C1b domains of PKC-\( \gamma \) showed different translocation patterns in the cell. A growing body of evidence indicates that C1a and C1b domains have distinct roles in the full-length PKC molecule. For instance, Slater et al. (29, 30) have reported that PKC-\( \alpha \) contains two distinct binding sites with low and high affinity for phorbol esters and that DAG and phorbol esters bind to the two discrete sites with opposite affinity. Although these *in vitro* and cell studies have demonstrated distinct properties and roles of the C1a and C1b domains, no direct correlation between the intrinsic properties of individual C1 domains and their specific roles in different PKC isoforms has been established. Our recent study of PKC-\( \alpha \) shed new light on the differential roles of C1a and C1b domain in the activation of conventional PKC (14).

The study showed that differential roles of the two C1 domains in DAG-induced PKC activation are correlated with their different membrane penetration behaviors. That is, the C1a domain plays an essential role because its hydrophobic residues can penetrate into the membrane to bind DAG whereas the C1b domain does not because of lack of membrane penetration. Differential effects of cationic residue mutations described in this report corroborate the critical involvement of C1a domain in the membrane binding and activation of PKC-\( \alpha \). Among three cationic residues in the C1a domain, Arg\(^{77}\) is most essential for anionic vesicle binding and Lys\(^{82}\) also makes considerable contribution to vesicle binding, suggesting that these residues make immediate contact with anionic membrane surfaces. Interestingly, an orientation of the membrane-bound C1a domain that allows the penetration of its hydrophobic residues into the hydrophobic core of the membrane would also permit favorable contact of the two cationic residues with anionic membrane surfaces (see Fig. 2). These interactions are nonspecific Coulombic interactions, as the mutations reduce the binding to PS- and PG-containing vesicles to comparable extents. This notion is consistent with our previous finding that the vesicle binding and monolayer penetration of the isolated C1 domains (i.e. C1a + C1b) showed no PS specificity (14). Unlike C1a domain, C1b domain appears to bind the membrane in an orientation that allows neither the penetration of its hydrophobic residues nor electrostatic interactions between its cationic residues with anionic membrane surfaces. Note that C1a domain mutants display much larger decreases in activity than expected from their reduced membrane affinity. In particular, R77A shows no activity even under the conditions where all enzyme molecules are vesicle-bound. Furthermore, R77A showed markedly reduced penetration into the POPC/POPS (6:4) monolayer \((\pi_c = 26 \text{ dyn/cm})\) when compared with wild type (data not shown). This indicates that the electrostatic interactions of the C1a cationic residues with anionic phospholipids are important not only for membrane binding but also for membrane penetration, DAG binding, and activation of PKC-\( \alpha \). It has been shown that multiple anionic phospholipids, including some PS molecules, are required for

**TABLE I**

**Binding parameters for PKC-\( \alpha \) and mutants determined from SPR analysis.**

| Enzymes       | \( k_a \) (M\(^{-1}\) s\(^{-1}\)) | \( k_d \) (s\(^{-1}\)) | \( K_d \) (M) |
|---------------|----------------------------------|------------------------|--------------|
| POPC/POPS/DOG (69:30:1) and 0.1 mM Ca\(^{2+}\) | | | |
| PKC-\( \alpha \) | (1.5 ± 0.5) × 10\(^6\) | (1.2 ± 0.3) × 10\(^{-4}\) | (8.0 ± 3.0) × 10\(^{-11}\) |
| D55A | (1.3 ± 0.2) × 10\(^6\) | (8.0 ± 0.8) × 10\(^{-5}\) | (6.2 ± 1.1) × 10\(^{-11}\) |
| D116A | (1.6 ± 0.4) × 10\(^6\) | (1.8 ± 0.4) × 10\(^{-4}\) | (1.1 ± 0.4) × 10\(^{-10}\) |
| POPC/POPG/DOG (69:30:1) and 0.1 mM Ca\(^{2+}\) | | | |
| PKC-\( \alpha \) | (3.3 ± 0.9) × 10\(^5\) | (3.5 ± 0.2) × 10\(^{-4}\) | (1.1 ± 0.3) × 10\(^{-9}\) |
| D55A | (4.2 ± 0.6) × 10\(^5\) | (1.6 ± 0.4) × 10\(^{-4}\) | (3.8 ± 1.1) × 10\(^{-10}\) |
| D116A | (4.0 ± 0.5) × 10\(^5\) | (4.1 ± 0.4) × 10\(^{-4}\) | (1.0 ± 0.2) × 10\(^{-9}\) |
| POPC/POPG/DOG (69:30:1) and 7 \( \mu \)M Ca\(^{2+}\) | | | |
| PKC-\( \alpha \) | (1.3 ± 0.2) × 10\(^5\) | (6.5 ± 0.6) × 10\(^{-4}\) | (5.0 ± 0.9) × 10\(^{-9}\) |
| D55A | (4.2 ± 0.7) × 10\(^5\) | (1.4 ± 0.2) × 10\(^{-4}\) | (3.3 ± 0.7) × 10\(^{-10}\) |
| D116A | (1.1 ± 0.3) × 10\(^5\) | (3.7 ± 0.5) × 10\(^{-4}\) | (3.4 ± 1.0) × 10\(^{-9}\) |
conventional PKC activation (31, 32). It is not likely that a conventional PKC molecule contains multiple specific binding sites for these anionic phospholipids. Our results indicate that the cationic residues in the C1a domain can provide nonspecific electrostatic interaction sites for the anionic phospholipids.

**The Role of C1 Domain Aspartates and the Origin of PS Specificity**—A consensus model of conventional PKC activation holds that the Ca\(^{2+}\)-dependent binding of PKC to PS and DAG (or phorbol esters) triggers conformational changes of PKC, resulting in the removal of the pseudosubstrate from the active site and PKC activation (2). Our previous studies provided specific mechanistic details for this general model: Ca\(^{2+}\) and PS induce the membrane binding of protein and the specific membrane penetration of the C1a domain of PKC-\(\alpha\) to allow its interactions with DAG, which also drives the removal of the pseudosubstrate from the active site. Based on cellular translocation studies, Oancea and Meyer (33) proposed that the DAG-binding site of PKC-\(\gamma\) (i.e. two C1 domains) is inaccessible to DAG in the resting state because it is clamped to the catalytic domain by the pseudosubstrate. The present study indicates that a single aspartate residue Asp\(^{55}\) is involved in tethering of the C1a domain of PKC-\(\alpha\) to the protein molecule in the resting state, thereby rendering the DAG-binding site inaccessible to DAG in the membrane. The mutation of Asp\(^{55}\) to alanine (or lysine) dramatically changed the membrane binding, activation, and monolayer penetration of PKC-\(\alpha\). In particular, D55A shows much higher vesicle affinity, activity, and monolayer penetration power than wild type under nonactivating conditions, i.e. with PG and in the absence of (or at low) Ca\(^{2+}\), indicating that D55A has enhanced conformational flexibility that allows it to be activated much more easily than wild type. As a result, D55A shows much reduced PS specificity, which is reminiscent of the isolated C1 domains (i.e. C1a + C1b) (14), suggesting that its behaviors are dictated mainly by the C1 domains due to the disruption of C1a domain tethering. A recent study by Johnson et al. (34) suggested that a PS-specific binding site is located in the C1 domain of PKC-\(\beta_2\), based on the finding that the isolated C1b domain has higher affinity for PS than for other anionic phospholipids. It should be noted that the reduced PS specificity of D55A cannot be accounted for by this model for at least two reasons. First, D55A has higher affinity for PS vesicles and higher activity in the presence of PS vesicles than wild type (see Fig. 7), which precludes the possibility that Asp\(^{55}\) is directly involved in PS binding. Second, no mutation of C1a domain cationic residues has a significant effect on PS specificity of binding and activity, showing that these residues do not serve as a PS-binding site. Thus, it is also unlikely that Asp\(^{55}\) indirectly influences PS specificity by interacting with the PS-binding site in the C1 domain. Furthermore, the model cannot fully explain the enhanced penetration of D55A into PG-containing monolayers and its elongated membrane residence time at PG-containing vesicles and at low Ca\(^{2+}\). These data are more consistent with the notion that the disruption of Asp\(^{55}\)-mediated tethering leads to the nonspecific membrane penetration of the C1a domain, which results in dramatically enhanced binding affinity for non-PS anionic phospholipid aggregates and much higher activity in the presence of nonspecific lipids. This in turn suggests that the specific Ca\(^{2+}\)- and PS-dependent membrane penetration and activation of PKC-\(\alpha\) involves the disruption of Asp\(^{55}\)-mediated C1a domain tethering. The role of Asp\(^{116}\) of the C1b domain in PKC-\(\alpha\) activation is less clearly defined. The wild type-like vesicle affinity and monolayer penetration of D116A indicate that Asp\(^{116}\) is not directly involved in PKC activation and PS specificity. D116A, however, shows consistently lower activity than wild type in the presence of POPC/POPS/DG vesicles and has significantly higher activity under nonspecific conditions, e.g. in the presence of POPC/POPG/DG vesicles (see Fig. 11). In particular, D116A has >5-fold higher basal activity than wild type in the absence of calcium and lipid cofactors (data not shown). Thus, Asp\(^{116}\) might be indirectly involved in PS specificity of PKC activation by suppressing the level of nonspecific activation. This would supplement the direct role of Asp\(^{55}\) of the C1a domain in the PS-specific PKC activation.

The understanding of the exact chemical nature of the C1a domain tethering and the identification of residues that interact with Asp\(^{55}\) would require high-resolution structural information of the full-length PKC molecule. Based on several lines of evidence supporting the importance of C1-C2 interdomain interactions in PKC regulation, we speculate that Asp\(^{55}\) interacts with a C2 domain residue in the calcium-binding loop. Although either the C1 or C2 domain alone is capable of recruiting conventional PKC to the membrane, a concerted action of both domains is absolutely required for full activation of the enzyme and, in particular, for its PS specificity. For instance, our previous study with the isolated C1 and C2 domains of PKC-\(\alpha\) indicated that a primary determinant of PS specificity resides in the C2 domain (14), yet the present study shows that the mutation in the C1a domain has a dramatic effect on the PS specificity. Furthermore, the full-length PKC-\(\alpha\), which shows much more pronounced PS specificity than does the isolated C2

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**Fig. 13. A proposed mechanism of the in vitro membrane binding and activation of conventional PKC.** In this model, the C1a domain and the C2 domain are tethered via hydrogen bond between Asp\(^{55}\) and a C2 domain residue (e.g. Asn\(^{189}\)). When the protein binds to PG-containing membranes (case A), the C1a-C2 tethering remains intact, and consequently PKC remains largely inactive. When the protein binds to PS-containing membranes (case B, see the inset), however, the carboxylate of PS releases Asp\(^{55}\) of the C1a domain from the tethering, resulting in the membrane penetration and DAG binding of the C1a domain and PKC activation.
domain, exhibits its full PS specificity only in the presence of C1 domain ligand, DAG, or phorbol esters. The close interaction of C1 and C2 residues has also been implicated in the regulation of a novel PKC from *Aplysia* (35). A recently determined crystal structure of the C2 domain of PKC-α-C1a domain specifically interacts with Asn 189 in the Ca²⁺ that might interact with Asp 55. Also, it remains to be seen whether or not the model can account for the activation of other PKCs. As such, the model provides a basis for further investigations of putative C1-C2 inter-domain interaction and the origin of PS specificity (7). In this structure, the phosphate oxygen of a PS molecule specifically coordinates with a calcium ion, while its carboxylate interacts with the backbone and side chain nitrogens of Asn 189 in the calcium binding pocket (see Fig. 13). The structure raises an intriguing possibility that Asp 55 in the C1a domain specifically interacts with Asn 189 in the Ca²⁺ binding pocket of the C2 domain in the resting state to keep the protein in an inactive conformation, as schematically illustrated in Fig. 13. We propose that upon membrane binding of PKC, which is driven by electrostatic interactions involving the C2 domain-bound calcium ions and cationic residues in the C1a domain, the carboxylate group of PS (one or more molecules) might unleash the putative tethering by replacing Asp 55. This might allow the C1a domain to penetrate into the membrane and bind DAG. The molecular motion accompanying the membrane penetration would then remove the pseudosubstrate from the active site, hence the activation. Undoubtedly, the corroborated hypothesis of this model entails further studies, including the mutation of Asn 189 and other C2 domain residues that might interact with Asp 55. Also, it remains to be seen whether or not the model can account for the activation of other PKCs. As such, the model provides a basis for further investigations of the molecular mechanisms underlying the subcellular targeting and activation of PKC isomers.

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Roles of Ionic Residues of the C1 Domain in Protein Kinase C-α Activation and the Origin of Phosphatidylerine Specificity

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