Residues in the Second Cysteine-rich Region of Protein Kinase C δ Relevant to Phorbol Ester Binding as Revealed by Site-directed Mutagenesis*

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Phorbol esters bind with high affinity to protein kinase C (PKC) isozymes as well as to two novel receptors, n-chimaerin and Unc-13. The cysteine-rich regions present in these proteins were identified as the binding sites for the phorbol ester tumor promoters and the lipophilic second messenger sn-diacylglycerol. A 50-amino-acid peptide comprising the second cysteine-rich region of PKC δ, expressed in Escherichia coli as a glutathione S-transferase (GST)-fusion protein, bound [3H]phorbol 12,13-dibutyrate (PDBu) with high affinity (Kd = 0.8 nM). Using the cDNA of that cysteine-rich region as a template, a series of 37 point mutations was generated by site-directed mutagenesis, and the mutated proteins were analyzed quantitatively for binding of [3H]PDBu and, as appropriate, for binding of the ultrapotent analog [3H]bryostatin 1. Mutants displayed one of three patterns of behavior: phorbol ester binding was completely abolished, binding affinity was reduced, or binding was not significantly modified. As expected, five of the six cysteines as well as the two histidines involved in Zn2+ coordination are critical for the interaction of the protein with the phorbol esters. In addition, mutations in several positions, including phenylalanine 3, tyrosine 8, proline 11, leucines 20, 21, and 24, tryptophan 21, glutamine 27, and valine 38 drastically reduced the interaction with the ligands. The effect of these mutations can be rationalized from the three-dimensional (NMR) structure of the cysteine-rich region. In particular, the C-terminal portion of the protein does not appear to be essential, and the loop comprising amino acids 20 to 28 is implicated in the binding activity.

The protein kinase C (PKC)1 isozymes are a family of related proteins that mediate one of the major cellular signal transduction pathways which utilizes lipophilic second messengers. PKC plays a central role in mediating the activation of growth factors, neurotransmitters, and hormones and has been implicated in cellular growth control and carcinogenesis (Weinstein, 1988). Diacylglycerol, one of the products of the hydrolysis of inositol 1,4,5-trisphosphate and phosphatidylcholine, was described as the endogenous activator for most of the PKC isozymes (Blumberg, 1991; Nishizuka, 1992). Both the classical isozymes (PKC α, PKC β1, PKC β2, and PKC γ) and novel isozymes (PKC δ, PKC ε, PKC η, and PKC ι) are responsive to diacylglycerol. In contrast, the atypical isozymes (PKC ζ and PKC λ) are unresponsive to this lipophilic second messenger (Ono et al., 1989b; Kazanietz et al., 1993; Akimoto et al., 1994). The phorbol esters, tumor-promoting diterpene derivatives from plants of the family Euphorbiaceae, bind in a phospholipid-dependent manner to the same site in PKC as do diacylglycerols and activate the enzyme (Sharkey et al., 1984). These compounds have become a valuable tool for studying the mechanism of PKC activation and for elucidation of the cellular pathways in which PKC is involved. Deletion analysis of PKC revealed that the cysteine-rich regions present in the regulatory domain are the sites of phorbol ester/diacylglycerol binding (Ono et al., 1989a; Kaibuchi et al., 1989). Each of these cysteine-rich regions is a 50- or 51-amino-acid domain possessing the motif HX12CX12CX13CX12CX12CX13CX12CX13CX, where H is histidine, C is cysteine, X is any other amino acid, and n is 13 or 14. These structures, also called zinc fingers, are coordinated by two Zn2+ ions (Quest et al., 1992). The motif is duplicated in tandem in both the classical and novel PKC isozymes and is present only once in the atypical PKC isozymes. Studies using recombinant cysteine-rich regions revealed that a single copy of the motif is sufficient for the binding of phorbol esters (Ono et al., 1989a; Burns and Bell, 1991; Quest and Bell, 1994; Quest et al., 1994a; Kazanietz et al., 1994a; Wender et al., 1995). This conclusion is corroborated by the ability of the novel non-kinase proteins n-chimaerin and Unc-13, both of which contain a single copy of the motif, to function as high affinity phorbol ester receptors (Hall et al., 1990; Ahmed et al., 1991, 1992; Maruyama and Brenner, 1991; Arecos et al., 1994). On the other hand, the proto-oncogenes vav and c-raf, although they have a single copy of the cysteine-rich domain that coordinates Zn2+ in their structure, do not bind phorbol esters (Ghosh et al., 1994; Kazanietz et al., 1994a).

As determined by NMR (Hommel et al., 1994), in solution the second cysteine-rich domain of rat PKC α adopts a globular fold, where the two Zn2+ ions are coordinated by two noncons-ecutive sets of Zn2+ -binding residues (three cysteines and one histidine in each case) to form two separate metal-binding sites. The topology can be described as two antiparallel β-sheets followed by a C-terminal helix. The two metal-binding sites are located at either end of one of the major β-sheets, with

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§ The abbreviations used are: PKC, protein kinase C; PDBu, phorbol 12,13-dibutyrate; GST, glutathione S-transferase.
the histidines involved in Zn$^{2+}$ coordination buried in a hydrophobic cavity. The postulated consensus sequence for binding of phorbol esters includes 13 amino acids (Kazanietz et al., 1994a). In PKC $\zeta$, one of the atypical isoforms lacking phorbol ester binding, a single amino acid differs in the motif (glycine instead of proline in position 11). However, restoration of the proline by site-directed mutagenesis did not restore binding (Kazanietz et al., 1994a). These results strongly suggested that other amino acids besides those postulated for the consensus must be necessary for ligand binding. Therefore, we decided to perform a more extensive mutagenesis study to evaluate residues within the cysteine-rich region that may be relevant for the interaction. Earlier studies indicated that double point mutations of cysteines in PKC $\gamma$ cysteine-rich regions (those corresponding to positions 31 and 34 in the consensus) abolished phorbol ester binding (Ono et al., 1989a). Further, an elegant deletional analysis (Quest et al., 1994b) clearly identified a 43-amino-acid fragment as the minimum necessary for the ligand interaction. In our study we expressed a single recombinant cysteine-rich region of PKC $\delta$ (50 amino acids) in E. coli as a GST-fusion protein. A series of 37 point mutations in 25 different positions were made, and the corresponding proteins were assayed for ligand binding using $[^3H]$PDBu and $[^3H]$bryostatin 1. A unique aspect of this study is that we were able to quantitate the interaction of the mutants with $[^3H]$PDBu by Scatchard analysis and thereby measure quantitatively the changes in binding affinity as a consequence of the point mutations. Our experiments led us to the identification of several amino acid residues within the phorbol ester binding domain that are critical for the interaction of the ligand with the receptor. Elsewhere we have described the modeling of the structure of the second cysteine-rich region domain of PKC $\delta$ to the known structure of the same domain in PKC $\alpha$. We were able to take advantage of this computer model together with our mutational analysis to develop insights into the regions regulating phorbol ester and phospholipid interactions.

**EXPERIMENTAL PROCEDURES**

Materials—$[^3H]$PDBu (20.0 Ci/mmol) was purchased from DuPont NEN. $[^3H]$Bryostatin 1 (4.8 Ci/mmol) was prepared as described previously for $[^3H]$Bryostatin 4 (Devries et al., 1988). PDBu was obtained from LC Services Corp. (Woburn, MA). Phosphatidylserine was purchased from Sigma. Reagents for expression and purification of recombinant proteins in Escherichia coli were obtained from Pharmacia Biotech Inc.

Expression of Recombinant Cysteine-rich Region of PKC $\delta$ in E. coli—A cDNA fragment encoding the second cysteine-rich region of PKC $\delta$ was generated by polymerase chain reaction, using the full-length mouse cDNA clone as a template (Mischak et al. 1991) and the following nucleotides: 5'TGAGGATCCACCGATTCAAGGTTTATACAGCTTTAGTTATAAC3' (sense) and 5'ATCGAGCTCACACAGTGTGGCCACCTTCTC3' (antisense), where the BamHI and EcoRI sites are underlined. The 160-base pair fragment was subcloned in the pCR1 vector using the TA cloning system (Invitrogen), and the BamHI-EcoRI fragment was then subcloned in-frame into the pGE2XTK vector (Pharmacia) to get the pGE$\delta$xi plasmid. The fragment was sequenced to confirm complete homology with the published sequence. Sequencing of this and the other constructs was performed by Bioserve Biotechnologies (Laurel, MD). XL1-blue E. coli cells (Stratagene) were transformed with the pGE$\delta$xi plasmid and then grown in LB media containing 50 $\mu$g/ml ampicillin. Expression of the GST-fusion protein was induced by the addition of 0.5 mM isopropyl-$\beta$-D-thiogalactopyranoside after the bacteria reached an A$_{600}$ of 0.5-0.7. After 5 h at 37°C, the cells (50 ml) were pelleted at 4000 x g, resuspended in 1 ml of phosphate-buffered saline, and disrupted by sonication. The GST-fusion protein was purified using glutathione-Sepharose 4B beads, according to the manufacturer's instructions (Pharmacia).

Site-Directed Mutagenesis of the GST-Fusion Proteins—Site-directed mutagenesis was performed with the Unique Site Elimination (U.S.E.) system (Pharmacia), using the pGEX$\delta$ plasmid as the template. The PstI site in the vector was eliminated by using the PstI/SacI pGEX U.S.E. primer from the same company. Mutagenic primers for the different mutants are described in Table I. Mutant plasmids were selected by restriction analysis, as described by the manufacturer, and confirmed by sequencing using the dyelex chain termination method (Sanger et al., 1977). The mutagenic primers were used to generate the corresponding mutant GST-fusion proteins, which were purified with the glutathione-Sepharose 4B beads, as described above.

$[^3H]$PDBu and $[^3H]$Bryostatin 1 Binding—$[^3H]$PDBu binding was measured using the polyethylene glycol precipitation assay described in our laboratory (Sharkey and Blumberg, 1985) in a total volume of 250 $\mu$L, using 1 $\mu$g of phosphatidylserine as described earlier (Kazanietz et al., 1992). Detailed descriptions for Scatchard assays can be found elsewhere (Kazanietz et al., 1993). $[^3H]$Bryostatin 1 binding was measured using a variant of the filtration assay described for $[^3H]$epibryostatin 4 (Lewin et al., 1991; Kazanietz et al., 1994a, 1994b) in a total volume of 250 $\mu$L, using 1 $\mu$g of GST-fusion protein and 100 $\mu$g/ml phosphatidylserine. All binding assays were performed at 18°C.

Binding using bacterial lysates was carried out using a fixed concentration of radioligand (10 nm for either $[^3H]$PDBu or $[^3H]$bryostatin 1). Pellets from 1.5-ml cultures were lysed by sonication in 500 $\mu$L of 50 mM Tris-HCl, pH 7.4, and 1 $\mu$g of EGTA. Fifty $\mu$L per tube of the total lysate were used both for the $[^3H]$PDBu and $[^3H]$bryostatin 1 binding assays.

**RESULTS**

The phorbol ester receptors share a high degree of homology in the cysteine-rich region. Alignment of these domains of the PKC isoforms, of n-chimaerin and of Unc-13 shows a consensus of 13 residues (Kazanietz et al., 1994a). These conserved amino acids include the cysteines and histidines involved in the coordination of Zn$^{2+}$ atoms, as well as two glycines (positions 23 and 28 in the consensus) and a glutamine (position 27). In PKC $\zeta$ and PKC $\lambda$, the atypical isoforms lacking phorbol ester binding, a single proline at position 11 in the consensus is replaced by glycine and arginine, respectively. The proto-oncogenes c-raf and vav share high homology with the phorbol ester receptors, and the degree of conservation among the consensus residues is 77% and 92%, respectively. Still, these two proteins are not able to bind phorbol esters either with high or low affinity (Ghosh et al., 1994; Kazanietz et al., 1994a).

Analysis of conservation of amino acids within the 50-amino-acid domain in the phorbol ester-binding proteins reveals regions with high and low degrees of variability. In addition to those residues absolutely conserved in the cysteine-rich region, several positions show very restricted changes. Positions 8, 13, and 22 are occupied by aromatic amino acids. Hydrophobic amino acids are present in positions 5, 20, 21, 24, 29, 38, and 46. Regions of hypervariability are present at both the N- and C-terminal ends (Fig. 1).

The second cysteine-rich region of PKC $\delta$ was expressed in E. coli as a GST-fusion protein. After isopropyl-$\beta$-D-thiogalactopyranoside induction of bacterial cultures, high levels of recombinant proteins were found in lysates as revealed by the appearance of a strong 33-kDa band in Coomassie Blue-stained SDS-polyacrylamide gels (Fig. 2A). Overexpression of the cysteine-rich region of PKC $\delta$ resulted in high binding activity of $[^3H]$PDBu and $[^3H]$bryostatin 1 in lysates (Fig. 2B and C). Mutants of the cysteine-rich region were likewise expressed as GST-fusion proteins in E. coli, and, in all cases, a 33-kDa band was observed in bacterial lysates. In all cases, the GST-fusion proteins were recovered predominantly from the soluble frac-

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2 It has been suggested recently (Gulbins et al., 1994) that the vav protooncogene product binds phorbol esters. Our results using the recombinant cysteine-rich region of vav or the full-length protein strongly argue against the presence of either a high or low affinity binding site. The basis for the discrepancy between the two studies is currently not known.
tion of the bacterial lysates (data not shown); they were purified from that fraction using glutathione-Sepharose 4B beads. The purity of the preparations was generally >80%. In some cases, a degradation product corresponding to GST could also be observed in the polyacrylamide gels (Fig. 2). The GST-fusion proteins could be detected easily with an anti-GST antibody in Western blots, and they showed a very high level of GST enzymatic activity (data not shown).

Bacterial lysates for the different mutants were evaluated for phorbol ester binding using [3H]PDBu as a ligand. In addition, and in order to detect low affinity interactions that might not have been detected with this classical ligand, a more sensitive assay using the ultrapotent analog [3H]bryostatin 1 in the presence of phosphatidylserine was also carried out (Fig. 2A). The GST-fusion proteins could be detected easily with an anti-GST antibody in Western blots, and they showed a very high level of GST enzymatic activity (data not shown).

Several hydrophobic amino acids in the cysteine-rich regions seem to be essential for phorbol ester binding. Replacement of tyrosine by glycine in position 8 reduced the binding affinity of [3H]PDBu to 60-fold. In contrast, the substitution of phenylalanine in position 13 by glycine did not change the affinity for the ligand. A highly lipophilic region located in the loop formed by amino acids 20 to 27 was very sensitive to mutations. Mutation of any of the three leucines in positions 20, 21, and 24 induced either reduction or complete loss of [3H]PDBu binding. Only the first of these leucine to glycine mutants gave measurable [3H]PDBu binding (Kd = 12 nM, 15-fold lower affinity than the wild type) (Fig. 3). Elimination of either leucine 21 or 24 induced a complete loss of [3H]PDBu binding, and only low levels of the ultrapotent ligand [3H]bryostatin 1 could be detected in lysates, suggesting a large reduction in ligand affinity. Mutation of tyrosine to glycine resulted in a reduction in the affinity for [3H]PDBu of 31-fold. A phenylalanine in that same position could still be tolerated with no significant changes in binding affinity. It is remarkable that in the non-phorbol ester receptors PKC ζ, c-raf, and vav, several changes are found in this region (Fig. 1) that could explain the lack of binding in these proteins. Mutation of valine 38 completely abolished phorbol ester binding.

The results suggest that the lack of phorbol ester binding by PKC ζ may be rationalized by the combined effects of the substitution of proline 11 to glycine and of leucine 20 to arginine. The former change caused a 125-fold decrease in affinity to the second cysteine-rich region of PKC δ. The conversion of leucine 20 to glycine caused a 15-fold decrease in binding; the additional effect when a positively charged residue is inserted in this site in the hydrophobic loop still requires experimental determination.

In addition to the cysteines and histidines, other amino acids in the consensus are essential for phorbol ester binding. Mutation of phenylalanine in position 3 to glycine completely abolished [3H]PDBu and [3H]bryostatin 1 binding. Substitution of the phenylalanine by another aromatic amino acid (tryptophan) preserved phorbol ester binding. Interestingly, a leucine was also tolerated in that position without any change in the binding affinity, suggesting that any hydrophobic amino acid in position 3, either aromatic or aliphatic, is sufficient to allow ligand binding. Glutamine in position 27 is also essential for phorbol ester binding. Mutation of this residue to glycine almost abolished [3H]PDBu binding, although binding of the ultrapotent analog [3H]bryostatin 1 was still detectable, indicating a large reduction in binding affinity. An introduction of a tryptophan in this position completely abolished binding of both [3H]PDBu and [3H]bryostatin 1. Mutation of glutamine 27 to either valine or threonine reduced the affinity of [3H]PDBu by factors of 28 or 131, respectively. Replacement of the proline in position 11 by glycine produced a large decrease in the affinity for [3H]PDBu, with a decrease in Kd of about 125-fold when compared to the wild-type cysteine-rich region (Fig. 3).

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PKC \(z\) and vav have an alanine in position 12. Mutagenesis analysis shows that this residue is not essential for phorbol ester binding, since no changes in \([\text{3H}]\text{PDBu}\) binding were found after replacing the threonine with glycine, serine, or valine. Another potential hydrogen bond acceptor is found in position 15 where either aspartic acid, glutamic acid, or serine is found in the phorbol ester receptors. A glycine occupies that position in PKC \(z\), and a lysine is found in vav. Replacement of the aspartic acid by glycine in the second cysteine-rich region of PKC \(\delta\) did not affect phorbol ester binding. Substitution of the aspartic acid by asparagine, serine, or tyrosine did not modify \([\text{3H}]\text{PDBu}\) binding either. Positions 30 and 39 are occupied by either a basic amino acid or glutamine, suggesting a potential role as a hydrogen bond donor. Mutation of the lysine 30 to asparagine did not affect phorbol ester binding. Moreover, introduction of a glutamic acid in that position produced only a minor reduction (about 4-fold) in the affinity for \([\text{3H}]\text{PDBu}\) binding. Similarly, substitution of lysine 41 by glycine did not affect phorbol ester binding. Mutation to valine of methionine 36, a residue conserved in the second copy of cysteine-rich regions of PKC, did not change the affinity for \([\text{3H}]\text{PDBu}\). No changes in binding were also observed after mutation of lysine 4 to valine.

**DISCUSSION**

Cysteine-rich regions in PKC, n-chimaerin, and Unc-13 are the binding sites for the second messenger diacylglycerol as well as for the phorbol ester tumor promoters. The second cysteine-rich region of PKC \(\delta\) expressed as a GST-fusion pro-
Site-directed mutagenesis was performed using the Unique Site Elimination (U.S.E.) system following the procedures described by the manufacturer (Pharmacia) and using the pGEX plasmid as the template. Mutagenic primers used in the mutagenesis reaction are shown in the table, and the mutated codon is underlined. The positions in the cysteine-rich region subjected to mutagenesis are indicated on the left, using the nomenclature as in Fig. 1. Shown in parentheses are the corresponding positions in the full-length mouse PKC δ. Plasmids were transformed into E. coli, and, after induction of the bacterial cultures with 0.5 mM isopropyl-thiogalactopyranoside (0.5 nm), bacterial pellets from 1.5-ml cultures were lysed and subjected to [3H]PDBu or [3H]bryostatin 1 binding, using 20 nm and 10 nm concentrations of radioligand, respectively. “ND” and “--” indicate, respectively, that binding was detected or not detected in the bacterial lysates. Under our assay conditions, “--” corresponds to a K < 0.5 nm. “--” indicates that low binding was detected in lysate (<5,000 cpm for [3H]PDBu and <1,000 cpm for [3H]bryostatin 1). K values for [3H]PDBu were obtained from Scatchard plots as shown in Fig. 3 and are expressed as the mean ± S.E. of 3-5 experiments in each case. ND, not determined.

| Amino acid number | Mutation | Oligonucleotide | [3H]PDBu binding | [3H]Bryostatin 1 binding | K < 3H>PDBu |
|-------------------|----------|----------------|-----------------|--------------------------|-------------|
| Wild-type         | G       | GGGTATAAAACTCTGATCGCTGGGACAGGATGC | +               | +                        | 0.8 ± 0.1    |
| 1 (231)           | H → G   | GGTATAAAACTCTGATCGCTGGGACAGGATGC | +               | ND                       |             |
| 3 (233)           | F → G   | GCTAATGATTAAACCTTCCGTGATGGACCTC | +               | ND                       |             |
| 3 (233)           | F → W   | GCTAATGATTAAACCTTCCGTGATGGACCTC | +               | ND                       |             |
| 3 (233)           | F → L   | GCTAATGATTAAACCTTCCGTGATGGACCTC | +               | ND                       |             |
| 4 (241)           | K → V   | GTCATGATCTAAATACCTTCCGTGATGGACCTC | +               | ND                       |             |
| 2 (283)           | Y → G   | GAAGTGGGCTTACATGGTATACCCGGTATGC | +               | ND                       |             |
| 11 (241)          | P → G   | GTGTCGCGAACAGCTTGCTGATGGACCTC | +               | 100 ± 33                 |             |
| 12 (242)          | T → G   | CCACATGCTGACAGAAGGTCATGATGC | +               | 1.7 ± 0.3                |             |
| 12 (242)          | T → S   | CCACATGCTGACAGAAGGTCATGATGC | +               | 2.6 ± 0.6                |             |
| 12 (242)          | T → V   | CCACATGCTGACAGAAGGTCATGATGC | +               | 0.9 ± 0.1                |             |
| 13 (243)          | F → G   | CCACATGCTGACAGAAGGTCATGATGC | +               | ND                       |             |
| 14 (244)          | C → G   | GCACATGCTGACAGAAGGTCATGATGC | +               | ND                       |             |
| 15 (245)          | D → G   | CAAATGCTGACAGAAGGTCATGATGC | +               | 0.5 ± 0.1                |             |
| 15 (245)          | D → N   | CAAATGCTGACAGAAGGTCATGATGC | +               | ND                       |             |
| 15 (245)          | D → S   | CAAATGCTGACAGAAGGTCATGATGC | +               | 0.9 ± 0.1                |             |
| 15 (245)          | D → Y   | CAAATGCTGACAGAAGGTCATGATGC | +               | 0.6 ± 0.1                |             |
| 17 (247)          | C → G   | CCACATGCTGACAGAAGGTCATGATGC | +               | ND                       |             |
| 20 (250)          | L → G   | TCACATGCTGACAGAAGGTCATGATGC | +               | 12 ± 1.8                 |             |
| 21 (251)          | L → G   | TCACATGCTGACAGAAGGTCATGATGC | +               | ND                       |             |
| 22 (252)          | W → F   | CCGTTGTCGCCATGCAGGACCCAGGACCCAC | +               | 0.9 ± 0.2                |             |
| 24 (254)          | L → G   | CCTATGCTGACAGAAGGTCATGATGC | +               | ND                       |             |
| 27 (257)          | Q → G   | TCACATGCTGACAGAAGGTCATGATGC | +               | ND                       |             |
| 27 (257)          | Q → V   | TCACATGCTGACAGAAGGTCATGATGC | +               | ND                       |             |
| 27 (257)          | Q → T   | TCACATGCTGACAGAAGGTCATGATGC | +               | ND                       |             |
| 27 (257)          | Q → T   | TCACATGCTGACAGAAGGTCATGATGC | +               | 105 ± 36                 |             |
| 27 (257)          | Q → W   | TCACATGCTGACAGAAGGTCATGATGC | +               | ND                       |             |
| 30 (260)          | K → N   | CCAATGCTGACAGAAGGTCATGATGC | +               | ND                       |             |
| 30 (260)          | K → E   | CCAATGCTGACAGAAGGTCATGATGC | +               | 3.5 ± 0.4                |             |
| 34 (264)          | G → C   | GTGACATGCTGACAGAAGGTCATGATGC | +               | ND                       |             |
| 36 (266)          | M → V   | GCTGTCGCTGACAGAAGGTCATGATGC | +               | 0.6 ± 0.1                |             |
| 38 (268)          | V → G   | CCGCGTCGCTGACAGAAGGTCATGATGC | +               | ND                       |             |
| 39 (269)          | H → G   | CCGCGTCGCTGACAGAAGGTCATGATGC | +               | ND                       |             |
| 41 (272)          | K → G   | CCGCGTCGCTGACAGAAGGTCATGATGC | +               | 100 ± 0.1                |             |
| 42 (272)          | C → G   | GCGCGTCGCTGACAGAAGGTCATGATGC | +               | ND                       |             |
| 46 (276)          | V → G   | CACGCGTCGCTGACAGAAGGTCATGATGC | +               | 0.7 ± 0.1                |             |
| 50 (280)          | C → G   | GTGACATGCTGACAGAAGGTCATGATGC | +               | 0.5 ± 0.1                |             |

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ond cysteine-rich region of PKC δ to that published for PKC α facilitated interpretation of the changes observed after mutation. For example, the side chain of phenylalanine 3 is involved in a hydrophobic interaction with the side chains of histidine 1 and leucine 29 (or, for other cysteine-rich domains, other hydrophobic amino acids in position 29), and, therefore, it is extremely important for the overall fold of the structure. This is supported by the finding that binding is completely abolished after mutation to glycine but still retained after mutation to tryptophan or leucine. Tyrosine 8 and proline 11 are part of a big loop (residues 7 to 12). The side chain in tyrosine 8 points toward the outside of the protein, and only hydrophobic residues are tolerated in this position, therefore suggesting a possible involvement in phospholipid interaction. The side chain of proline 11 has some hydrophobic interactions with leucine 20. Furthermore, since proline changes the angle of the peptide backbone, the conformational change produced as a consequence of its mutation to glycine may be responsible for the large loss in phorbol ester binding affinity. Although the PKC ζ has a glycine in this position, its mutation to proline did not restore binding (Kazanietz et al., 1994a), suggesting that further differences in other amino acids must contribute to the lack of binding in this PKC isozyme. Glutamine 27 is conserved in all the PKC isozymes and buried deep in the protein. Although its backbone does not seem to have strong interactions with other residues, its side chain folds inside the protein, interacts with the side chains of asparagine 7, proline 11, and leucine 21, and also forms a weak hydrogen bond with the backbone of tyrosine 8. Changes in position 27 were not tolerated, and binding was totally lost or substantially reduced after mutation, suggesting that the size and nature of the glutamine are extremely important to keep the structure in the right conformation for the interaction with the phorbol esters. Valine 38, a conserved amino acid (only in the first cysteine-rich region of PKC δ and PKC ζ is an isoleucine found), is critical for phorbol ester binding. Its hydrophobic side chain is buried deep inside the protein and interacts with the side chains of phenylalanine 3, leucine 29, cysteine 42, and valine 46 to form a hydrophobic core inside the protein.

The loop of amino acids 20 to 27 is an essential part of the structure necessary for phorbol ester binding. Mutations of residues within that loop strongly affect the activity. Position 20 is occupied by leucine or other hydrophobic amino acids in

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Fig. 3. Representative [3H]PDBu Scatchard plots for the second cysteine-rich domain of PKC δ (wild-type) and mutants. The recombinant GST-fusion proteins were incubated for 5 min (18°C) with increasing concentrations of [3H]PDBu in the presence of 100 μg/ml phosphatidylglycerol and 1 mM EGTA. Binding was measured using the polyethylene glycol precipitation assay. Representative experiments for the wild-type and 3 representative mutants as indicated in the graph are shown. Each point represents the mean of three experimental values, generally with a standard error of <2%. Note different scales for [3H]PDBu for the different mutants. For each of the GST-fusion proteins shown, similar results were obtained in at least two additional experiments.
Fig. 4. Three-dimensional structure of the second cysteine-rich domain of PKC δ. The specific residues analyzed by mutagenesis are indicated in the figure. Phorbol ester binding was completely abolished, red; binding affinity was reduced, blue; or binding affinity was not significantly modified, green. The two Zn$^{2+}$ ions are shown in yellow.

the phorbol ester receptors (an arginine is found in PKC δ). Its hydrophobic chain points outside toward the protein surface and does not seem to be very important for the overall structure of the protein. Its mutation to glycine produce a 15-fold loss in binding activity, probably a consequence of a reduced interaction with the lipid. The side chain of leucine 21 folds inside the protein and interacts with the side chains of threonine 12, glutamine 27, and asparagine 37. Its backbone forms a hydrogen bond with the backbone of threonine 12. The loss in binding after mutation to glycine clearly supports its importance in the protein folding. In the case of tryptophan 22 and leucine 24, no strong backbone or side chain interactions are found. Both side chains point toward the outside of the protein and, therefore, may be critical for the interaction with either the phospholipid or the phorbol ester lipophilic chains. The mutant tryptophan 22 to glycine, in contrast to the one to phenylalanine, is not able to interact physically with phospholipid vesicles in an ultracentrifugation assay similar to the one described by Quest et al. (1994a), suggesting that a hydrophobic residue in position 22 may be involved in the interaction of PKC with membrane phospholipids. In PKC δ, the positive charge of the arginine in position 20 may be disruptive to the interaction of this region with the phorbol esters and phospholipids.

Fig. 4 shows a three-dimensional view of the second cysteine-rich region of PKC delta and a summary of the amino acids within the domain relevant for phorbol ester binding. The residues analyzed by mutagenesis displayed one of three patterns of behavior: phorbol ester binding was completely abolished, binding affinity was reduced, or binding affinity not significantly modified. Our experimental results support the importance of the loop of amino acids in positions 20 to 27 in phorbol ester binding. Others (Ichikawa et al., 1995) have postulated a role for this loop in phorbol ester binding based on the exposed hydrophobic surface of the hairpin loop which presumably interacts with the membrane phospholipids. As described in detail elsewhere, computer modeling provides strong support for this region being the site of phorbol ester binding. Finally, crystallographic analysis of the second cysteine-rich region of PKC δ has demonstrated directly that phorbol ester binds in the absence of phospholipid (Zhang et al., 1995) in a narrow groove at the top of the domain, near the loop created by amino acids 20–27. Bound phorbol esters replace bound water molecules which form bridging hydrogen bonds in the β-sheets and create a hydrophobic cap that stabilizes the membrane-bound form of the enzyme.

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