Role of Hydrophobic Residues in the C1b Domain of Protein Kinase C δ on Ligand and Phospholipid Interactions*

The C1 domains of conventional and novel protein kinase C (PKC) isoforms bind diacylglycerol and phorbol esters with high affinity. Highly conserved hydrophobic residues at or near the rim of the binding cleft in the second cysteine-rich domain of PKC-δ (PKC-δC1b) were mutated to probe their roles in ligand recognition and lipid interaction. [3H]Phorbol 12,13-dibutyrate (PDBu) binding was carried out both in the presence and absence of phospholipids to determine the contribution of lipid association to the ligand affinity. Lipid dependence was determined as a function of lipid concentration and composition. The binding properties of a high affinity branched diacylglycerol with lipophilicity similar to PDBu were compared with those of PDBu to identify residues important for ligand selectivity. As expected, Leu-20 and Leu-24 strongly influenced binding. Substitution of either by aspartic acid abolished binding in either the presence or absence of phosphatidylserine. Mutation of Leu-20 to Arg or of Leu-24 to Lys caused a dramatic (340- and 250-fold, respectively) reduction in PDBu binding in the presence of lipid but only a modest reduction in the weaker binding of PDBu observed in the absence of lipid, suggesting that the main effect was on C1 domain-phospholipid interactions. Mutation of Leu-20 to Lys or of Trp-22 to Lys had modest (3-fold) effects and mutation of Phe-13 to Tyr or Lys was without effect. Binding of the branched diacylglycerol was less dependent on phospholipid and was more sensitive to mutation of Trp-22 to Tyr or Lys, especially in the presence of phospholipid, than was PDBu. In terms of specific PKC isoforms, our results suggest that the presence of Arg-20 in PKC-γ may contribute to its lack of phor- bol ester binding activity. More generally, the results emphasize the interplay between the C1 domain, ligand, and phospholipid in the ternary binding complex.

The protein kinase C (PKC) family of serine/threonine ki-

nases plays a central role in mediating the signals that lead to divergent cellular functions (1, 2). The structure of the PKCs is composed of an N-terminal regulatory region and a C-terminal catalytic region. The regulatory region modulates enzymatic activities by interacting with endogenous and exogenous activators and cofactors of PKCs through subdomains such as the pseudosubstrate region and the C1 and C2 domains (3, 4). The twin C1 domains, a tandem repeat of a cysteine-rich, finger structure, are the binding sites for the endogenous PKC ligand sn-diacylglycerol (DAG) and for the phorbol ester tumor promoters (5, 6).

The C1 domain consists of a conserved 50 amino acid sequence possessing the motif HX_CX_HX_CX_C (C, cysteine; H, histidine; X, any other amino acid) and coordinating two Zn2+ ions (7). The solution structure of the C1b domain of PKC-α was determined by NMR. The domain adopts a globular fold allowing two non-consecutive sets of residues to form the two separate zinc-binding sites (8). The x-ray crystallographic structure of the C1b domain of PKC-δ in complex with phorbol 13-acetate in the absence of phospholipid revealed that phorbol 13-acetate binds in a hydrophilic groove between two pulled-apart β strands at the tip of the domain. Phorbol ester binding caps the hydrophilic groove and generates a contiguous hydrophobic surface covering one-third of the domain, thereby facilitating the membrane insertion of the domain (9). These conclusions are supported by subsequent NMR analysis of the C1b domain of rat PKC-γ in solution titrated with lipid micelles in the presence and absence of phorbol ester (10).

The residues critical for maintaining the overall structure and ligand binding by the C1 domain have been explored in several studies (9, 11–14). The 2 histidines and all but 1 of the 6 conserved cysteines that coordinate the two Zn2+ ions, namely the residues at positions 3, 8, 11, 21, 24, 27, and 38, are vital for structural integrity and the interaction with ligands. Two loops at positions 7–12 and 20–27, which comprise most of the β2 and β3 segments, constitute the phorbol ester binding site. Mutations within this region drastically affect the activity of the C1 domain (12).

In addition to ligand binding, the C1 domain also contributes to membrane interaction as does the C2 domain of the classical PKCs and the pseudosubstrate region (15, 16). Less is known about the residues responsible for membrane interaction. Since its discovery as a conserved structural module, the C1 domain has been found to be present in a range of novel proteins, distinct from the PKCs, which constitute the superfamily of phorbol ester/DAG receptor proteins. The chimaeiras, Munc-13, PKD/PKC-μ, and RasGRP exemplify subgroups within this emerging superfamily. The chimaeiras, RasGRP, and PKC isoforms differ in their ligand recognition, reflecting

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† To whom correspondence should be addressed: Bldg. 37, Rm. 3A01, National Cancer Institute, 37 Convent Dr., MSC 4255, Bethesda, MD 20892-4255. Tel.: 301-496-3189; Fax: 301-496-8709; E-mail: blumberp@dc37a.nci.nih.gov.

‡ The abbreviations used are: PKC, protein kinase C; PKD, protein kinase D; DAG, diacylglycerol; PAG, polycladamide gel electrophoresis; PDBu, phorbol 12,13-dibutyrate; LUV, large unilamellar vesicle; POPs, sn-1-palmi
toyl-2-oleoylphosphatidylserine; POPC, sn-1-palmityl-2-oleoylphosphati
dyholine; GST, glutathione S-transferase; IPTG, isopropyl-1-thio-
opyranoside; PS, phosphatidylserine.
Role of the Hydrophobic Residues in PKC-δ/γ/C1 Domains

both differences in lipid requirements as well as in intrinsic specificity. Understanding the structural basis for ligand selectivity remains largely unresolved. Phe-20 had been suggested to be involved in differential affinity of β2-chimaerin to constrained DAG analogs and thymeleatoxin (17); the same residue was also suggested to be responsible for weaker phorbol ester binding of the C1α domains of PKCs (13). Arg-20 in PKC-ζ and -η may contribute, along with Gly at position 11, to its lack of phorbol ester recognition. Trp-22 was implicated in mediating differential lipid and ligand binding in PKC-α and PKD/ PKC-µ (15). However, few studies had thoroughly examined the roles of these residues in C1 domain function.

Guided by computer modeling, NMR, and x-ray crystallography, we sought in the present study to use site-directed mutagenesis to explore three aspects of C1 domain functions in parallel: ligand recognition, lipid interaction, and ligand selectivity. We mainly focused on four residues at positions 13, 20, 22, and 24 along the rim of the ligand-binding pocket that are exposed to the surface and positioned toward the membrane (9). Residues of varying hydrophilicity such as Tyr, Asp, Lys, and Arg were introduced at these positions in order to change the overall surface hydrophobicity around the binding cleft, therefore affecting their interaction with lipids and ligands. In order to explore residues potentially important for determining the specific binding activity of different C1 domains, we also mutated select residues in PKC-δ/γ/C1b to the unique residues appearing in the C1 domains of PKC-ζ, PKC-α, and n/β-chimaerin, as well as in PKD/PKC-µ, which were candidates for their differential binding activity and ligand selectivity, as suggested by sequence comparisons and our structural modeling studies. The binding activities of wild type and mutant PKC-δ/γ/C1b domains for phorbol ester and DAG were characterized in the presence and absence of phospholipid or in the presence of lipid vesicles of different compositions. Our results further our insight into the structural basis of C1 domain function.

EXPERIMENTAL PROCEDURES

Materials—[20-3H]Phorbol 12, 13-dibutyrate (PDBu) (20 Ci/mmol) was purchased from PerkinElmer Life Sciences. PDBu was obtained from Alexis Biochemicals (Pittsburgh, PA). Synthesis of 1-(4-methyl-3-(methylpentanoyl)-2-(3-methylbut-2-enoyl)-sn-glycero (97F31, the branched DAG) will be reported elsewhere. Phosphatidylserine was purchased from Sigma. sn-1-Palmitoyl-2-oleoylphosphatidylserine (POPS) and sn-1-palmitoyl-2-oleoylphosphatidylcholine (POPC) were purchased from Avanti Polar Lipids (Alabaster, AL). Regents for expression and purification of glutathione S-transferase (GST) fusion proteins were obtained from Amersham Pharmacia Biotech.

Expression and Purification of the C1b Region of PKC-δ/γ/C1b in Escherichia coli—The C1b domain of PKC-δ was generated by polymerase chain reaction using the full-length mouse PKC-δ cDNA as template and was subcloned into a pGEX-2TK vector (Amersham Pharmacia Biotech). The recombinant plasmid was expressed in E. coli XL1-blue cells and purified to homogeneity as described previously (18). Site-directed mutagenesis of the GST-C1b fusion proteins was performed with the Unique Site Elimination (U.S.E.) system (Amersham Pharmacia Biotech) as described previously (12). Briefly, the pGEX-C1b plasmid was used as the template and a PstI selection primer was used to convert a PstI site to a SacII site within the pGEX plasmid. The mutated residues are shown in Fig. 1. After two consecutive cycles of restriction digestion and bacteria expansion, the full-length sequences of the mutant plasmids were verified by sequencing, which was performed by the DNA Minicore, Division of Basic Sciences, NCI, National Institutes of Health (Bethesda, MD). The wild type and mutant pGEX-C1b plasmids were transformed into E. coli XL1-blue (Stratagene), and the positive clones were subsequently picked and grown in LB medium supplemented with 100 μg/ml ampicillin. The expression of GST fusion protein was induced by the addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The bacteria was harvested after 5 h of induction at 37 °C and the GST-C1b protein was purified using glutathione-Sepharose 4B beads (following the manufacturer’s recommendation (Amersham Pharmacia). The purity of the protein after eluting from the beads was verified by SDS-PAGE and staining with Coomassie Blue.

[^1H]PDBu Binding Assay—[^1H]PDBu binding to the wild type and mutant GST-C1b domains was measured using the polyethylene glycol precipitation assay developed in our laboratory (19) with minor modifications. The assay mixture (250 μl) contained 50 mM Tris-Cl (pH 7.4), 100 μg/ml phosphatidylserine, 4 mg/ml bovine immunoglobulin G, and [^1H]PDBu, and variable concentrations of competing ligand. Incubation was carried out at 37 °C for 5 or 30 min. Samples were centrifuged at 0 or 5°C for 10 min, 200 ml of 35% polyethylene glycol in 50 mM Tris-Cl (pH 7.4) was added, and the samples were incubated at 0°C for an additional 15 min. The tubes were centrifuged in a Beckman 12 microcentrifuge at 4°C (12,000 rpm, 15 min). A 100-μl aliquot of the supernatant was removed for the determination of the free concentration of[^1H]PDBu, and the pellet was carefully dried. The tip of the centrifuge tube containing the pellet was cut off and transferred to a scintillation vial for the determination of the total bound[^1H]PDBu. Aquasol was added both to an aliquot of the supernatant and to the pellet. The activity was measured by scintillation counting. Nonspecific binding was measured using an excess of nonradioactive PDBu (30 μM). Specific binding was calculated as the difference between total and nonspecific binding (20). For the determination of dissociation constants (Kd) and number of binding sites (Bmax), typical saturation curves with increasing concentrations of[^1H]PDBu (between 0.125 and 4 nM) were performed in triplicate. Dissociation constants (Kd) of the branched DAG 97F31 were determined by competition of[^1H]PDBu binding to GST-C1b using 6–8 increasing concentrations of the 97F31. ID50 values were determined from the competition curve, and the Kd for the competing ligand was calculated from its ID50 using the relationship Kd = ID50/(1 + L/Kd), where L is the concentration of free[^1H]PDBu and Kd is the dissociation constant for PDBu. Approximately 30 ng/tube GST-C1b domain was used for each assay. In cases in which the Kd of PDBu for the mutant PKC-δ/C1b domain was >20 nM, dissociation constants for PDBu were determined by competition using non-radioactive PDBu competing with[^1H]PDBu (specific activity 870.2 dpm/pmol). Values represent the mean of n experiments, as indicated, with triplicate determinations for each concentration curve in each experiment. Mutant protein (4 μg/tube) was used for each competition assay. The amount of protein was adjusted for each assay so that the specific binding was over 60–80% of total binding and the total bound[^1H]PDBu was below 30% of the total amount of[^1H]PDBu in the assay. For measuring[^1H]PDBu binding in the absence of phospholipids, the Kd was determined by competition using nonradioactive PDBu as described previously with modifications. The competition concentrations of unlabeled PDBu (10 nM to 10 μM) were used to compete 100 nM[^1H]PDBu (specific activity 870.2 dpm/pmol) in a typical 250-μl reaction mix. 4–10 μg/tube PKC-δ/C1b domain was used in each assay, and the amount was adjusted so that the specific binding was over 60–80% of total binding and the total bound[^1H]PDBu was below 30% of the total amount of[^1H]PDBu in the assay. Nonspecific binding, determined in the presence of 100 μM PDBu, was 20–40% of total bound in the absence of unlabeled PDBu. The data were fitted to the theoretical inhibition curve, and the Kd was calculated from the equation Kd = ID50 – L, where ID50 is the concentration of the nonradioactive PDBu that displaced the binding of the[^1H]PDBu by 50% and L is the concentration of free radioligand at the ID50. Since some of the C1 domain bound to the walls of the assay centrifuge tube, both the pellet and tube were counted.

Large Unilamellar Vesicles (LUV)—The preparation of the sucrose-loaded vesicles was adopted, with minor modification, from the procedure of Mosior and Epand (21). Aliquots of lipids, e.g. POPS and POPC, in chloroform were mixed and dried under a stream of N2. The lipids were subsequently resuspended in 170 mM sucrose in 20 mM Tris-Cl (pH 7.4) and were subjected to five freeze-thaw cycles by placing in a 42 °C water bath and in dry ice alternately. LUV were obtained by 40 rounds of extrusion through a 100-nm polycarbonate membrane in a LipoFast liposome “factory” (Sigma). Lipid concentrations were monitored by including a trace amount of[^1H]HPCPC before extrusion to ensure an accurate final concentration after extrusion.

Vesicle Binding Assay—An experimental approach similar to that

[^2] Q. J. Wang, T.-W. Fang, V. E. Marquez, and P. M. Blumberg, manuscript in preparation.

[^3] K. Nacro, D. M. Signo, S. Yan, M. C. Nicklaus, L. L. Pearce, N. E. Lewin, S. H. Garfield, F. M. Blumberg, and V. E. Marquez, manuscript in preparation.
described above was used for the determination of \(^{[3}\text{H}]\text{PDBu}\) binding in the presence of LUV. Briefly, wild type and mutant GST-\(\alpha\text{C1b}\) domains were incubated with \(^{[3}\text{H}]\text{PDBu}\) (2 nM), sucrose-loaded vesicles comprising POPS and POPC in the presence of 50 mM Tris buffer (pH 7.4), 100 mM KCl, and 5 mg/ml γ-globulin. The concentration of the total phospholipid in the assay was 200 mM unless otherwise indicated. Incubations were carried out at 22 °C for 5 min, followed by polyethylene glycol precipitation as described above for the \(^{[3}\text{H}]\text{PDBu}\) binding assay. 15 ng of protein was used in each assay, and the amount was optimized so that the specific binding was over 60–80% of total binding and the total bound \(^{[3}\text{H}]\text{PDBu}\) was below 30% of the total amount of \(^{[3}\text{H}]\text{PDBu}\) in each binding assay.

RESULTS

\(^{[3}\text{H}]\text{PDBu}\) Binding to PKC-\(\alpha\text{C1b}\) Mutants in the Presence or Absence of Lipid—Mutations were introduced in PKC-\(\alpha\text{C1b}\) using site-directed mutagenesis as described under “Experimental Procedures.” The mutated residues and their positions are indicated in Fig. 1A. The three-dimensional structure of the four hydrophobic residues in relation to the orientation of PDBu in complex with PKC-\(\alpha\text{C1b}\) is illustrated in Fig. 1B.

The wild type and mutant PKC-\(\alpha\text{C1b}\) domains were expressed in E. coli as GST fusion proteins. Protein expression was induced by the addition of IPTG. In initial experiments, the expression level after induction with IPTG was monitored every hour. An incubation time of 4–5 h after the addition of IPTG was found to be optimal and was then used subsequently for all GST PKC-\(\alpha\text{C1b}\) fusion proteins. The fusion proteins were predominantly expressed in the cytoplasm and were isolated from the soluble fraction by binding to glutathione-Sepharose 4B beads. The purified proteins were detected on SDS-PAGE with an apparent molecular mass of 33 kDa. All mutant proteins appeared the same size on SDS-PAGE as the wild-type protein. No shift in molecular weight was observed (data not shown).

We first determined the apparent \(^{[3}\text{H}]\text{PDBu}\) binding affinities of wild type and mutant PKC-\(\alpha\text{C1b}\) domains in the presence of phosphatidylinerine (Table I). \(^{[3}\text{H}]\text{PDBu}\) bound to the wild type PKC-\(\alpha\text{C1b}\) domain with a \(K_d\) of 0.49 nM, in good agreement with our previous value (12, 18). The mutants varied markedly in their affinities for PDBu. Replacement of Leu-20 or Leu-24 with Asp led to complete loss of measurable binding. Several hundred-fold loss of activity was observed for L20R and for L24K. In contrast, only a 3-fold effect was observed for L20K, comparable to that for W22K. The other mutations caused less than a 2-fold change. The \(B_{\text{max}}\) values for all the mutants were reduced compared with the wild type, with the L20K, L20F, and L24K mutants being 3–6-fold lower. This reduction presumably reflects stability or ease of folding and did not correlate with the changes in binding affinity. Due to the variable \(B_{\text{max}}\) the amount of protein used was optimized to give above 60–80% specific binding for \(^{[3}\text{H}]\text{PDBu}\) and below 30% of total \(^{[3}\text{H}]\text{PDBu}\) bound in each binding assay.

Analysis of \(^{[3}\text{H}]\text{PDBu}\) binding in the absence of PS permits the effects of the mutations on the interaction of the C1 domain with ligand to be separated from those on the interaction of the C1 domain with lipid or of the C1-ligand complex with lipid (Table II). In the absence of PS, the wild-type PKC-\(\alpha\text{C1b}\) domain showed a 70-fold decrease in \(^{[3}\text{H}]\text{PDBu}\) binding affinity. This value agrees well with that reported previously (18). As in the presence of lipid, neither the L20D nor L24D mutants displayed detectable \(^{[3}\text{H}]\text{PDBu}\) binding. Dramatically, the L20R and L24K mutants, which showed 340- and 250-fold weaker binding than the wild type in the presence of lipid, showed decreases of only 6.7- and 2.9-fold, respectively, compared with wild type in the absence of phospholipid; indeed, their absolute binding affinities were similar in the presence or absence of phospholipid. It thus appears that these mutations have limited effect on the ligand binding per se but interfere with formation of the complex with phospholipid. The L20K mutant showed a modest (3.2-fold) decrease in binding affinity in the absence of lipid, similar to its decrease in the presence of phospholipid (3.2-fold), suggesting that the effect was on the ligand binding. Conversely, the W22K mutant showed virtually no decrease in the absence of phospholipid (1.1-fold) compared with a 2.9-fold decrease in the presence, suggesting that its influence was on lipid interaction.

Since only properly folded protein is capable of binding to PDBu, reflected by the changes of \(B_{\text{max}}\) value, we were only determining the binding properties of mutant protein that had retained an intact structure. Meanwhile, the \(^{[3}\text{H}]\text{PDBu}\) binding curves for the wild-type and mutant PKC-\(\alpha\text{C1b}\) domain are consistent with homogeneous binding kinetics. Computer modeling studies showed that mutations introduced at the hydrophobic residues at the rim of the binding cleft are unlikely to introduce structural changes to the overall folding of the zinc finger protein.

Lipid Dependence of PKC-\(\alpha\text{C1b}\) Mutants—To evaluate interaction of the C1 domain-ligand complex with phospholipids directly, we determined the dose dependence of \(^{[3}\text{H}]\text{PDBu}\) binding to the wild type and mutant PKC-\(\alpha\text{C1b}\) domains as a function of phospholipid concentration, using LUV (Fig. 2). The mutants examined were those whose binding affinities were measurable and dependent on the presence of lipid. The F13K and F13Y and L20K mutants showed either no or little shift, whereas the W22K and W22Y mutants showed intermediate shifts in the dose-response curves. The results of the lipid reconstitution thus show good qualitative agreement with the conclusions from the binding analysis in the presence and absence of phospholipid.

The binding analysis ± lipid was carried out in the presence of 100% phosphatidylserine (100 mg/ml). Using POPS:POPC mixed vesicles, we compared the lipid dependence of the wild type and the W22K mutant of PKC-\(\alpha\text{C1b}\) as a function of the mol% POPS at a constant total concentration of lipid of 0.2 mM (Fig. 3A). The mol% POPS for 50% reconstitution under these conditions shifted from 13.5% for the wild type to 46.5% for the W22K mutant. Lipid conditions that are marginal for reconstitution of ligand binding should be reflected in a decreased apparent affinity for ligand binding. Thus, at POPS:POPC (50:50), which is saturating for binding of PDBu to the wild type but not for the W22K mutant of PKC-\(\alpha\text{C1b}\), the \(K_d\) for the mutant was 6.38 ± 1.11 nM compared with 0.35 ± 0.15 nM for the wild type, a difference of 20-fold, whereas no or less difference in relative \(K_d\) value was seen in the absence of lipid or under lipid conditions that gave rise to maximal reconstitution (Fig. 3B). We have described elsewhere similar differential effects of lipid composition on ligand binding to different phospholipid receptors with different lipid requirements, namely PKC-α and RasGRP (22).

Binding of DAG to PKC-\(\alpha\text{C1b}\) Mutants in the Presence or Absence of Lipid—9TF31 is a synthetic unconstrained branched DAG with hydrophobicity similar to that of PDBu (log \(P = 3.88\) compared with 3.43 for PDBu). It was selected for the current study from a series of branched DAGs based on its low log \(P\) value with retention of PKC binding affinity. We determined the binding to wild type and mutant PKC-\(\alpha\text{C1b}\) domains of this DAG in the presence and absence of lipid and compared these results with those for PDBu (Table III). Whereas PDBu binding affinity to wild type PKC-\(\alpha\text{C1b}\) decreased 68-fold in the absence of phosphatidylserine, the binding affinity of the DAG only decreased by 20-fold. Thus, in the absence of phospholipids, the

\(^4\) Pak, Y., Enyedy, I. J., Varady, J., Kung, J. W., Lorenzo, P. S., Blumberg, P. M., and Wang, S. (2001) J. Med. Chem., in press.
FIG. 1. A, the mutagenic scheme on PKC-δC1b and the sequence alignment of the cysteine-rich regions from different classes of DAG/phorbol ester receptors. The sequence of the second cysteine-rich domain of PKC-δ, PKC-δC1b, is shown together with the mutated amino acids and the sites of mutation as marked with *. The elements of secondary structure are shown above the sequence. In the consensus, the PKC-α and -δ sequences are from bovine (b) and the PKC-μ and chimaerin sequences are from human (h). Residues which are the same as the mutations made in PKC-δC1b are shown in bold. B, the modeled complex structure of PDBu in complex with the C1b domain of PKC-δ (see Footnote 4). It is of note that based upon our predicted model for PDBu and the x-ray structure for phorbol 13-acetate, PDBu and phorbol 13-acetate form the same hydrogen bonding network with PKC-δC1b.
DAG bound with only 15-fold weaker affinity than did PDBu. These results suggest a weaker contribution of the phospholipid to the DAG binding. Second, the W22K or W22Y mutants showed reduced affinity in the absence of phospholipid as well as a reduction in the presence of phospholipid, suggesting an effect of this residue on the C1 domain-DAG interaction, which was not seen for PDBu. Finally, the L24K mutant appeared to have a substantial effect on DAG binding even in the absence of phospholipid.

**TABLE I**

| 3C1b mutants | [3H]PDBu Kd | Ratio | [3H]PDBu B<sub>max</sub> | B<sub>max</sub> % |
|--------------|-------------|------|----------------|------------|
|              | nM          | mut./3C1b | pmol/mg | mut./3C1b |
| Control      | 0.49 ± 0.03 (3) | (1.00) | 13400 ± 2800 (3) | (1.00) |
| F13Y         | 0.78 ± 0.02 (3) | 1.60 | 7050 ± 350 (3) | 52.73% |
| F13K         | 0.75 ± 0.14 (3) | 1.53 | 9020 ± 880 (3) | 67.39% |
| L20D         | ND | ND | ND | ND |
| L20K         | 1.54 ± 0.50 (4) | 3.15 | 1740 ± 410 (4) | 13.01% |
| L20R         | 165.9 ± 7.6 (3) | 339.26 | 10140 ± 830 (3) | 75.76% |
| L20F         | 0.56 ± 0.09 (3) | 1.15 | 4360 ± 570 (3) | 32.61% |
| W22Y         | 0.66 ± 0.22 (3) | 1.35 | 8700 ± 1600 (3) | 65.26% |
| W22K         | 1.42 ± 0.13 (3) | 2.90 | 11790 ± 500 (3) | 88.16% |
| L24D         | ND | ND | ND | ND |
| L24K         | 122 ± 23 (4) | 250.36 | 5050 ± 470 (4) | 37.73% |

**TABLE II**

| 3C1b mutants | [3H]PDBu Kd | Ratio | [3H]PDBu B<sub>max</sub> | B<sub>max</sub> % |
|--------------|-------------|------|----------------|------------|
|              | nM          | mut./3C1b | pmol/mg | mut./3C1b |
| Control      | 33.4 ± 5.6 (4) | 1.03 | 5020 ± 470 (4) | 80.75 |
| F13Y         | 34.3 ± 6.7 (3) | 0.66 | 4050 ± 140 (3) | 66.16 |
| F13K         | 22.1 ± 1.8 (3) | 0.66 | 3320 ± 220 (3) | ND |
| L20D         | ND | ND | ND | ND |
| L20K         | 107 ± 13 (3) | 3.19 | 2860 ± 410 (3) | 57.04 |
| L20R         | 223 ± 62 (3) | 6.66 | 740 ± 180 (3) | 14.78 |
| W22Y         | 38.1 ± 6.6 (3) | 1.14 | 4300 ± 1100 (3) | 85.64 |
| W22K         | 38.1 ± 2.8 (3) | 1.14 | 3040 ± 550 (3) | 60.53 |
| L24D         | ND | ND | ND | 1800 ± 300 |
| L24K         | 98 ± 18 (3) | 2.94 | 35.87 |

**FIG. 2.** [3H]PDBu binding to PKC-δC1b and its mutants as a function of concentration of phospholipid. Binding was carried out as described under “Experimental Procedures.” The final concentration of phospholipid in the form of LUVs in the reaction was 0.2 mM and the vesicles were composed of 70 mol% POPS and 30 mol% POPC. Values represent the mean ± S.E. of at least three experiments per mutant.
Role of the Hydrophobic Residues in PKC-δC1b Domain

Dissecting the mechanisms contributing to the selective activation of members of the DAG/phorbol ester receptors is critical to the understanding of the signaling through DAG and phorbol esters. Studies on the PKC family showed that the C1 domain and pseudosubstrate region, along with the C2 domain in Ca²⁺-dependent classical PKCs, act as membrane targeting modules to trigger the membrane association of PKC and subsequent conformational change that activates the kinases (24). Several regulatory factors have been described that can contribute to isoform selectivity: 1) the differential lipid requirements of members of this family (22, 24, 25); 2) the differential localization induced by the C1 domain ligands and cofactors (26); 3) proteins that interact with PKCs, such as RACKs, RICKs, and STICKs (3, 4); 4) regulation through intramolecular and intermolecular phosphorylation (27, 28). Our current study is focused on the interactions within the ternary complex formed between the C1 domain, the membrane, and the ligands for the C1 domain. Since detailed structural information exists for the binary complex formed between the C1b domain of PKC and its ligand as determined by NMR and x-ray crystallography (8, 10, 14), we sought to use this domain as a model to address the effect of lipid on the C1 domain and ligand interaction with particular emphasis on the hydrophobic interactions within the ternary complex.

The lipophilicity of C1 ligands is one element contributing to the pattern of translocation of GPP-PKC-δ in vivo, reflecting the importance and specificity of hydrophobic interactions between the C1 ligand and the membrane (29). The C1 domain itself makes important contributions to hydrophobic interactions both with the ligand and the membrane. Data from NMR and x-ray crystallographic studies indicated a number of conserved hydrophobic residues: Tyr-8, Met-9, Phe-13, Leu-20, Tyr-22 (in PKC-α) or Trp-22 (in PKC-δ, ε, η, θ), Leu-24, and Ile-25, which are exposed at the surface of the protein and form a hydrophobic cap to facilitate the membrane insertion of the protein (14). Our previous molecular modeling studies showed that Leu-20, Leu-24, and Trp-22 contributed to the hydrophobic interactions between PDBu and PKC-δC1b, but Phe-13 did not have any significant hydrophobic interaction with PDBu (30). To further evaluate the role of these hydrophobic residues in the membrane binding and the ligand selectivity, we mutated these four highly conserved hydrophobic residues at the rim of the binding cleft. Our results indicated that the [H]PDBu binding to mutants in which Leu-20 and Leu-24 were changed to hydrophilic residues (with the exception of L20K) were greatly reduced in the presence of lipids but showed little changes from wild type in the absence of lipids, reflecting a greater contribution to the interaction with the membrane. In contrast, the binding affinities of the mutants at residues Phe-13 and Trp-22 were less affected. The order of their overall impact on lipid interaction was Leu-24 > Leu-20 > Trp-22 > Phe-13. The nature of the substitution, as expected, greatly influenced the outcome. The order of the impact of the residues introduced at these positions was Asp > Arg > Lys > Trp > Phe. Introducing the negatively charged aspartic acid fully abolished ligand binding either in the presence or absence of phospholipids. In contrast, mutating to a hydrophobic residue,
phenylalanine, did not change the affinity of PKC-δC1b. In an initial study probing the structural determinants in PKC-δC1b for ligand recognition by site-directed mutagenesis, a L24G mutation completely abrogated the [3H]PDBu binding and W22G and L20G mutants displayed partial activity, whereas W22F and F13G mutants showed affinity similar to that of wild type PKC-δC1b (12). Molecular dynamics simulations showed that mutation of Leu-24 to Gly significantly affected the backbone conformation of Gly-23 and residue 24 and mutation of Leu-20 to Gly significantly affected the backbone conformations of Val-25 and Leu-26. These results suggest a role for Leu-20 and Leu-24 in maintaining the binding site conformation in addition to their role in ligand-PKC and lipid-PKC interactions. Molecular dynamics simulation showed that mutation of Phe-13 to Gly did not significantly alter the conformation of the binding site. Taken together with the present study, our results showed that Phe-13 played a minimal role in ligand-PKC and lipid-PKC interactions. In comparison, a recent report by Medkova and Cho (15) on the interplay of the C1 and C2 domains of PKC-α in its membrane binding and activation indicated a significant role for the hydrophobic residues Trp-58 and Phe-60 (equivalent to the Trp-22 and Leu-24 in PKC-δC1b) in the C1a domain of PKC-α for the membrane penetration and activation of PKC-α, whereas those in the C1b domain of PKC-α were found to be not directly involved in these processes. Both we and others have described the non-equivalent roles of the C1a and C1b domains as well as marked differences in the C1a and C1b domains of different isoforms (15, 31, 32).

The ability of phorbol esters and related compounds to bind to C1 domains in the absence of phospholipids may have significant implications in the selective activation of the soluble, non-membrane-associated PKCs. PKC is known to interact with both cytoskeletal and nuclear proteins in the absence of lipids. However, the mode of their binding and activation is less known. Prekeris et al. (33) showed that PKC-δ bound to actin in a phorbol ester-dependent manner. Similarly, F-actin also interacts with other members of PKC family, and the isoform specificity is associated with their differential dependence on phorbol esters and Ca2+. STICKs, RACKs, and RICKs were also described to recruit the inactive or activated non-membrane-bound PKCs to the different intracellular locations (3, 4). Here, a 70- and 20-fold weaker binding affinity in the absence of lipids was detected for the wild-type C1 domain by 97F31 and the DAG. Design of ligands that are selective for either protein-associated or free PKC could cause antagonism of typical responses modulated by membrane-associated PKC.

We probed the determinants in the C1 domain for the selective recognition of DAG versus phorbol esters. Although both DAG and phorbol ester are thought to bind to the same binding sites in the C1 domain, differences in their mechanisms of interaction have been described. The low and high affinity binding sites within the twin C1 domains of PKC-α, described by Slater et al. (35), were reported to show opposite affinities for DAG and phorbol ester. Two modes of interaction between DAG and the C1 domain, namely sn-1 and sn-2, were proposed based on computer-guided, molecular docking analysis (36, 37). Strikingly, constrained DAG analogs developed from this modeling with branched side chains to maximize the hydrophobic interaction between DAG and the hydrophobic

### Table III

| 8C1b mutants | K<sub>s</sub> (mM) | K<sub>d</sub>/K<sub>pDBu</sub> | K<sub>s</sub> (mM) | K<sub>d</sub>/K<sub>pDBu</sub> |
|--------------|----------------|----------------|----------------|----------------|
| Control      | 24.1 ± 2.3 (3) | 49.12          | 486 ± 47 (3)   | 14.56          |
| F13Y         | 24.2 ± 1.0 (3) | 31.06          | 418 ± 51 (3)   | 12.18          |
| F13K         | 17.8 ± 2.2 (3) | 23.68          | 315 ± 18 (3)   | 14.26          |
| L20D         | ND             |                | 1130 ± 260 (3) | 10.63          |
| L20K         | 42.4 ± 8.2 (3) | 27.50          | 1860 ± 210 (3) | 8.35           |
| L20R         | 687 ± 69 (4)   | 4.14           | 1120 ± 170 (3) | 29.41          |
| W22Y         | 149.5 ± 6.0 (3)| 226.5          | 2760 ± 340 (3) | 72.37          |
| W22K         | 361 ± 44 (4)   | 254.3          | 15600 ± 3100 (3)| 158.23        |
| L24D         | ND             |                | ND             |                |
| L24K         | 1555 ± 92 (4)  | 12.54          | 15600 ± 3100 (3)| 158.23        |

### Table IV

Comparison of the binding activities of PKC-δC1b mutants and wild type protein with relevance to differential C1 domain functions

| Ligand | PKC-δ | C1 domain | Full-length protein |
|--------|-------|-----------|---------------------|
| PDBu (K<sub>d</sub>) | Mutant L20R/PKC-δC1b | 165.9 ± 7.6 (3) / 0.49 ± 0.03 (3) | PKC-δ/PKC-δ |
| PDBu (K<sub>d</sub>) | Mutant L20F/PKC-δC1b | 162.1 ± 1.1 (3) / 1.31 ± 0.22 |
| Thymeleatoxin (K<sub>d</sub>) | Mutant K41Q/PKC-δC1b | 0.9 ± 0.1 (10) / 11.6 ± 3.06 (3) |
| BS-DL-B8 (K<sub>d</sub>) | Mutant V38Y/PKC-δC1b | 0.80 ± 0.35 (3) / 0.75 ± 0.08 (5) |
| PDBu (K<sub>d</sub>) | Mutant W22K/PKC-δC1b | 1.54 ± 0.02 (3) / 0.75 ± 0.08 (5) |
| PDBu (K<sub>d</sub>) | Mutant L24D/PKC-δC1b | 0.80 ± 0.35 (3) / 0.75 ± 0.08 (5) |
| PDBu (K<sub>d</sub>) | Mutant L24K/PKC-δC1b | 1.7 ± 0.78 / 0.49 ± 0.03 (3) |

Inhibition of [3H]PDBu binding to mutants in the C1b domain of PKC-δ by 97F31

Binding by the branched DAG 97F31 in the presence or absence of 100 μg/ml PS was determined by competition of [3H]PDBu binding. Values represent the mean ± S.E. of the number of experiments in parentheses. ND, not detected.
residues near the binding cleft exhibited binding potencies approaching that of PDBu (36). Our results demonstrated significant differences in the affinities of the mutants for the DAG versus PDBu, reflecting the existence of specific determinants for the recognition of DAG. Specifically, mutant L24K showed a unique pattern of recognition of 9TPrF1. In addition, the mutants at Trp-22 exhibited a large reduction in their binding affinity to DAG but not to PDBu. Our molecular dynamics simulation revealed that the side chain of Trp-22 was very flexible, which would allow the side chain to easily adopt different conformations when interacting with different ligands. Thus, introducing different side chains at this position could affect the ligand selectivity. The fact that the same mutated residue (Lys-22) occurs in all members of PKD/PKC-μ family implies a potential different selectivity for DAG and consequently a possible functional difference compared with the other members of the PKC family. This hypothesis is currently under investigation.

Members of the DAG/phorbol ester receptors exhibit different patterns of ligand selectivity and lipid requirements (13, 23). However, the structural features that account for selective PKC isozyme modulation are less known. To further our understanding of the structure activity relations of other classes of DAG/phorbol ester receptors, we probed the role of specific residues in other C1 domains by mutating the corresponding residues in PKC-δC1b. We found that L20R resulted in >300-fold loss of apparent affinity toward PDBu and >30-fold loss of affinity to the branched DAG in the presence of PS, as well as a 10-fold shift in lipid dependence. The atypical PKCs, PKC-ζ and PKC-λ/θ, are unable to bind C1 domain ligands despite the high homology to other C1 domains. An attempt at restoring the DAG binding activity of PKC-ζ by mutating Gly at position 11 to Pro was unsuccessful (11). Here, we identified another possible site that may account for the inactivity of PKC-ζ. Efforts to introduce a second mutation, R20L, in addition to G11P in order to restore the activity of PKC-ζ, are under way.

By mutating hydrophobic residues located at the rim of the ligand binding cleft in PKC-δC1b to hydrophilic residues, we demonstrated the critical role played by the hydrophobic residues in the C1 domain for C1 domain/lipid interaction and for ligand recognition. Our findings provide further insight into the structural basis for regulation of DAG/phorbol ester-generated signaling pathways, enhancing our ability both to predict behavior from sequence as well as exploit sequence differences for design of selective ligands.

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Qiming J. Wang, Tzan-Wei Fang, Kassoum Nacro, Victor E. Marquez, Shaomeng Wang and Peter M. Blumberg

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