Protein Kinase C Domains Involved in Interactions with Other Proteins†

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ABSTRACT: We have used a blot overlay assay to detect protein kinase C (PKC) interactions with other proteins. In many cases, the PKC binding proteins are also PKC substrates [Chapline et al. (1993) *J. Biol. Chem. 268*, 6858]. The purpose of the current studies was to characterize the PKC domains involved in the interactions with other proteins. α, β, and ϵ isoforms of PKC interact with the same binding proteins in fibroblast cell extracts. These results indicate that constant rather than isozyme-specific (variable) regions are the major determinants of the interactions studied. PKC binding required phosphatidylserine (PS), indicating that the PS binding regulatory domain of PKC is involved in the interactions. The PKC pseudosubstrate peptide sequence, which is contained within the regulatory domain, also showed PS-dependent binding to the PKC binding proteins. To further investigate the role of the pseudosubstrate peptide in promoting PKC–protein interactions, an N-terminal truncation mutant lacking the pseudosubstrate sequence was prepared. Binding of the mutant α-PKC was diminished compared to wild-type α-PKC, although some binding was still apparent. These results indicate that the pseudosubstrate sequence contributes to, but is not the sole determinant of, PKC binding activity.

Protein kinase C (PKC)[1] is a family of phospholipid-dependent serine/threonine kinases that play a major role in regulation of cell growth and differentiation (Nishizuka, 1992; Stabel & Parker, 1991). The reasons for kinase heterogeneity are not known; however, differences in tissue distribution have been noted which suggest isozyme-specific functions. Differences among the isozymes in substrate specificity and subcellular localization have also been noted, which suggests that PKCs contain unique determinants which regulate their interactions with other proteins. We have used an overlay assay to study PKC interactions with other proteins (Hyatt et al., 1990). Comparison of the properties of REF52 cell PKC binding proteins and PKC substrates indicated that the two major binding proteins are also substrates.[2] The two major binding proteins/substrates were not found in SV40-transformed REF52 cells (Hyatt et al., 1990). Recently, we reported that the overlay assay can be used to screen expression libraries to isolate novel PKC substrates (Chapline et al., 1993). These results emphasize that the overlay assay is a valuable method for studying PKC interactions with binding proteins/substrates. The purpose of the present study was to identify the PKC domains involved in the interactions detected by this assay.

Initial definition of the PKC binding domains was found by comparing binding proteins for α-, β- and ϵ-PKCs. Similarities in binding profiles for each isozyme indicated that constant rather than variable regions contain the primary determinants of the binding activity. In fact, the binding protein profile detected by overlaying with α-PKC was mimicked by overlaying with an α-PKC sequence from the first constant region of the regulatory domain, namely, the pseudosubstrate peptide. A similar, but distinct, profile was also found by overlaying with [^{14}C]phosphatidylserine (PS), indicating that many PS binding proteins are also PKC binding proteins. The results suggest that the interaction of α-PKC with phospholipid binding proteins is mediated at least in part through the PKC pseudosubstrate domain.

MATERIALS AND METHODS

Cell Culture. REF52 and SV40-REF52 cells were obtained from D. McClure (Eli Lilly Co., Greenfield, IN). Cells were passaged twice weekly and grown in a 3:1 mixture of Dulbecco's modified Eagle's and Ham's F12 media containing 10% fetal bovine serum and other additions as described (McClure et al., 1982).

Preparation of Cell Lysates. Cells were grown in 100-mm dishes to near-confluence. For collection of cell lysates for biochemical assays, cells were washed twice with 50 mM Tris-HCl (pH 7.4) which contained 2.5 mM magnesium chloride and 0.25 M sucrose (buffer A). Cells were washed once and then scraped into buffer A containing 2.5 mM EGTA, 1 mM phenylmethanesulfonyl fluoride, and 10 μg/mL leupeptin (lysis buffer). Cells were sonicated 6 times for 5 s each time at 5-s intervals. Samples were centrifuged at 100000*g* for 60 min to collect what is referred to as the cytosol (soluble) and membrane (particulate) fractions.

Immunoblots. Samples were prepared for electrophoresis, separated on 7.5 or 10% sodium dodecyl sulfate–polyacrylamide gels (SDS–PAGE) (Laemmli, 1970), and electrophoretically transferred to nitrocellulose. Nitrocellulose sheets were blocked with 5% instant milk in 50 mM Tris-HCl (pH 7.4) containing 0.5 M sodium chloride (TBS) and then washed twice with TBS.

Overlay Assay. Blocked nitrocellulose sheets were cut into strips corresponding to individual lanes. The strips were washed twice in 3 mL of TBS. The standard assay conditions employed a 1-h incubation in 10 μg/mL partially purified rabbit brain PKC (an isozyme mixture containing α-, β-, and smaller amounts of ϵ-PKCs), 10 mg/mL bovine serum albumin, 20 μg/mL phosphatidylserine (PS), 1 mM EGTA,

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[1] Abbreviations: PS, phosphatidylserine; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PDBu, phorbol dibutyrate; PKC, protein kinase C; PBS, phosphate-buffered saline; MSB, microtubule stabilization buffer; TBS, Tris-buffered saline.

[2] S. L. Hyatt, L. Liao, and S. Jaken, in preparation.

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1.2 mM calcium, 10 μg/mL leupeptin, and 10 μg/mL aprotinin in TBS. After incubation with PKC, samples were washed twice briefly in phosphate-buffered saline (PBS) containing cofactors. After being washed, samples were fixed in 0.5% formaldehyde in PBS for 20 min at room temperature and then incubated in 2% glycine in PBS for 20 min to block reactive aldehyde groups. After being washed 3 times in TBS, samples were processed with isozyme-specific anti-PKC antibodies and second antibody as described for immunoblots. Bound α-PKC was detected with monoclonal antibody M6 (Leach et al., 1988). Bound ε-PKC was detected with C-terminal anti-peptide antibody which had been purified on a peptide affinity column.

Where indicated, the pseudosubstrate peptide sequence (α-PKC 19–31) or an unrelated basic peptide sequence (RARKFHVGRAYELLRGYVNFRLQYP), which is referred to as R-25-P, was used in the overlay assay at 10–40 μM. Antibodies to each of these KLH-conjugated peptides were prepared in rabbits according to standard protocols. Briefly, glutaraldehyde was used to couple each of the peptids to KLH. Initial injections included 1 mg of conjugated peptide in complete Freund's adjuvant. Animals were boosted at 2–3-week intervals with 200–500 μg of conjugated peptide. Antiserum to the pseudosubstrate peptide was affinity-purified against the appropriate peptide conjugated to agarose.

The phosphatidylserine (PS) overlay was done with 10 μg/mL PS (1 μCi/75 μg) in TBS containing 10 mg/mL BSA, 1 mM EGTA, and 1.2 mM calcium where indicated. After a 1-h incubation at room temperature, the nitrocellulose was briefly rinsed 3 times with PBS containing 0.9 mM calcium and 0.5 mM magnesium at 4 °C, dried, and exposed to film for 1–5 days. No differences in [^{14}C]PS binding were observed if the brief rinses were done at 23 °C. However, prolonged washing did decrease PS binding. PS binding appeared to be coordinately (i.e., not selectively) lost from the PS binding proteins.

Preparation of α-PKC Pseudosubstrate Deletion Mutant. Rat α-PKC originally obtained from Y. Nishizuka (Kobe) was subcloned into pBluescript SK (Stratagene). A fragment was excised using the *Pst*I site at 370 in the α-PKC sequence and the *Hind*III site in the multiple cloning sequence of SK. The fragment was subcloned into pQE10 (Qiagen) for expression in bacteria and into pBlueBacHis (Invitrogen) for expression in Sf9 insect cells. Cell lysates were prepared in 6 M guanidine hydrochloride, and 6X-His-tagged proteins were purified by nickel affinity chromatography in 8 M urea.

Expression and Purification of PKC Binding Proteins. Two cDNAs isolated by interaction cloning of PKC binding proteins (Chapline et al., 1993) were subcloned into pQE vectors (Qiagen). The expressed proteins were purified by nickel affinity chromatography. The purified proteins were used to study PKC interactions with binding proteins. Clone 35A is the rat homologue of MARCKS related protein (Chapline et al., 1993). Clone 35F is a partial cDNA containing unique sequences that is still being characterized.

PKC Denaturation. To determine if PKC binding required native protein, rabbit brain PKC was diluted into 8 M urea to a final concentration of 130 μg/mL and incubated for 7 days (4 °C). PKC was then diluted into TBS containing PS and calcium for the overlay assays shown in Figure 9; 8 M urea was used because these were the conditions required to solubilize and purify the α-PKC truncation mutant described above.

Materials. Electrophoresis reagents were from Bio-Rad (Richmond, CA). [3-^{14}C]PS (1,2-dioleoyl) was from New England Nuclear (Boston, MA). Alkaline phosphatase-conjugated goat anti-mouse IgG and substrate were from Promega (Madison, WI). Peptides were synthesized in the Protein Chemistry Laboratory of the W. Alton Jones Cell Science Center. Peptide R-25-P and corresponding antisera were a gift of Dr. John Crabb (Lake Placid, NY). Antibody to β-PKC and myelin basic protein substrate peptide were purchased from Upstate Biotech (Lake Placid, NY).

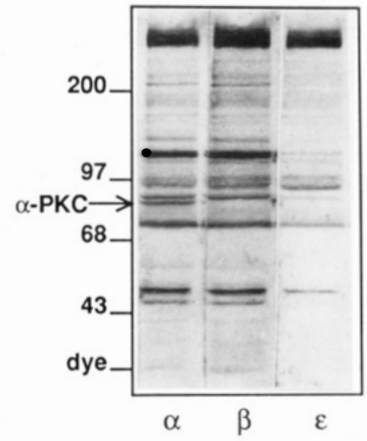

FIGURE 1: Comparison of α-, β-, and ε-PKC binding proteins. Nitrocellulose strips of REF52 cell cytosol (100 μg) were overlayed with a purified rabbit brain PKC preparation containing α-, β-, and smaller amounts of ε-PKC. Bound PKCs were detected with isozyme-specific antibodies.

RESULTS

Isozyme Specificity of Binding Protein Interactions. PKCs are a family of proteins which are characterized by an N-terminal regulatory domain and a C-terminal catalytic domain (Jaken, 1990). The isozymes are highly homologous in four conserved (constant, C) regions which are separated at defined intervals by five isozyme-specific (variable) regions. We reasoned that if constant regions were important for mediating the interactions with binding proteins, overlay assays with different PKC isozymes would be similar. Overlay assays with α-, β-, and ε-PKCs were compared (Figure 1). α- and β-PKC binding proteins were assayed in the presence of calcium and were essentially identical. Because there is no endogenous β-PKC in REF52 cells, the overlays with β-PKC clearly reveal an additional binding protein that migrates just above α-PKC. ε-PKC binding to the major α- and β-PKC binding proteins was also observed (see also Figure 2), although the band intensities were weaker. This is partially due to the lower amounts of ε-PKC in our preparations. ε-PKC binding to the major α- and β-PKC binding protein at 114 kDa could be detected in the presence of calcium (see Figure 2), which was not included in the assay buffer used in the experiment shown in Figure 1. PS was required for binding of each isozyme (data not shown).

ε-PKC differs from α- and β-PKCs with regard to substrate specificity and calcium dependence (Schaap et al., 1989) due to deletion of the C2 domain. Whereas α-PKC binding required either calcium or phorbol ester, ε-PKC binding occurred in the absence of calcium and phorbol ester (Figure 2). PDBu slightly augmented ε-PKC binding. Although calcium inhibited ε-PKC binding activity, inhibition was overcome in the presence of PDBu. Binding of both α- and ε-PKCs to a 114-kDa protein required calcium. This may be due to the calcium requirement for PS binding to this protein (discussed in Figure 3). These results indicate that constant regions other than the C2 region are the major determinants of the interactions measured in this assay system.

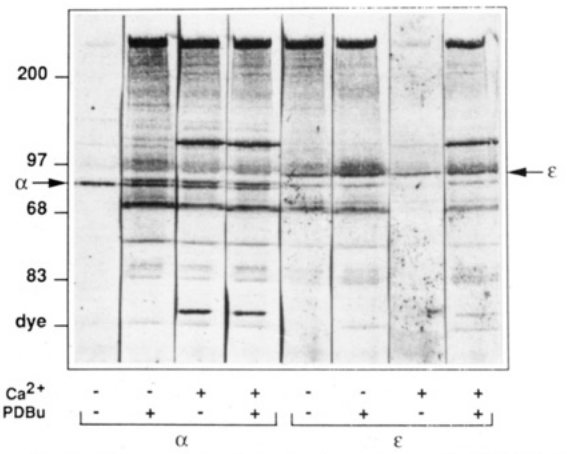

FIGURE 2: Calcium and phorbol ester dependence of ε-PKC binding. Nitrocellulose strips of REF52 cell cytosol (100 μg) were overlayed with a brain PKC/isozyme mixture in the presence and absence of calcium (1 mM EGTA and 1.2 mM calcium) and PDBu (200 nM) as indicated. Bound ε-PKC was detected with immunopurified anti-C-terminal antibody.

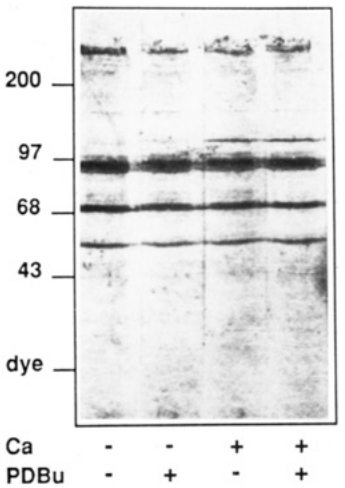

FIGURE 3: Calcium and phorbol ester dependence of PS binding. PS binding proteins in REF52 cytosols (100 μg) were detected by overlaying with [^{14}C]PS as described under Materials and Methods. Other additions are as described in the legend to Figure 4.

One major 32-kDa calcium-dependent α-PKC binding protein was only barely detectable as an ε-PKC binding protein. The significance of this cannot be evaluated at this time because detection of the 32-kDa α-PKC binding protein is variable and depends on the partial renaturation of the blotted protein.

Calcium Dependence of PS Binding. Previous studies have demonstrated that all PKC binding proteins are also PS binding proteins[2] (Wolf & Sahyoun, 1986; Chapline et al., 1993). [^{14}C]PS overlays were used to determine if the effects of calcium and PDBu on PKC binding activity could be attributed to effects on the binding proteins rather than the PKCs (Figure 3). Phorbol esters did not influence PS binding to the immobilized proteins. PS binding to most of the proteins was calcium-independent with the exception of a 114-kDa protein (Figure 3). This appears to be the same protein as the calcium-dependent α- and ε-PKC binding protein seen in Figure 2. Thus, the calcium requirement for PKC binding to p114 was linked to its calcium requirement for PS binding.

α-PKC Binding Proteins Are Also Pseudosubstrate Binding Proteins. The PS dependence of the α-PKC–protein interactions suggests that the PKC regulatory domain plays an important role in mediating these interactions. Constant regions within the regulatory domain include C1, C2, and

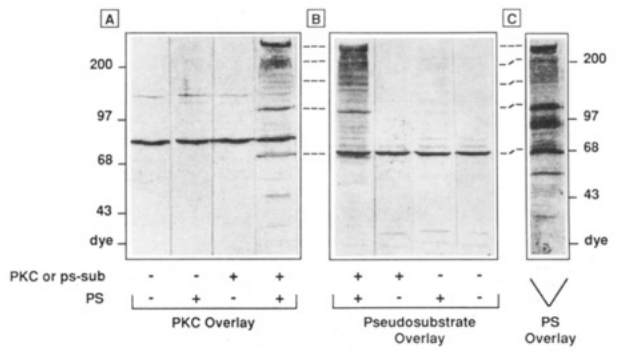

FIGURE 4: α-PKC binding proteins are also pseudosubstrate binding proteins. REF52 soluble proteins (100 μg/lane) were assayed for α-PKC (A) or pseudosubstrate (ps-sub, B) binding proteins as described under Materials and Methods. The prominent endogenous immunoreactive protein in (B) has not been identified but does not appear to be related to PKC. Assays were done in the presence (+) or absence (–) of PS. PS binding proteins (C) were detected by overlaying with [^{14}C]PS as described under Materials and Methods.

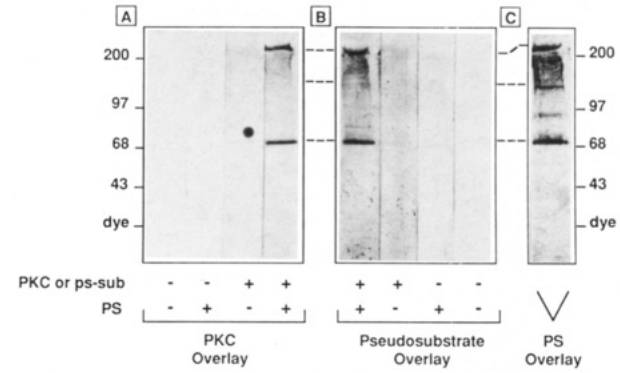

FIGURE 5: Heat-soluble α-PKC binding proteins. REF52 cell cytosol (100 μg/lane) was heat-denatured. Soluble proteins were collected and assayed for PKC and pseudosubstrate binding as described in the legend to Figure 4.

part of C3. To further define the PKC domain involved, we prepared the C1 α-PKC pseudosubstrate peptide and corresponding antibodies. Pseudosubstrate peptide binding proteins were detected under the same assay conditions described for the α-PKC overlay. Five major pseudosubstrate binding proteins were detected (Figure 4A,B). The molecular weights of these proteins correlated with the molecular weights of α-PKC binding proteins, although an endogenous pseudosubstrate antibody immunoreactive band partially obscures the p71 binding protein. Heat denaturation was used to eliminate this difficulty. The two major heat-soluble α-PKC binding proteins were also pseudosubstrate binding proteins (Figure 5A,B). Pseudosubstrate interactions with these proteins were PS-dependent (Figures 3 and 4). Pseudosubstrate binding was not dependent on either phorbol esters or calcium, with the exception of calcium dependence for p114 binding described above (data not shown). Relatively high concentrations of peptide were required to detect binding. This may reflect both the low affinity of the antipeptide antisera for the peptide and the lower affinity of the peptide for the binding proteins compared to PKC. The peptide (10 μM) did not effectively compete for α-PKC (24 nM) binding, implying that peptide binding affinity is much less than the holoenzyme.

The PS dependence of binding raised the question of whether PKC was recognizing free PS or PS bound to the nitrocellulose-immobilized proteins. An overlay assay with [^{14}C]PS was used to detect PS binding proteins from REF52 cells (Figures 4C and 5C). All of the α-PKC/pseudosubstrate binding proteins correlated with PS binding proteins. However, two

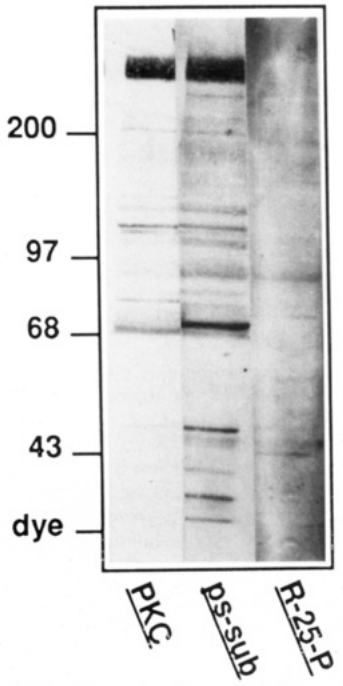

FIGURE 6: Specificity of pseudosubstrate binding. Soluble proteins from REF52 cells were overlayed either with the pseudosubstrate peptide or with R-25-P peptide described under Materials and Methods. Binding proteins were detected with the appropriate anti-peptide antibodies. Differences in antibody sensitivities do not account for the absence of R-25-P binding proteins. The R-25-P antibody was nearly 100-fold more potent than the anti-pseudosubstrate antibody in ELISA comparisons.

major PS binding proteins (around 97 and 50 kDa in Figures 4C and 5C) were not detected as major PKC/pseudosubstrate binding proteins.

Specificity of Pseudosubstrate Peptide Binding. Interaction of the pseudosubstrate peptide with binding proteins depended on phospholipid (Figures 4B and 5B), but did not depend on calcium or PDBu. We considered that the basic character of the pseudosubstrate peptide would favor electrostatic interactions with the anionic PS bound to proteins. To reduce the contribution of simple ionic interactions, all assays were done in the presence of 0.5 M sodium chloride and 10 mg/mL bovine serum albumin. To demonstrate the specificity of the interaction between the pseudosubstrate peptide and the PS binding proteins, overlays with another basic peptide were compared. R-25-P, which has a net positive charge of 6, did not bind to the PKC and pseudosubstrate binding proteins (Figure 6). Thus, although the basic character of the pseudosubstrate peptide is likely to contribute toward interacting with PS, basic peptides in general do not share this property under the assay conditions used.

The specificity of α-PKC pseudosubstrate peptide binding was also demonstrated in competition experiments. The ϵ-PKC pseudosubstrate peptide effectively competed for α-PKC peptide binding (Figure 7A). In contrast, the substrate peptide sequence from myelin basic protein did not compete, and in fact potentiated α-PKC peptide binding (Figure 7B). These results indicate that pseudosubstrate peptides share the unique property of interacting with PKC binding proteins. The potentiation observed with the myelin basic protein peptide also indicates that more complex, higher order interactions may also occur.

Binding Characteristics of the α-PKC N-Terminal Truncation Mutant. To better define the contribution of the pseudosubstrate domain to the interactions studied, we prepared an α-PKC mutant in which the N-terminal region

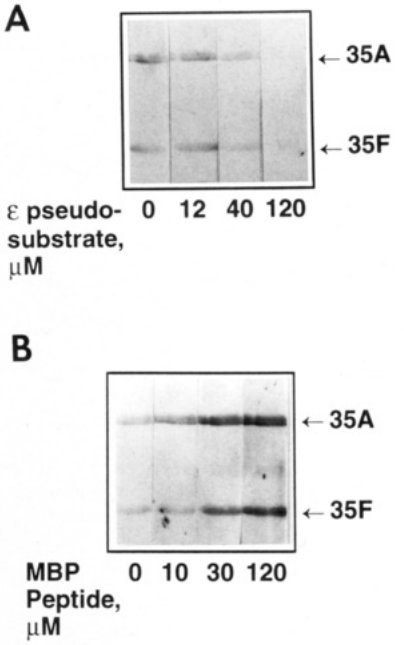

FIGURE 7: ϵ-PKC pseudosubstrate peptide competes for α-PKC pseudosubstrate peptide binding. Two PKC binding proteins, designated 35A and 35F, were overlayed (as described) with 10 μM α-PKC pseudosubstrate peptide that was competed with increasing molar concentrations of either the serine-substituted ϵ-PKC pseudosubstrate peptide (ERMRPRKRQG(S)VRRRV] or myelin basic protein substrate peptide [QKRPSQRSKYL) (B). α-PKC pseudosubstrate binding was measured using an antibody against the peptide.

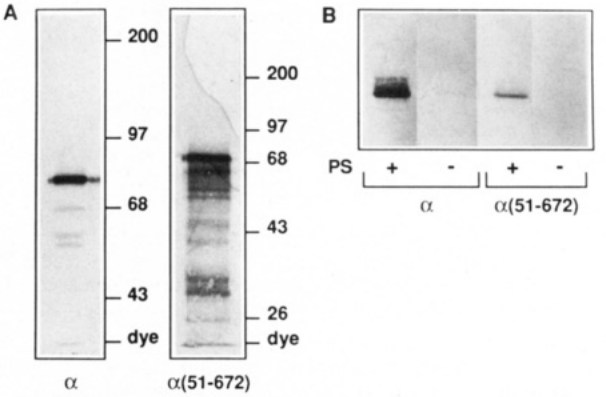

FIGURE 8: Comparison of wild-type and N-terminal truncation mutant α-PKC binding activities. (A) Aliquots of wild-type and mutant α-PKCs were electrophoresed and immunoblotted. Blots were stained with the α-PKC-specific monoclonal antibody M6. (B) The PKC binding protein 35H (Chapline et al., 1993) was blotted to nitrocellulose which was overlayed with comparable amounts of wild-type or mutant α-PKC. Binding activity was detected by staining with M6.

containing the pseudosubstrate sequence was deleted. Binding properties of the bacterially expressed fusion protein were compared to wild-type α-PKC expressed in Sf9 cells. The bacterially expressed mutant protein was used in these studies because, unlike wild-type α-PKC, the mutant protein expressed in Sf9 cells was recovered totally in the particulate fraction and resisted solubilization with nonionic detergents and high salt. Insolubility of N-terminal PKC truncation mutants has been observed by others (James & Olson, 1992; Muramatsu et al., 1992). Wild-type and mutant PKCs were both recognized by the α-PKC-specific antibody M6 (Figure 8). Overlay assay results with comparable amounts of immunoreactive wild-type and mutant α-PKC demonstrated that the deletion mutant retains binding activity but that this

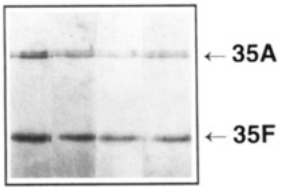

FIGURE 9: Effect of denaturing solutions on α-PKC binding. Two PKC binding proteins, designated 35A and 35F, were overlayed (as described) with two concentrations of either native PKC (N) or denatured PKC (D). PKC was denatured in 8 M urea as described under Materials and Methods. Blots were developed with the α-PKC-specific monoclonal antibody M6.

activity is relatively less than that of wild-type α-PKC (Figure 8). Denaturation of wild-type α-PKC in the solutions used to prepare the bacterial mutant α-PKC did not significantly inhibit binding (Figure 9). Thus, the pseudosubstrate sequence contributes to PKC binding but is not the sole determinant of PKC binding activity.

DISCUSSION

The PS dependence and phorbol ester regulation of α-PKC binding demonstrate that the regulatory domain contains important determinants of the PKC interactions. Furthermore, the similarity of α- and β-PKC binding protein profiles suggests that constant rather than variable domains mediate the interactions. Constant regions within the regulatory domain include C1, C2, and part of C3. The major α- and β-PKC binding proteins were also detected with ε-PKC, indicating that the C2 region (which is not present in ε-PKC) is not the primary determinant of PKC binding. On the other hand, the PKC binding protein profile was mimicked by the pseudosubstrate peptide found in the C1 region. These preliminary structure–function studies allowed us to focus our attention on the C1 region for further analysis of PKC domains involved. The α-PKC mutant lacking the pseudosubstrate sequence still interacted with binding proteins; however, this interaction was attenuated compared to wild-type α-PKC. These results suggest that PKC interactions are mediated both by the pseudosubstrate and by other PKC domains. Although our *invitro* assays did not indicate isozyme specificity, it is still possible that under appropriate conditions mimicking the cellular environment, isozyme-specific binding protein interactions may occur.

The PS binding region within the PKC regulatory domain has not yet been localized. The pseudosubstrate is an autoinhibitory sequence within the regulatory domain that is thought to interact with the substrate binding region in the catalytic domain (House & Kemp, 1987). In the presence of PKC cofactors (PS), a conformational change occurs which relieves the autoinhibition and allows PKC to interact with substrates. The pseudosubstrate sequence is very basic which would promote electrostatic interactions with anionic phospholipids. Mosior and McLaughlin (1991) recently demonstrated that the pseudosubstrate peptide directly interacts with acidic lipids. The interaction was of sufficient strength to provide a significant amount of the free energy needed for stabilizing the active form of PKC. Our results demonstrate that the pseudosubstrate binds to PS binding proteins and is important for maximal interactions of PKC with other proteins. However, the high concentration of peptide used to detect binding also indicates that this binding may be of relatively low affinity. Furthermore, the peptide did not effectively compete for α-PKC binding. It is most likely that the pseudosubstrate represents only one component required for high-affinity binding of the holoenzyme.

The interaction of binding proteins with PS raises the question of whether PKC is recognizing protein sequences, PS, or both. Careful comparison of [^{14}C]PS and PKC overlays (Figures 4 and 5) indicated that not all PS binding proteins are PKC binding proteins.[2] Thus, in addition to PS binding, certain protein sequences may be necessary for functional binding to PKC. We have used the overlay assay to screen expression libraries and have cloned additional PKC binding proteins/substrates (Chapline et al., 1993). Comparison of the translated sequences of the clones isolated indicates that proteins with high positive charge density domains interact with PKC. Phosphorylation decreased both PKC and PS binding to the expressed proteins. The strong correlation between PS and PKC binding emphasizes the importance of PS bridging for determining PKC interactions. However, since not all PS binding proteins are efficient PKC binding proteins, additional protein–protein interactions may be required to stabilize the ternary complex. This hypothesis can now be tested by studying the interactions of PKCs with the cloned binding proteins/substrates.

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