Identification and Characterization of α-Protein Kinase C Binding Proteins in Normal and Transformed REF52 Cells†

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ABSTRACT: Immunocytofluorescence studies demonstrated that α-PKC is concentrated in focal contacts of REF52 cells but not in their SV40-transformed derivatives [Jaken et al. (1989) *J. Cell Biol. 109*, 697–704; Hyatt & Jaken (1990) *Mol. Carcinog. 3*, 45–53]. Discrete localizations imply that PKC is targeted to these areas possibly via protein–protein interactions. We have used an overlay assay to detect α-PKC binding proteins. The molecular interactions between α-PKC and the binding proteins depended on phospholipid and either calcium or phorbol esters. Unlike the kinase activity, binding activity was detected in the absence of added calcium, indicating that calcium, which is necessary for phosphorylation of most substrates, is not required for binding. Vinculin and talin, two focal contact proteins, bound α-PKC. REF52 cells express several annexins (I, II, and VI) which bind PKC. Both annexin I expression and vinculin expression were decreased in SV40-REF52 cells. The two major REF52 cell binding proteins (p71 and p>200 kDa) were also down-regulated in the transformed cells, indicating transformation-sensitive regulation of PKC binding protein activity.

Protein kinase C (PKC)[1] is a family of phospholipid-dependent kinases that are important mediators of cell growth and differentiation (Nishizuka, 1992). PKCs are the major receptors for tumor-promoting phorbol esters and, therefore, are thought to play an important role in the later stages of tumor development. Although all of the PKCs require anionic phospholipid such as phosphatidylserine (PS) for catalytic activity, the isozymes differ in their requirements for additional cofactors. Group A PKCs (α, β, and γ) are calcium-dependent, whereas group B (δ, ε, and η) and group C (ζ) PKCs are calcium-independent. The activity of group A and group B PKCs is stimulatable by diacylglycerol (DAG) or phorbol esters. ζ-PKC activity is not regulated by these compounds and is not a phorbol ester receptor.

DAG appears to be the major cellular regulator of PKC activity (Nishizuka, 1992). Agonist-stimulated increases in DAG activate PKC and cause redistribution from soluble to particulate fractions. *In vitro* studies have demonstrated that DAG (or phorbol esters) increases the affinity of PKC for PS. Increased PS binding may account for the observed DAG-dependent redistribution of PKC to membrane fractions. Since many other proteins including several cytoskeletal (CSK)-associated proteins (Jaken, 1992) also interact with PS, redistribution of PKC could lead to close associations between PKC and other PS binding proteins. These phospholipid-dependent protein–protein interactions could serve to stabilize PKC membrane and/or cytoskeletal associations. In this study, we have used a blot overlay assay to study the interactions between PKC and other proteins and to identify some of these PKC binding proteins. In order to understand the *in vivo* relevance of the *in vitro* overlay assay, it is necessary to define conditions that support binding. Our results indicate that PKC binding is supported by conditions that do not support catalytic activity, indicating that activated PKC conformations are not necessary for the measured interactions.

Within the detergent-insoluble CSK fraction of REF52 cells, α-PKC is concentrated in focal contacts and, in fact, colocalizes with talin (Jaken et al., 1989). Focal contacts are specialized areas that mediate attachment of the actin CSK with the plasma membrane and interaction of the cell with the extracellular matrix (Burridge et al., 1988; Geiger et al., 1987). In contrast to normal REF52 cells, α-PKC is not associated with cell adhesion sites in SV40-transformed REF52 cells (Hyatt et al., 1990). The discrete localization in normal REF52 cells, and the difference in localization between normal and transformed cells, suggests that there are cellular determinants of α-PKC localization. In this study, we have compared the expression of PKC binding protein activities in normal and SV40-REF52 cells. Our results demonstrate that the content of several PKC binding proteins is reduced in the transformed cells. These results are consistent with the idea that PS-dependent protein–protein interactions may influence PKC subcellular location *in vivo*.

EXPERIMENTAL PROCEDURES

Cell Culture. REF52 and SV40-REF52 [WT6 Ag6 (McClure et al., 1982, 1984)] 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).

Preparations 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 then washed once and scraped into buffer A containing 2.5 mM EGTA, 1 mM phenylmethanesulfonyl fluoride, and 10 μg/

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[1] Abbreviations: DAG, diacylglycerol; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PDBu, phorbol dibutyrate; PBS, phosphate-buffered saline; PKC, protein kinase C; TBS, Tris-buffered saline.

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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 soluble and particulate fractions.

Immunoblots. Samples were prepared for electrophoresis, separated by 7.5 or 10% 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.

PKC 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 PS, 1 mM EGTA, 1.2 mM calcium, 10 μg/mL leupeptin, and 10 μg/mL aprotinin in TBS. Because binding is reversible, in initial experiments PS and calcium were retained throughout the washes and incubations with antibodies. However, we have found that fixing the PKC to the binding proteins eliminates this requirement. After incubation with PKC, samples were briefly washed twice in phosphate-buffered saline (PBS) containing cofactors. Proteins were then 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).

Phosphatidylserine Overlay Assay. Nitrocellulose sheets were overlayed with [^{14}C]PS (10 μg/mL, 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. Similar results were obtained with PBS washes at 23 °C.

Vinculin Immunoprecipitation. Cytosol was adjusted to 50 mM Tris-HCl (pH 8.5), 0.15 M sodium chloride, 1 mM EDTA, 1% deoxycholate, 1% Triton X-100, and 0.1% sodium dodecyl sulfate (RIPA buffer) and precleared with formalin-fixed Staph A cells. The precleared sample was incubated with 3 μL of rabbit anti-vinculin or control serum. Immunocomplexes were collected on protein A–Sepharose, eluted in SDS–PAGE sample preparation buffer (Laemmli, 1970), resolved on 7.5% SDS–PAGE, and transferred to nitrocellulose.

PS Affinity Chromatography and Microsequencing. An excess of phospholipid vesicles containing PS, phosphatidylcholine, and PE (2:2:1) was coupled to Affi-gel 10 (Bio-Rad, Richmond CA) according to the method of Meers et al. (1987). Soluble fractions from REF52 or SV40-REF52 cells were applied to the column after the lysis buffer was adjusted to 2.5 mM calcium in excess of EDTA. After the column was washed, PS binding proteins were eluted in 50 mM Tris, pH 7.4, containing 300 mM sodium chloride and 2.5 mM EDTA (Meers et al., 1987). Eluted proteins were resolved by 10% SDS–PAGE. Protein bands were identified after soaking the gel in 4 M sodium acetate. Proteins from excised bands were electroeluted and concentrated by acetone precipitation. Proteins were digested with 1% trypsin in 0.25 M Tris-HCl, pH 7.4, containing 2 M urea. Peptides were separated on a Vydac C18 column using a trifluoroacetic acid and acetonitrile gradient. After the solvent was removed, peptides were sequenced on an Applied Biosystems 470/120A protein sequencer.

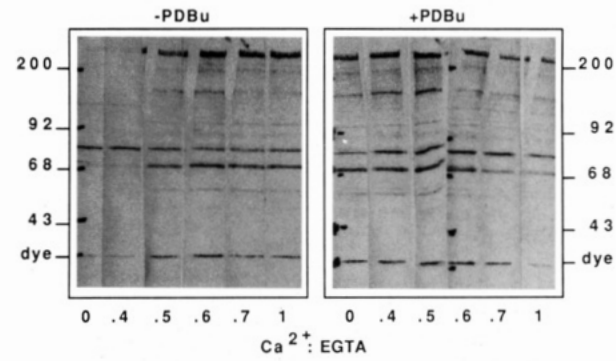

FIGURE 1: Phorbol esters decrease the calcium requirement for α-PKC binding to cellular proteins. Membranes (100000*g* pellets) were collected, separated by SDS–PAGE, transferred to nitrocellulose, and incubated in the presence of PKC, PS, 1 mM EGTA, and various calcium concentrations to produce the calcium:EGTA ratios shown. Blots were developed with the α-PKC specific monoclonal antibody M6.

Materials. Electrophoresis reagents were from Bio-Rad. [^{32}P]ATP and [3-^{14}C]PS (1,2-dioleoyl) were from New England Nuclear (Boston, MA). Alkaline phosphatase-conjugated goat anti-mouse IgG and substrate were from Promega (Madison, WI). Formalin-fixed Staph A cells were from BRL (Gaithersburg, MD). Chicken vinculin and talin and anti-vinculin antibody were kind gifts from K. Burridge (Chapel Hill, NC). Bovine α-PKC was expressed in Sf9 cells and was a gift from P. J. Parker (London, U.K.). In some experiments, human α-PKC was expressed in Sf9 cells using recombinant virus obtained from Dr. Robert Bell (Durham, NC). Human annexins I and II were a gift from Dr. M. J. Bienkowski (Kalamazoo, MI). Antibodies to various annexin species were a gift from Dr. John Dedman (Cincinnati, OH).

RESULTS

Phospholipid and Calcium Dependence of α-PKC Interactions. Binding of α-PKC to REF52 cell particulate proteins was detected using an overlay assay system as described under Experimental Procedures. In the presence of PS, but in the absence of either calcium or PDBu, only the endogenous 80-kDa α-PKC immunoreactive band was observed (Figure 1, lane 1). Additional immunoreactive bands appeared when the nitrocellulose was overlayed with purified PKC and PS in the presence of either calcium or PDBu. The concentration dependence for calcium was determined with a calcium/EGTA buffer (Figure 1). In the absence of PDBu, calcium was required for PKC binding. Binding proteins were detected at a calcium:EGTA ratio of 0.5–0.6, which corresponds to approximately 200 nM calculated free calcium. This is less than the calcium requirement for detecting maximal α-PKC kinase activity in our assay system (Jaken & Kiley, 1987). In the presence of PDBu, calcium was not required for PKC binding. Binding occurred in the absence of added calcium and in the presence of 1 mM EGTA. Although PDBu decreases the calcium requirement for α-PKC phosphotransferase activity, some calcium is still required for catalytic activity (Jaken & Kiley, 1987). The differences in calcium requirements for binding and phosphorylating activities indicate that a fully active PKC conformation is not required for interactions with these binding proteins.

The interaction of α-PKC with the nitrocellulose-immobilized proteins was reversible. When the cofactors (PS and

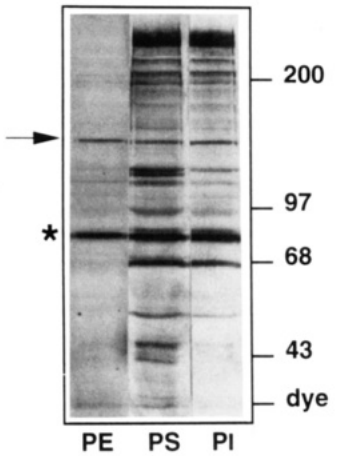

FIGURE 2: Phospholipid dependence of PKC binding. REF52 cell soluble proteins (75 μg/lane) were electrophoresed and transferred to nitrocellulose. PKC binding was measured in the presence of various phospholipids (100 μg/mL). PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine. Asterisk, endogenous α-PKC; arrow, unrelated cross-reacting protein apparent in some preparations.

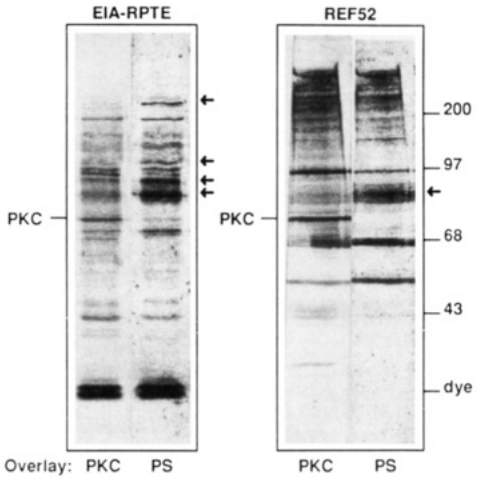

FIGURE 3: Comparison of PKC and PS binding proteins. Cell lysates (100 μg of protein) from E1A-immortalized renal proximal tubule epithelial cells (RPTE) and REF52 cells were electrophoresed and blotted to nitrocellulose. PKC and PS binding proteins were detected in adjacent lanes using the overlay assays outlined under Experimental Procedures. PKC indicates the endogenous α-PKC in the samples. Arrows indicate prominent PS binding proteins which do not correspond to prominent PKC binding proteins.

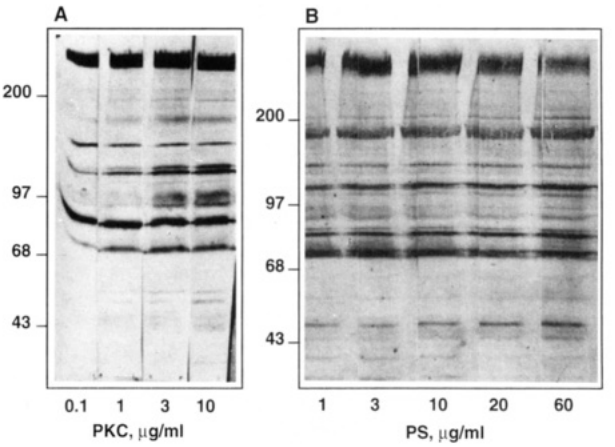

FIGURE 4: Saturability and PS concentration dependence of PKC binding. (A) Soluble fractions of REF52 cells (100 μg/lane) were electrophoresed, transferred to nitrocellulose, and overlayed with the indicated concentrations of α-PKC in the presence of PS (20 μg/mL), calcium (1.2 mM), and EGTA (1 mM). In this experiment, α-PKC purified from Sf9 cells infected with recombinant human α-PKC baculovirus was used. In (B), overlays were done with 2 μg/mL α-PKC with the indicated concentrations of PS.

calcium) were omitted during the washes and antibody incubation, α-PKC binding proteins were no longer detected. This procedural difficulty was overcome by fixing the bound PKC to the binding proteins with formaldehyde as described under Experimental Procedures.

PKC binding required PS; no binding proteins were detected in the absence of PS. The neutral phospholipids phosphatidylcholine and phosphatidylethanolamine, which do not support PKC or phorbol ester binding, did not substitute for PS (Figure 2, lane 1, and data not shown). PI supported binding to nearly the same extent as PS. This is of interest because PI is only 40% as effective as PS in supporting the catalytic activity of α-PKC (Huang et al., 1988; Sekiguichi et al., 1987). In contrast, PI has been reported to be nearly as effective as PS in supporting PKC binding to phospholipid vesicles (Bazzi & Nelsestuen, 1987a,b). Mixed micelles of PS and phosphatidylcholine fully supported binding (data not shown), indicating that dilution of the PS negative charge with the neutral lipid did not inhibit binding.

Comparison of PKC and PS binding proteins has been used to demonstrate that all PKC binding proteins are also PS binding proteins[2] (Wolf & Sahyoun, 1986). Thus, it is possible that PKC is merely recognizing the PS bound to the proteins, and not the proteins themselves. However, not all PS binding proteins are PKC binding proteins (Figure 3). PKC and PS binding proteins were directly compared on adjacent lanes of blotted proteins from lysates of E1A-immortalized renal proximal tubule epithelial cell and REF52 cells. All of the PKC binding proteins closely aligned with a PS binding protein; however, several prominent PS binding proteins did not align with prominent PKC binding proteins (arrows in Figure 3). Thus, although PS bridging is necessary for PKC binding, it appears that additional interactions are required to form complexes of sufficient affinity to be detected in the blot overlay.

Two other lines of evidence also suggest that the observed PS-dependent interactions are specific. First, a homogeneous preparation of α-PKC (produced by overexpressing bovine α-PKC in Sf9 cells) was used to determine the concentration dependence of α-PKC binding. Binding was saturable with a maximum reached at 3.0 μg/mL α-PKC for most of the binding proteins (Figure 4A). Second, we reasoned that if PKC were nonspecifically associating with protein-bound PS, then excess free PS would compete for PKC binding to proteins. On the other hand, if PS "bridged" PKC to other PS binding proteins and additional protein–protein interactions stabilized the complexes, excess free PS should not inhibit PKC binding. Binding at 1 and 60 μg/mL PS was similar (Figure 4B). Thus, PKC binding to the nitrocellulose-bound proteins was not competed by high concentrations of free PS. These data suggest that PKC accumulates with the immobilized protein-bound PS rather than the free PS vesicles.

Comparison of PKC Binding Proteins in Normal and Transformed REF52 Cells. Binding proteins in soluble and particulate fractions of normal and transformed REF52 cells were compared in PKC blot overlays. In general, many more binding proteins were apparent in REF52 cells than in their transformed counterparts (Figure 5). In particular, the two major PKC binding proteins in the particulate fraction of normal cells were not detected in either the soluble or the

[2] Liao et al. (1994).

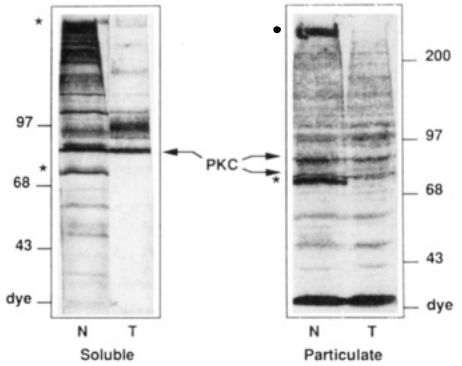

FIGURE 5: PKC binding proteins in normal and transformed REF52 cells. REF52 and SV40-REF52 cell lysates were collected and centrifuged at 100000*g* to produce soluble and particulate fractions. Binding proteins in aliquots (100 μg of protein) were detected by PKC blot overlay. Arrows indicate endogenous PKC immunoreactive proteins in these samples at 80 kDa in the soluble fraction and at 80 and 74 kDa in the particulate fraction. Asterisks indicate the major PKC binding proteins of 71 and >200 kDa.

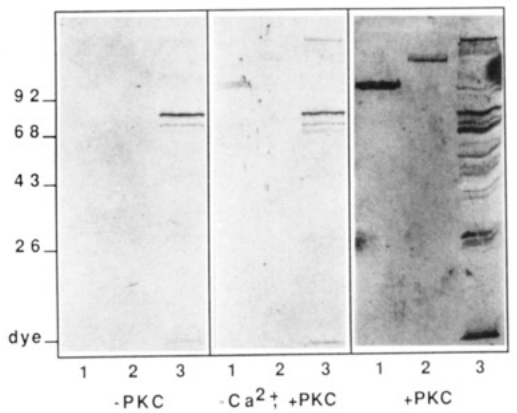

FIGURE 6: α-PKC binds to vinculin and talin. Vinculin (1 μg, lane 1), talin (1 μg, lane 2), and particulate proteins (75 μg, lane 3) were tested in the overlay assay described under Experimental Procedures. The blots were developed with M6 after incubating in the presence of calcium and PS without PKC (left panel), with PS and PKC but without calcium or PDBu (middle panel), or with PKC, calcium, and PS (right panel).

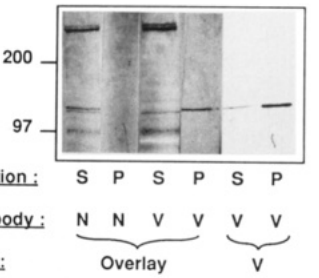

FIGURE 7: Identification of vinculin as an α-PKC binding protein. Vinculin was immunoprecipitated from REF52 cytosols as described under Experimental Procedures. Lanes 1, 3, and 5, supernatants containing nonimmunoprecipitated proteins (S); lanes 2, 4, and 6, eluates from the immune complexes (P). Overlay assays were performed on lanes 1–4. Lanes 1 and 2 show that nonimmune rabbit serum (N) does not promote the appearance of α-PKC binding proteins in immunoprecipitates. In contrast, vinculin antiserum (V) removes a 116-kDa binding protein from the cytosol (compare lanes 1 and 3). This 116-kDa binding protein appears in the immunoprecipitate (lane 4). Most of the vinculin was depleted from the cytosol by immunoprecipitation and was collected in the immune complex as shown in the immunoblots stained with anti-vinculin antibody (lanes 5 and 6).

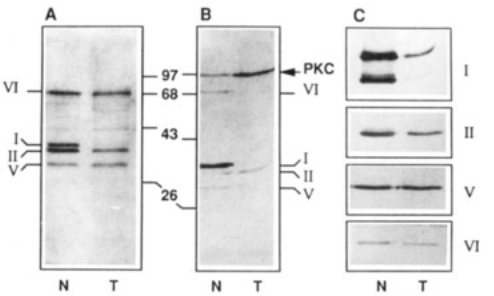

FIGURE 8: Comparison of annexins as binding proteins in normal and transformed REF52 cells. Soluble fractions from normal (N) and SV40-transformed (T) REF52 cells were chromatographed on a PS affinity column. In (A), eluted proteins were stained with Coomasie Blue. In (B), eluted proteins were blotted and assayed for PKC binding activity. Endogenous PKC in the cytosols is also partially purified on the PS column (arrow). In (C), eluted proteins were blotted and incubated with antibodies specific for annexins I, II, V, or VI.

particulate fractions of transformed cells. These results demonstrate a general down-modulation of PKC binding protein activity in SV40-REF52 cells, although one binding protein of approximately 97 kDa was more abundant in soluble fractions of transformed cells.

α-PKC Binds to Vinculin and Talin. We have previously shown that α-PKC colocalizes with focal contacts in REF52 cells (Jaken et al., 1989). We, therefore, investigated whether the focal contact proteins vinculin and talin were α-PKC binding proteins. The overlay assay shown in Figure 6 indicates that both chicken vinculin and chicken talin bind α-PKC in the presence of calcium and phospholipid. The molecular weight of vinculin corresponds to the molecular weight of a minor binding protein band in the particulate fraction of REF52 cells (Figure 6, lane 3). Several other PKC substrates (histone, myelin basic protein, and myosin light chain) did not bind α-PKC in this assay.

We subsequently determined by immunoblot analysis with anti-vinculin antibodies that most (>90%) of the vinculin in REF52 cells was recovered in the soluble fraction (100000*g* supernatant). Therefore, the soluble fraction was used as the source of vinculin for further experiments. To determine if vinculin accounted for a binding protein in REF52 cytosol, we used immunoprecipitation with anti-vinculin antibodies to deplete the cytosol of vinculin. Results (Figure 7) show the loss of a 116-kDa α-PKC binding protein from the vinculin-depleted cytosol. Furthermore, a 116-kDa binding protein and immunoreactive vinculin were both recovered in the immunoprecipitate. Thus, vinculin represents one of the minor binding protein bands in REF52 cytosol. Comparison of the vinculin content in normal and transformed REF52 cells by immunoblots was used to determine that the vinculin content was decreased approximately 50% in SV40-REF52 cells (data not shown). However, vinculin was still concentrated in the cell–substratum adhesion sites of the transformed cells.

α-PKC Binds to Annexins. Because of the close correlation between PKC binding proteins and PS binding proteins, we used PS affinity chromatography to purify PKC binding proteins and compare binding protein content between normal and transformed cells. Four major protein bands were eluted from the column (Figure 8A). After electrophoretic separation, each of the protein bands was electroeluted from the polyacrylamide gel. Microsequencing analysis of peptide fragments was used to tentatively identify the four bands as annexin VI, annexin I, annexin II, and annexin V (Table 1). The band corresponding to annexin 1 was only barely detectable in extracts from the SV40-REF52 cells. Levels of annexins II and VI were slightly diminished. Immunoblot analysis with antisera specific for individual annexins was used to further compare the annexin content of the normal

Table 1: Tryptic Peptide Sequences of PS Affinity-Purified Proteins[a]

band	molecular mass (kDa)	sequence	annexin peptide
1	71	XINEAYKEDDY	h-annVI (148–157)
2	36	GVDEATTI	r-annI (59–66)
		AAYLQETGKPLDETLK	r-annI (82–97)
3	35	GVXXVTIVNILTNR	h-annII (50–63)
		LXXXXGDHSTPPSAYGSVKPYTNFDAERDAL	h-annII (11–40)
4	32	QAYEEEEYGSNLXDDVVGD	r-annV (125–142)
		GLGXDED	r-annV (28–34)

[a] Calcium-dependent PS binding proteins from REF52 cells were isolated by PS affinity chromatography. Four polypeptides were separated by electrophoresis, electroeluted, and digested with trypsin for microsequencing as described under Experimental Procedures. The peptide sequences and the corresponding annexin sequences are listed. r, rat; h, human.

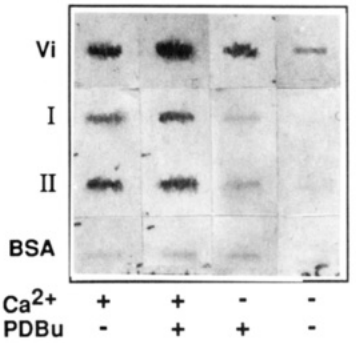

FIGURE 9: Comparison of the calcium dependence for PKC binding to annexins and vinculin. Annexin I or II (100 ng) or vinculin (1 μg) was directly applied to nitrocellulose using a slot-blot manifold. PKC overlay assays were performed in the presence of EGTA (1 mM) and PS (20 μg/mL). Calcium (1.2 mM) and PDBu (200 nM) were included where indicated.

and transformed cells. Whereas nearly equal amounts of immunoreactive annexins II, V, and VI were found, annexin I was specifically decreased in the transformed cells (Figure 8C).

Overlay assays of the PS column eluates demonstrated that annexin I was the predominant PKC binding protein (Figure 8B). Only weak bands corresponding to annexins II and VI were observed. In contrast, when the electroeluted proteins were directly applied to nitrocellulose rather than electrophoretically transferred from denaturing gels, annexin VI bound PKC much better than annexins I and II (data not shown). Native annexins I and II bound equally well when they were directly applied to nitrocellulose slot blots (see Figure 9). These results demonstrate that sample preparation can influence the ability of annexins to act as PKC binding proteins.

Comparison of Calcium Requirements for Vinculin and Annexin Binding. Comparison of PKC binding to vinculin and annexins I and II revealed a difference in calcium requirements among the binding proteins (Figure 9). PKC bound to both vinculin and annexins in the presence of PS and calcium. PDBu did not enhance binding at the excess calcium concentrations used. In the absence of added calcium, PDBu supported binding to vinculin but not to the annexins. Annexins are calcium-dependent PS binding proteins, whereas PS binding to vinculin is not calcium-dependent. Thus, the calcium dependence for PKC binding to annexins reflects properties of the annexins rather than of α-PKC itself.

DISCUSSION

PKC is a large family of phospholipid-dependent kinases that are important for cell growth and differentiated functions (Nishizuka, 1992). Most cells express more than one type of PKC; however, the reason for PKC heterogeneity is not yet known. Isozyme-specific properties could potentially lead to activation by different signaling pathways, different substrate specificities, and/or different subcellular locations. The discrete localization of α-PKC in focal contacts of REF52 cells indicates that PKCs may be targeted to distinct subcellular sites via specific interactions (Jaken et al., 1989). The loss of targeting of α-PKC to cell adhesion sites in SV40-REF52 cells demonstrates that there are cellular determinants of α-PKC localization (Hyatt et al., 1990). To test this hypothesis, we have pursued identification of binding proteins and comparison of their expression in normal and SV40-transformed REF52 cells using a blot overlay assay.

The relevance of the *in vitro* assay depends on the specificity of the interactions measured. In agreement with previous studies (Wolf & Sahyoun, 1986), we have also found that all PKC binding proteins are PS binding proteins. However, not all PS binding proteins are PKC binding proteins,[2] indicating that PS bridging alone is not sufficient to support stable PKC binding. Preliminary analyses indicated that PKC preferentially recognized protein-bound rather than free PS since PKC binding was relatively constant over a 60-fold range of PS concentrations (Figure 4B). These results demonstrate that PS bridging is essential for PKC protein recognition and, furthermore, suggest that additional protein–protein interactions stabilize the phospholipid-dependent binding.

We have also used the overlay assay to clone PKC binding proteins by screening expression libraries (Chapline et al., 1993). At the present time, it appears tht most if not all of the cloned binding proteins are also PKC substrates. Furthermore, comparison of binding proteins and substrates in REF52 cells indicates that the major binding proteins and substrates copurify.[3] Many PKC substrates interact with PS (Jaken, 1992), and this may be important for PKC–substrate interactions (Bazzi & Nelsestuen, 1987a; Newton & Koshland, 1990). Our results indicate that in the presence of PS, PKC interactions with substrate proteins are of sufficiently high affinity to be detected in an overlay assay. We propose that PS bridges PKC to other PS binding proteins and that the phospholipid-dependent interactions are stabilized by protein–protein interactions. It is now possible to explore these ideas in more detail by studying the interactions of PKCs with the binding proteins/substrates that have been cloned from expression libraries by overlay assay screening (Chapline et al., 1993).

Close analysis of the cofactor requirements for α-PKC binding indicated a difference in calcium requirement for binding and kinase activities. PDBu supported binding in the absence of calcium, which is required for α-PKC kinase activity. Furthermore, PI, which does not efficiently support catalytic activity (Huang et al., 1988; Sekiguchi et al., 1987; Bazzi & Nelsestuen, 1987b), supported binding activity. In our hands, calcium was not required for binding to vinculin

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

and was required for binding to the annexins, which are a group of calcium-dependent PS binding proteins. Thus, the calcium dependence for binding to annexins reflected the PS binding properties of the annexins and not a requirement for PKC catalytic activity. In this regard, the binding proteins we are studying are distinct from the recently described Receptors for Activated C Kinase (RACKS) (Mochly-Rosen et al., 1991a,b). The PS dependence for PKC binding to RACKS was attributed to a requirement for catalytically active PKC. In our studies, PS appeared to be required for PS bridging to other PS binding proteins/substrates. These results still leave open the possibility that PS-induced conformational changes (although not necessarily to the fully active conformation) are also important.

Our results clearly demonstrate that PKC can bind to certain cytoskeletal-associated proteins including vinculin and annexins in vitro. These results do not yet determine if these interactions occur in vivo. In general, PKC binding proteins were not as abundant in SV40-REF52 cells as in normal REF52 cells. In particular, the two major REF52 cell PKC binding proteins (p71 and p>200 kDa), vinculin, and annexin I were all decreased in SV40-REF52 cells, indicating that transformation down-regulates binding protein expression and/or activity. Although α-PKC colocalizes with vinculin and talin in focal contacts of REF52 cells, vinculin and talin are also found in the less stable adhesion plaques formed in SV40-REF52 cells which do not contain α-PKC (Jaken et al., 1989; Hyatt et al., 1990). Thus, vinculin and talin cannot be the primary determinants of PKC association with focal contacts. The difference in annexin I content between the normal and transformed cells allows for a potential difference in targeting PKC location; however, annexin I did not localize to focal contacts of REF52 cells (data not shown). Several other binding proteins have now been identified by using the overlay assay to screen rat kidney and REF52 cell expression libraries (Chapline et al., 1993). These studies also demonstrated that increased phosphorylation of PKC binding proteins/substrates coordinatively decreases PKC and PS binding to these target proteins. The possibility that differences in the expression and/or phosphorylation state of PKC binding proteins may contribute to the differential localization of PKCs between normal and transformed cells requires further investigation of additional PKC binding proteins.

REFERENCES

Bazzi, M. D., & Nelsestuen, G. L. (1987a) *Biochemistry 26*, 1974.

Bazzi, M. D., & Nelsestuen, G. L. (1987b) *Biochemistry 26*, 115.

Burridge, K., Fath, K., Kelly, T., Nuckolls, G., & Turner, C. (1988) *Annu. Rev. Cell Biol. 4*, 487.

Chapline, C., Ramsay, K., Klauck, T., & Jaken, S. (1993) *J. Biol. Chem. 268*, 6858.

Geiger, B. T., Volk, T., Volberg, T., & Bendori, R. (1987) *J. Cell Sci. 8* (Suppl.), 252.

Huang, K.-P., Huang, F. L., Nakabayashi, H., & Yoshida, Y. (1988) *J. Biol. Chem. 263*, 14839.

Hyatt, S. L., Klauck, T., & Jaken, S. (1990) *Mol. Carcinog. 3*, 45.

Jaken, S. (1992) in *Protein kinase C: Current concepts and future perspectives* (Epand, R., & Lester, D., Eds.) Ellis Horwood, Chichester, U.K.

Jaken, S., & Kiley, S. C. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 4418.

Jaken, S., Leach, K., & Klauck, T. (1989) *J. Cell Biol. 109*, 697.

Laemmli, U. K. (1970) *Nature 227*, 680.

Leach, K. L., Powers, E. A., McGuire, J. C., Dong, L., Kiley, S. C., & Jaken, S. (1988) *J. Biol. Chem. 263*, 13223.

Liao, L., Hyatt, S. L., Chapline, C., & Jaken, S. (1994) *Biochemistry* (following paper in this issue).

McClure, D. B., Hightower, M. J., & Topp, W. C. (1982) in *Growth of cells in hormonally defined media* (Sato, G. H., Pardee, A. B., & Sirbasku, D. A., Eds.) pp 345–364, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY.

McClure, D. B., Dermody, M., & Topp, W. C. (1984) in *Cancer Cells 1/The transformed phenotype* (Levine, A. J., Van de Woude, G. F., Topp, W. C., & Watson, J. D., Eds.) pp 17–23, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Meers, P., Ernst, J. D., Duzgunes, N., Hong, K., Fedor, J., Goldstein, I. M., & Papahadjopoulos, D. (1987) *J. Biol. Chem. 262*, 7850.

Mochly-Rosen, D., Khaner, H., & Lopez, J. (1991a) *Proc. Natl. Acad. Sci. U.S.A. 88*, 3997.

Mochly-Rosen, D., Khaner, H., Lopez, J., & Smith, B. L. (1991b) *J. Biol. Chem. 266*, 14866.

Newton, A. C., & Koshland, D. E. (1990) *Biochemistry 29*, 6656.

Nishizuka, Y. (1992) *Science 258*, 607.

Sekiguchi, K., Tsukuda, M., Ogita, K., Kikkawa, U., & Nishizuka, Y. (1987) *Biochem. Biophys. Res. Commun. 145*, 797.

Wolf, M., & Sahyoun, N. (1986) *J. Biol. Chem. 261*, 13327.