Molecular Analysis of the Interactions between Protein Kinase C-ε and Filamentous Actin*

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Protein kinase C-epsilond (PKC-ε) contains a putative actin binding motif that is unique to this individual member of the PKC gene family. We have used deletion mutagenesis to determine whether this hexapeptide motif is required for the physical association of PKC-ε and actin. Full-length recombinant PKC-ε, but not PKC-βII, -δ, -η, or -ζ, bound to filamentous actin in a phorbol ester-dependent manner. Deletion of PKC-ε amino acids 222-230, encompassing a putative actin binding motif, completely abrogated this binding activity. When NIH 3T3 cells overexpressing either PKC-ε or the deletion mutant of this isoform were treated with phorbol ester only wild-type PKC-ε colocalized with actin in zones of cell adhesion. In binary reactions, it was possible to demonstrate that purified filamentous actin is capable of directly stimulating PKC-ε phosphotransferase activity. These and other findings support the hypothesis that a conformationally hidden actin binding motif in the PKC-ε sequence becomes exposed upon activation of this isoform and functions as a dominant localization signal in NIH 3T3 fibroblasts. This protein-protein interaction is sufficient to maintain PKC-ε in a catalytically active conformation.

Protein kinase C-epsilond (PKC-ε)1 was the first member of the novel (classified AGC-IIB) subfamily of lipid-regulated protein serine/threonine kinases to be characterized (1, 2) and remains the most extensively studied Ca2+-insensitive isoform of the PKC family. It is presently understood that PKC-ε may participate in the regulation of diverse functions in cells of various origins, including the modulation of gene expression (3), Raf-1 mitogenicity (4), neoplasic transformation (5, 6), cell adhesion (7), extension and maintenance of motile cellular protrusions (8), contraction in smooth muscle cells (9) and cardiomyocytes (10, 11), and secretory vesicle trafficking (12, 13).

PKC-ε is a typical multidomain protein in which the overall structural organization has been conserved in orthologous genes from yeast to mammals (2, 22). However, in mammals PKC-ε has acquired short sequence motifs in the regulatory N-terminal region that are not evident in invertebrates (ApII of Aplysia and PKC d98F of Drosophila; Ref. 15) and are postulated to function as localization signals in the subcellular targeting of this protein kinase. These putative targeting signals include peptide motifs reported to be capable of anchoring PKC-ε within the cytoskeletal matrix (10, 11, 13) and domains which appear to support interactions with either the Golgi or plasma membranes of mammalian cells (16, 17). This diversification may potentially recruit PKC-ε into distinct, and spatially segregated, multimeric signaling complexes in a species- and cell-specific manner and contribute to the striking functional versatility of this isoform.

Mammalian PKC-η is most closely related to PKC-ε in overall sequence identity and similarity (66 and 82% in human Swiss-Prot numbers P24723 and Q02156, respectively) and yet each displays a distinct pattern of distribution within the same cell, with PKC-η being concentrated in the Golgi apparatus and PKC-ε accumulating at areas of cell-cell contact in NIH 3T3 fibroblasts (18). For this reason, relatively small sequence motifs or signal patches may be the primary determinants of the disparity in their topogenic fate. Alignment of the amino acid sequences for human PKC-ε and PKC-η reveals that the principal difference between the two polypeptides can be attributed to an insertion of 56 additional residues within the hinge region of PKC-ε. The PKC-ε hinge region contains two consensus peptide motifs, a PEST sequence (19) and a sequence that is quite similar to the “destruction box” of mitotic cyclins (RLGL-DEPNF, residues 402–410 in human PKC-ε), that are known to target cytosolic proteins for ubiquitin-dependent destruction by the 26 S proteasome (19, 21). In addition, in vivo sorting assays conducted using truncated fragments of PKC-ε suggest that upon activation the hinge region may be involved in targeting the protein to the plasma membrane (16). The N- and C-terminal variable extensions of PKC-ε and -η show a significant degree of sequence identity (53 and 57%, respectively) and only the N terminus contains stretches of three or more residues in which nonconservative substitutions are encountered (residues 28–36 and 139–154 in human PKC-ε). The only remaining stretch of amino acids in which sequence divergence between PKC-ε and -η is apparent can be found within the conserved C1 domain, where a putative actin-binding motif that is unique to PKC-ε has recently been identified (13). We
have now investigated the functional importance of this putative actin-binding site by deletion mutagenesis and expression of the mutagenized PKC-ε cDNA in the Spodoptera frugiperda (Sf9) insect cell line and cultured NIH 3T3 fibroblasts.

Binary interactions between purified actin and a variety of recombinant PKCs were performed under in vitro conditions designed to provide direct tests of our hypotheses that filamentous actin (Aβp): 1) represents a novel and specific PKC-ε-binding protein that, 2) recognizes and physically interacts with the previously identified actin-binding motif in PKC-ε, and 3) is capable of maintaining this kinase in a catalytically active conformation (13). These analyses revealed that interactions between purified Aβp and full-length recombinant PKC-ε were quite specific, because PKC-βII,-δ, -η, and -ζ did not bind Aβp at a physiological ionic strength. Deletion of amino acid residues 222–230 in mouse PKC-ε completely abrogated this binding activity without altering the affinity of the deletion mutant (ΔPKC-ε222–230) for 4β-phorbol 12,13-dibutyrate (4β-PDBu). Moreover, immunofluorescence microscopy of PKC overexpressing NIH 3T3 cells confirmed that PKC-ε, but not ΔPKC-ε222–230, colocalized with actin. Finally, assays of in vitro phoshophotransferase activity indicated that Aβp may also function as an agonist of PKC-ε, in the absence of lipid metabolites or membrane phospholipids.

**EXPERIMENTAL PROCEDURES**

**Materials**—All reagents used in this study were purchased from Sigma, unless otherwise specified. Mouse PKC-ε or PKC-δ cDNAs inserted into the pLXSN retroviral vector (originally obtained from A. D. Miller) were constructed by D. Kirk Wies (East Carolina University, Greenville, NC). Antipeptide antiserum against the C-terminal of PKC-ε and PKC-δ were also provided by Drs. Wies. Antipeptide antiserum against the N-terminal of PKC-ε and PKC-δ were purchased from Transduction Laboratories (Lexington, KY). Anti-mannosidase II antiserum was purchased from BabCo (Richmond, CA). N-iodoacyetyl-N-ethylmaleimide (EDANS) was obtained from Molecular Probes (Eugene, OR). Recombinant purified PKC isozymes were purchased from Panvera (Madison, WI) and Oxford Biomedical Research (Oxford, MI). Nickel-nitrilotriacetic acid (Ni-NTA)-agarose was obtained from Biosynthesis Inc. (Lewisville, TX). Nickel-nitrilotriacetic acid (Ni-NTA)-agarose was obtained from Bio-Rad (Hercules, CA) for 20 cycles and the PCR product was gel purified and circularized by blunt-end ligation. Clones were screened by PCR amplification using the Bio-Rad protein assay. PKC-resistant NIH 3T3 cells were obtained from Life Technologies (Gaithersburg, MD). [3H]PDBu and [32P]ATP were purchased from Amersham International (Amersham, United Kingdom).

**Actin Preparations**—Rabbit skeletal muscle a-actin was purified as described (22) and polymerized to generate Aβp by dialysis against 4 mM imidazole, pH 7.5 containing 2 mM MgCl2 and 0.5 mM ATP. Monomeric actin (Aβ) was generated by dialyzing Aβp for 48 h against G-buffer (5 mM Tris-HCl, pH 7.6, containing 0.2 mM CaCl2, 0.2 mM ATP, and 0.5 mM β-mercaptoethanol). Cy324 of actin was labeled with the dye EDANS as described in Ref. 23 and a stable and soluble derivative of Aβp was generated using the heterobifunctional reagent 3-maleimidobenzoic acid N-hydroxysuccinimide ester (BSE) according to Betteche et al. (24). The two C-terminal residues of actin (Cy594 and Phe737) were selectively removed by digestion of AEDANS-labeled Aβp with bovine pancreatic type I trypsin according to the procedure described in Ref. 25.

**Subcellular Fractionation and Generation of Tritic PKC-ε Fragments**—Cytosolic fractions were obtained from rat cerebral cortex (13). This cytosolic fraction contained an average of 7 ng of PKC-ε/mg of protein. Cytosolic fractions containing trypsic PKC-ε fragments were generated by predigesting cytosol with trypsin at a protein to enzyme mass ratio of 40:1 for 5 min at room temperature before adding soybean trypsin inhibitor at four times the trypsin concentration.

**Subcloning and Baculovirus Expression of PKC Isozymes**—Mouse PKC-ε and PKC-δ cDNAs were originally subcloned into the pFastBac1 donor vector (Invitrogen) as p5/PKC-ε and p4/PKC-δ, respectively, and expressed in Sf9 cells, a clonal isolate of the S. frugiperda cell line IPLB-S2-2E, using the Bac-to-Bac™ baculovirus expression system (Life Technologies, Inc., Gaithersburg, MD). The p3/PKC-ε cDNA was then recloned into the pFastBac Hta vector for expression of polyhistidine-tagged (6His) PKC-ε using NcoI and KpnI, 5’-3’ respectively. The NcoI digest cleaved just downstream of the 5’ untranslated region allowing for the correct fusion insertion into the pFastBac HTa vector, according to the manufacturer’s instructions (Life Technologies, Inc.). To optimize efficiency of protein production, Sf9 cells were conditioned to grow in suspension using serum-free growth media SF-900 II SFM (Life Technologies, Inc.). Sf9 cells harboring PKC-δ or PKC-ε (v3/PKC-δ, v3/PKC-ε) were injected at 0.7% and subsequently stored at −70 °C in storage buffer (26) containing 16% glycerol (v/v). Each enzyme preparation was analyzed using SDS-PAGE and protein staining with Coomassie Brilliant Blue, immunoblotting using isozyme-specific antisera with recombinant PKC-ε and PKC-δ standards, and quantitated using the Bio-Rad protein assay.

**Deletion Mutagenesis**—Polymerase chain reaction (PCR)-based deletion mutagenesis was performed on p3/PKC-ε in order to generate a PKC-ε mutant lacking amino acid residues 222–230 that is designated ΔPKC-ε222–230. Forward and reverse primers (5’-CTCGAGCAGTTGGT-GCTCC-3’ and 5’-AGCCGACTTTGTAATAATGAGC-3’, respectively) were used with Vent DNA polymerase (New England Biolabs, Beverly, MA) for 20 cycles and the PCR product was gel purified and circularized by blunt-end ligation. Clones were screened by PCR amplification using the primers (5’-TCATAAGGCACAAAGGTTGAGC-3’ and 5’-AGCA-TGTTGCTTCATCAT-3’, respectively). DNA sequencing, using the Sequenase 7-deaza-dGTP DNA Sequencing kit (U. S. Biochemical Corp., Cleveland, OH), confirmed deletion of amino acids 222–230 and the absence of any secondary mutations being introduced as a result of the PCR amplification. ΔPKC-ε222–230 was subcloned from this plasmid (p5ΔPKC-ε222–230) into pFastBac HTa using the same strategy as described in the previous section to give p8/ΔPKC-εHT. The ΔPKC-ε222–230 cDNA (p5ΔPKC-ε222–230 and p8/ΔPKC-εHT) were then expressed in Sf9 cells, and purified, as described above. In addition, ΔPKC-ε222–230 (without the 6His tag) was subcloned from p5ΔPKC-ε222–230 into pLXSN to allow its expression in mammalian cells.

**Generation of Overexpressing Cell Lines**—NIH 3T3 fibroblasts were purchased from the American Type Culture Collection (Rockville, MD) and infected with pLXSN recombinant retrovirus or pLXSN harboring the genes for p3/PKC-ε or p5/ΔPKC-ε222–230. Plasmid pLXSN constructs were transfected into the amphotrophic packaging line PAA317 using Lipofectin and selecting with 400 μg/ml G418. The titer of the resulting retrovirus was amplified by serial passage between the p2 and pAA317 packaging cell lines (26). The 3T3 cells were infected with NIH 3T3 cells as described in Ref. 27. Stably expressing cell lines were selected and cloned by limiting dilution (28) in 500 μg/ml G418 to yield 16 subclones derived from single cells. The PKC-ε and ΔPKC-ε222–230 overexpressing NIH 3T3 cells were screened for PKC protein expression by Western blot analyses and representative clones were used for immunofluorescence studies.

**Immunofluorescence Microscopy**—Indirect immunofluorescence localization was performed as described in Ref. 29. Where specified, vector controls and PKC-ε overexpressing NIH 3T3 clones were treated with 100 nM phorbol 12-myristate 13-acetate (PMA) for 10 min. Cells were rinsed twice with phosphate-buffered saline, fixed with 4% paraformaldehyde in phosphate-buffered saline for 30 min, washed twice with phosphate-buffered saline containing 0.1 M glycine, and permeabilized with blocking buffer (phosphate-buffered saline containing 1% bovine serum albumin, and 2% normal goat serum, and 0.4% saponin) for 5 min. Cells were incubated with either anti-PKC-ε (diluted 1:500 for vector transfected cells or 1:2,000 for PKC-overexpressors) or anti-mannosidase II (diluted 1:500) in blocking buffer for 1 h, washed five times, and then incubated with an appropriate secondary antibody (fluorescein isothiocyanate-conjugated anti-rabbit IgG when costained with Texas red-conjugated phallolidin or Texas red-conjugated anti-mouse IgG when costained with anti-mannosidase II monoclonal antibody) in blocking buffer for 2 h.

**Actin Binding**—Actin binding assays were performed in Aβp buffer (4 mM Tris-HCl, pH 7.5, containing 10 μM CaCl2, 30 mM KCl, 120 mM potassium proprionate, 1 mM MgCl2, 150 μM ATP, and 100 μM ZnCl2) as described previously (13). Actin binding curves were obtained for each gel and used to estimate the percentage of Aβp-dependent codestimation (Aβp + PKC-ε). Where indicated MBS-actin was added to Aβp at the specified mass ratios. Samples were incubated for 15 min at room temperature in the presence of either 500 nM 4β-PDBu or an equimolar concentration of the inactive phorbol ester, 4c-PDBu, unless otherwise specified. Phospholipids were not
FIG. 1. Cosedimentation of A<sub>f</sub> and PKC-ε in the presence of MBS-actin and high salts. Panel A, recombinant full-length PKC-ε was coincubated with 500 nM 4<sup>-PDBu</sup> and the specified mass ratio of A<sub>f</sub> and MBS-stabilized monomeric actin. After sedimentation, actin pellets were analyzed by SDS-PAGE and immunoblotting for bound PKC-ε. The data reported are the mean ± S.E. of three independent experiments. Panel B, A<sub>f</sub> (40 μg) and PKC-ε (0.35 μg) were coincubated for 30 min in the presence of 500 nM 4<sup>-PDBu</sup> before adding KC1 to the specified final concentration. After incubating for an additional 5 min, all samples were sedimented and analyzed as above. The data reported are the mean ± S.E. of four independent experiments.

FIG. 2. Analysis of A<sub>f</sub> cosedimentation with tryptic fragments of PKC-ε. Panel A, native cytosolic PKC-ε was partially digested with trypsin producing one N-terminal fragment (left inset, lane 1) and two C-terminal fragments (right inset, lane 1) of PKC-ε (see arrowheads on left). Tryptic PKC-ε fragments that cosedimented with purified A<sub>f</sub> in the absence or presence of the synthetic peptide LKKQET (40 μM), were detected by Western blot analysis using antisera specific for either the N terminus (left inset, lane 2 versus 3) or C terminus (right inset, lane 2 versus 3). Panel B, A<sub>f</sub> cosedimentation assays performed using cytosolic PKC-ε in the presence of a specified concentration of a given synthetic peptide (amino acid sequence given by the single letter designation). The data reported are the mean ± S.E. of three independent experiments.
possibly due to relatively low affinity interactions between this enzyme and MBP-Ac. Similar competition studies, performed using synthetic peptides and cytosolic PKC-ε, indicate that hydrophilic forces may contribute to the high affinity interactions between PKC-ε and Aα (13). To more systematically examine the nature of this protein-protein interaction, Aα cosedimentation assays were performed using purified PKC-ε in the presence of either Triton X-100 or salt (KCl). While increasing concentrations of Triton X-100 (0.01–1.0%, v/v) had no effect on the amount of PKC-ε that cosedimented with purified Aα (data not shown), high concentrations of salt completely disrupted this protein-protein interaction (IC50 = 375 mM, Fig. 1B).

The C-terminal Half of PKC-ε (Residues 320–785) Does Not Cosediment with Aα—Limited trypsinization of PKC-ε results in the formation of three proteolytic fragments that can be separated by SDS-PAGE (Fig. 2A, lane 1, of left and right panels). These tryptic cleavage sites have been mapped to the hinge region of PKC-ε and shown to yield one N-terminal (1–319) and two C-terminal (320–785 and 364–785) fragments of the holoenzyme (32). When these tryptic fragments of PKC-ε were incubated with Aα in the presence of 500 nM 4β-PDBu, only undigested PKC-ε and its N-terminal fragment (PKC-ε1–319) cosedimented with Aα (Fig. 2A, lane 2 of left panel). It was also apparent that both PKC-ε and PKC-ε1–319 had become more concentrated in the Aα pellets (compare lanes 1 and 2 of Fig. 2A, left panel) and that this interaction was effectively reversed by the addition of a synthetic peptide that is identical to the PKC-ε putative actin-binding motif (LKKQET; Fig. 2A, lane 3 of left panel). Neither C-terminal peptide (PKC-ε320–785 or PKC-ε364–785) could be detected using anti-C-terminal antibodies in the same actin pellets (Fig. 2A, lane 2 of right panel). Additional Aα cosedimentation assays indicated that the synthetic hexapeptide LKKQET competes with intact, cytosolic, PKC-ε for Aα binding in a relatively specific manner. In these assays, 4β-PDBu (500 nM) increased the binding of PKC-ε to Aα by more than 3-fold (Fig. 2B, inset) and increasing concentrations of the synthetic peptide LKKQET completely inhibited this protein-protein interaction (Fig. 2B). Scrambling the amino acid sequence of this synthetic peptide to QKLKTE resulted in a loss of inhibition and a synthetic peptide reported to inhibit the binding of PKC-ε to the PKC anchoring protein RACK1 (DIINALCF; see Ref. 33) also failed to interfere with the cosedimentation of PKC-ε and Aα (Fig. 2B). Yet another synthetic peptide that proved to be ineffective in these competition assays (EAVSLKPT; data not shown) was identical to PKC-ε222–230 and reportedly inhibits the association of activated PKC-ε with cross-striated structures in saponin-permeabilized cardiomyocytes (10).

Identification of an Actin Binding Motif in PKC-ε—We expressed in Sf9 cells the full-length PKC-δ and PKC-ε, as well as a deletion mutant of PKC-ε (ΔPKC-ε222–230), that lacks a sequence motif previously implicated in the in vitro binding of PKC-ε to Aα (13). These kinases and Aα were purified from Sf9 cell lysates and rabbit skeletal muscle, respectively, to greater than 85% homogeneity (Fig. 3A). Although the nonapeptide deleted from ΔPKC-ε222–230 is located between the C1A and C1B phorbol ester-binding domains of this protein (34), this mutation did not significantly alter the specific binding of [3H]PDBu to PKC-ε in the absence or presence of phosphatidylserine (Fig. 3B). Intact and mutated PKC-ε bound to [3H]PDBu, only undigested PKC-ε and shown to yield one N-terminal (1–319) and two C-terminal (320–785 and 364–785) fragments of the holoenzyme (32). When these tryptic fragments of PKC-ε were incubated with Aα in the presence of 500 nM 4β-PDBu, only undigested PKC-ε and its N-terminal fragment (PKC-ε1–319) cosedimented with Aα (Fig. 2A, lane 2 of left panel). It was also apparent that both PKC-ε and PKC-ε1–319 had become more concentrated in the Aα pellets (compare lanes 1 and 2 of Fig. 2A, left panel) and that this interaction was effectively reversed by the addition of a synthetic peptide that is identical to the PKC-ε putative actin-binding motif (LKKQET; Fig. 2A, lane 3 of left panel). Neither C-terminal peptide (PKC-ε320–785 or PKC-ε364–785) could be detected using anti-C-terminal antibodies in the same actin pellets (Fig. 2A, lane 2 of right panel). Additional Aα cosedimentation assays indicated that the synthetic hexapeptide LKKQET competes with intact, cytosolic, PKC-ε for Aα binding in a relatively specific manner. In these assays, 4β-PDBu (500 nM) increased the binding of PKC-ε to Aα by more than 3-fold (Fig. 2B, inset) and increasing concentrations of the synthetic peptide LKKQET completely inhibited this protein-protein interaction (Fig. 2B). Scrambling the amino acid sequence of this synthetic peptide to QKLKTE resulted in a loss of inhibition and a synthetic peptide reported to inhibit the binding of PKC-ε to the PKC anchoring protein RACK1 (DIINALCF; see Ref. 33) also failed to interfere with the cosedimentation of PKC-ε and Aα (Fig. 2B). Yet another synthetic peptide that proved to be ineffective in these competition assays (EAVSLKPT; data not shown) was identical to PKC-ε222–230 and reportedly inhibits the association of activated PKC-ε with cross-striated structures in saponin-permeabilized cardiomyocytes (10).

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DBu with a $K_d$ of 2.3 ± 0.6 nM ($n = 3$) in the presence of phosphatidylinerse (Fig. 3B, left saturation curves). Phosphatidylinerse was required for the high affinity binding of $[^3H]$PDBu (10 nM) to both PKC-ε and ΔPKC-ε222–230, as the omission of this phospholipid decreased specific $[^3H]$PDBu binding by about 90% (Fig. 3B). The ability of ΔPKC-ε222–230 to bind Aγ was measured using cosedimentation assays and compared with that of the full-length recombinant PKC-ε. Recombinant

**Fig. 4. Immunofluorescent analysis of NIH 3T3 cell lines overexpressing PKC-ε or ΔPKC-ε222–230 recombinant proteins.** Cells that overexpress full-length PKC-ε (A-D) or ΔPKC-ε222–230 (E-H) were simultaneously costained for PKC-ε (left panels) and filamentous actin (right panels) after incubating for 10 min in the absence (A, B, E, and F) or presence (C, D, G, and H) of 100 nM PMA, as described under “Experimental Procedures.” White bar indicates 20 μm.
PKC-δ was also used as a negative control, since it has high sequence homology with PKC-ε (66% similarity and 45% identity) but does not contain the consensus actin-binding motif (13, 35). PKC-δ did not co-exist with Aβ in the present experiments, in the absence or presence of 4β-PDBu (Fig. 3C). In contrast, treatment with 4β-PDBu resulted in more than a 7-fold enhancement of PKC-ε binding to Aβ (Fig. 3C). Interestingly, the PKC inhibitor GF109203X (1 μM) had no effect on this protein-protein interaction (data not shown). While ΔPKC-ε(222–230) consistently displayed a weak association with Aβ (0.063 ± 0.008 PKCbound/PKCTotal), this interaction was not enhanced by increasing concentrations of 4β-PDBu (Fig. 3C). Fig. 3D shows a more extensive comparison of the Aβ binding properties of PKC-ε to those members of the PKC family having the highest degree of overall sequence identity to PKC-ε (PKC-δ and -η) or that have previously been reported to associate with Aβ (PKC-βII and -ζ). Only recombinant PKC-ε and ΔPKC-ε(222–230) reliably cosedimented with Aβ under the in vitro conditions used in these experiments (Fig. 3D).

Colocalization of Activated PKC-ε and Actin in PKC-ε Overexpressing NIH 3T3 Cell Lines—Immunofluorescence microscopy was used to examine the redistribution of activated PKC-ε in G418-resistant NIH 3T3 clonal derivatives overexpressing either the intact enzyme or ΔPKC-ε(222–230). Wild-type NIH 3T3 cells and cells transfected with an empty vector did not reliably stain for PKC-ε (not shown), due to a low level of expression for the endogenous isozyme (18). Staining of nonstimulated and PMA-treated PKC-ε overexpressing NIH 3T3 cells is shown in Fig. 4, A and C, respectively. The cellular morphology and distribution of PKC-ε staining was reminiscent of that previously reported (18). Staining for PKC-ε was diffuse throughout the cytoplasm of unstimulated cells, where it showed a punctate pattern that extended from around the nucleus toward the cell periphery and into long cytoskeletal extensions (Fig. 4A, arrow). In these resting cells, there was a convincing superimposition of PKC-ε and Aβ, obtained using phallolidin-Texas Red (Fig. 4, compare A and B), within the long cellular processes that were frequently observed. A small portion of PKC-ε also appeared to colocalize with a mannansidase II-rich component of the Golgi (data not shown). These observations support the conclusion that PKC-ε is capable of binding to both skeletal Aβ in vitro and nonmuscle Aβ in cultured fibroblasts. After 10 min in 100 nM PMA, the long cytoskeletal extensions of PKC-ε overexpressing cells retracted as the cells flattened out and displayed prominent ruffling of the plasma membrane. We observed a dramatic redistribution of activated PKC-ε and Aβ to the cell margins, typically in areas of cell-cell contact, and dissolution of actin stress fibers (Fig. 4, C and D). In contrast, clones overexpressing ΔPKC-ε(222–230) showed a predominantly perinuclear staining pattern in the absence of PMA that was not associated with Aβ (Fig. 4, compare E and F). Upon PMA treatment, ΔPKC-ε(222–230) became more diffusely distributed throughout the cell, where it showed a coarse punctate texture that was not associated with Aβ (Fig. 4, compare G and H). Moreover, membrane ruffling and the dissolution of actin stress fibers was no longer apparent in ΔPKC-ε(222–230) overexpressing fibroblasts, although cortical Aβ staining did appear to become more intense in the presence of PMA (Fig. 4H).

Aβ Binding Increases the Proteolytic Sensitivity of PKC-ε—We have hypothesized that hydrophilic interactions between Aβ and the C1 region of PKC-ε may anchor this kinase in an active conformation within microfilamentous structures (13). To investigate this possibility, we probed the topology of PKC-ε by performing protease sensitivity assays. The hinge region of PKCs becomes markedly more susceptible to trypsinization through a conformational change that accompanies activation (31). Indeed, the susceptibility of cytosolic PKC-ε to trypsinic cleavage was increased by roughly an order of magnitude in the presence of 500 nM 4β-PDBu (Fig. 5B). Digestion with increasing concentrations of trypsin resulted in three proteolytic fragments of PKC-ε of the predicted molecular masses (Figs. 2A and 5A). Here we report that the pattern of PKC-ε proteolysis observed in the presence of purified Aβ was essentially equivalent to that observed in the presence of 4β-PDBu (Fig. 5B), consistent with an Aβ-induced exposure of the PKC-ε hinge region.

Purified Aβ Is Sufficient to Stimulate Recombinant PKC-ε Phosphotransferase Activity—Although provocative, the equivocal nature of protease accessibility assays makes the use of independent assays obligatory. For this reason, we performed phosphotransferase assays to directly examine the binary interactions between purified Aβ and recombinant PKC-ε, in the complete absence of any classical PKC activators. Titration of Aβ, in the presence of PKC-ε (20 ng) and the substrate peptide myelin basic protein fragment 4–14 (MBP4–14), showed that Aβ physically interacts with this kinase to produce a 4-fold increase in kinase activity (Fig. 6A). Maximal in vitro activity

![Figure 5](image-url)
substrate preference of this kinase: AF-induced PKC-phorylation of these substrates by PKC-
level of maximal kinase activity produced by AF alone (Fig. 6 using a HCl) as substrates. While AF significantly enhanced the phosphotransferase activity in the absence and presence of AF (Fig. 6).

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occurred at an AF concentration of approximately 20 μg/ml (Fig. 6A, EC50 = 4.7 μg/ml). Importantly, the addition of 500 nM 4β-PDBu to this protein mixture significantly increased the level of maximal kinase activity produced by AF alone (Fig. 6A). This additive effect of 4β-PDBu on the Vmax of recombinant PKC-epsilon may reflect the existence of two independent mechanisms for activation and this possibility merits further investigation. In parallel assays, it was confirmed that purified AF was not an effective agonist of recombinant PKC-beta1 when measured using MBP4-14 as a substrate in the absence (Fig. 6A) or absence of 4β-PDBu and Ca2+ (Ref. 36 and data not shown).

Competition assays performed using synthetic peptides identical to PKC-epsilon pseudosubstrate peptide and MBP4-14 indicated that AF interacts with the former hexapeptide motif to stimulate PKC-epsilon phosphotransferase activity (Fig. 6B). As previously mentioned (10), an octapeptide derived from PKC-epsilon C-terminal of AF proved to be essential for AF-induced PKC-epsilon activity and it is shown here that equimolar concentrations of rVI-RACK1234-242 (DINALCF) was also an ineffective antagonist. The effect of AF-binding on the substrate specificity of PKC-epsilon was examined because chimeric studies have shown that the N-terminal regulatory domain of PKC-epsilon restricts the substrate specificity of this isozyme (37) and AF has been reported to disrupt the ability of PKC-epsilon to phosphorylate a variety of substrates (36). In the present study, we measured PKC-epsilon phosphotransferase activity in the absence and presence of AF using two synthetic peptides (δ peptide and MBP4-14) and three purified proteins (histone III, calponin, and protamine HCl) as substrates. While AF significantly enhanced the phosphorylation of these substrates by PKC-epsilon, it had no effect on the substrate preference of this kinase: δ peptide > protamine > MBP4-14 > histone III > calponin (Fig. 6C).

Interaction of PKC-epsilon with the C Terminus of Actin—The flexible C terminus of α-actin contains the four cleavage sites most accessible to proteolysis (25). Here we have used limited tryptic digestion to selectively remove the C terminus dipeptide of AF (Cy374-Phe375) to examine the importance of the C terminus in interactions with PKC-epsilon. The minimal time required for tryptic cleavage of the Lys373-Cys374 bond was established by monitoring the disappearance of AEDANS, a fluorescent stain known to specifically label Cy374 of AF (Fig. 7A). Equivalent amounts of PKC-epsilon co sedimented with intact and truncated AF (Fig. 7B). In contrast, the C-terminal of AF proved to be essential for the AF-induced stimulation of PKC-epsilon phosphotransferase activity (Fig. 7C). Finally, titrating the ratio of truncated to intact AF gradually led to a complete reversal of PKC-epsilon stimulation by intact AF (Fig. 7D).

DISCUSSION

A recurring theme in topological biochemistry is that cytosolic protein kinases may be recruited to perform distinct functions based on the localization signals they have acquired and their microenvironment at the time of activation. Thus, spatio-temporal gradients of required cofactors may activate multiple PKC isozymes within a common subcellular domain and further select for the isozyme(s) capable of anchoring to a cognate binding partner. It is presently understood that the resulting segregation of PKC isozymes directs the assembly of a coherent signaling apparatus in which the kinase(s) becomes colocalized with its intended substrates. Here we report two substantive findings that should contribute to continuing advances in our understanding of such isozyme-specific PKC signaling cascades. First, AF recognizes and binds directly to a hexapeptide motif that is unique to the regulatory C1 domain of PKC-epsilon. Second, physical interactions between AF and PKC-epsilon are dynamic and capable of stimulating PKC-epsilon kinase activity in the absence of lipid metabolites. It has previously been demonstrated that the activation of PKC-epsilon exposes an actin binding motif that binds AF and that a hexapeptide derived from the C1 domain of PKC-epsilon competitively inhibits this protein-protein interaction (13). Because several actin-binding proteins have conserved homologous peptide motifs (35), we hypothesized that this cluster of highly charged amino acids in PKC-epsilon may function as an actin-binding site. While putative PKC localization signals have commonly been deduced from such peptide competition studies (10, 36, 40) or binding assays using fragments of the full-length isozyme (16, 17, 41), the limitations inherent to each experimental approach argue in favor of circumspection. Here we have used PCR-based mutagenesis to delete the putative actin binding motif in PKC-epsilon in order to more directly examine its functional importance for the in vitro interactions between PKC-epsilon and AF. Our data suggest that the interface between PKC-epsilon and AF was

FIG. 6. Effects of actin on PKC phosphotransferase activity and substrate preference. Panel A, recombinant PKC-beta1 or -epsilon was preincubated with the substrate peptide MBP4-14 and the concentration of AF indicated in the presence of either 500 nM 4β-PDBu or 4β-PDBu. Phosphotransferase activity was initiated by addition of [γ-32P]ATP. Data shown represent the mean ± S.E. of three independent experiments. Curve fitting was performed using Sigma Plot Scientific Graphing Software 2.0 (Jandel Scientific, Inc.). Panel B, PKC-epsilon phosphotransferase activity was measured in the presence of 500 nM 4β-PDBu, AP (100 μg/ml), MBP4-14 (200 μg/ml), and increasing concentrations of either a hexapeptide (LKKQET) or octapeptide (DIINALCF) sequence derived from PKC-epsilon pseudosubstrate peptide and are the averages of two independent experiments. Panel C, recombinant PKC-epsilon phosphotransferase activity measured in the presence (control) or presence of AP (100 μg/ml) and 200 μg/ml of the specified substrate. Data are expressed as a percent of the maximal activity measured using a δ-pseudosubstrate peptide and are the averages of two independent experiments.
PKC-ε, the disparity in the amounts of recombinant PKC-ε previously been reported to bind actin (36, 42). In this respect, the mean activity was measured in the presence of 4β-PDBu, MBP4–14, 100 μg/ml intact A_F, and varying concentrations of truncated actin. Data are the mean ± S.E. of at least three independent experiments.

Panel D, PKC-ε phosphotransferase activity was measured in the presence of 4β-PDBu, MBP4–14, 100 μg/ml intact A_F, and varying concentrations of truncated actin. Data are the mean ± S.E. of at least three independent experiments.

Protein-protein interactions. In the case of PKC-ε, low affinity interactions with A_F were detected in the absence of phorbol esters using the deletion mutant ΔPKC-ε222–230. In contrast, the physical association of A_F with the intact enzyme was of a higher affinity (13) and significantly enhanced in the presence of 4β-PDBu. These findings suggest that interactions between A_F and its cognate binding site in PKC-ε may be positively regulated by allosteric interactions between diacylglycerol and the C1 homology domains of PKC-ε. The finding that arachidonic acid synergistically interacts with diacylglycerol to promote A_F binding to PKC-ε (13) is consistent with this model.

Multiple localization signals have been tentatively identified in the PKC-ε sequence (10, 11, 13, 16, 17). Two of these signal motifs have been mapped to the C1 domain of PKC-ε, the actin binding motif discussed here and a Golgi localization signal that reportedly dominates all other localization signals when the enzyme is in an inactive conformation (16). Importantly, deletion of the actin binding motif did not alter the Golgi localization signal that has been observed in this protein overexpressing NIH 3T3 cells (13).

While only vertebrate homologues of PKC-ε contain the consensus actin binding motif LKX2EX, both PKC-δ1 and -ζ have previously been reported to bind actin (36, 42). In this respect, the disparity in the amounts of recombinant PKC-δ1, -ε, and -ζ that cosedimented with A_F in a physiologically relevant buffering system, was unexpected and striking. It seems likely that the evolution of PKC actin binding motifs may have emerged, in both vertebrates and invertebrates, through divergent pathways and that distinct cofactors are required to support these protein-protein interactions. In the case of PKC-ε, low affinity interactions with A_F were detected in the absence of phorbol esters using the deletion mutant ΔPKC-ε222–230. In contrast, the physical association of A_F with the intact enzyme was of a higher affinity (13) and significantly enhanced in the presence of 4β-PDBu. These findings suggest that interactions between A_F and its cognate binding site in PKC-ε may be positively regulated by allosteric interactions between diacylglycerol and the C1 homology domains of PKC-ε. The finding that arachidonic acid synergistically interacts with diacylglycerol to promote A_F binding to PKC-ε (13) is consistent with this model.

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dominant localization signal when PKC-ε is activated and that Aβ-PKC-ε interactions may play an important role in regulating normal cell-cell as well as cell-substrate cohesion. A potential role of PKC-ε in regulating cell adhesion has recently been highlighted by the finding that this isozyme becomes selectively activated in HeLa cells during spreading (7). PKC activation has also been shown to be required to initiate fibroblast spreading on a fibronectin substrate (38) and to precede actin polymerization in the adhesion of HeLa cells to a gelatin substrate (39).

The finding that Aβ binding makes PKC-ε substantially more vulnerable to proteolytic degradation raised the possibility that this protein-protein interaction may lead to relief of the inhibition exerted by the pseudosubstrate domain on the kinase domain. On a structural level, it remains to be convincingly demonstrated that Aβ is actually capable of disrupting this interdomain interaction. However, it has now been established that Aβ is sufficient to stimulate PKC-ε phosphotransferase activity without drastically altering its substrate specificity. In contrast, Aβ apparently inhibits the phosphorylation of PKC-βII substrates by promoting the autophosphorylation of one or more Ser/Thr residues in this conventional isozyme (36). Preliminary evidence indicates that the C terminus of actin may play an important role in the Aβ-induced stimulation of PKC-ε activity, although this remains to be proven since removal of the C-terminal dipeptide of actin can cause significant changes in the overall topology of actin filaments (20). The present results, together with our previous studies (13), indicate that Aβ may be a bifunctional anchoring protein that maintains PKC-ε in an active conformation within cytoskeletal structures and appear to be assembled during cell spreading. Further studies involving a comparison of PKC-ε and ΔPKC-ε222-230 overexpressing NIH 3T3 cell lines should reveal whether Aβ-PKC-ε interactions participate in the regulation of cell adhesion or the oncogenic cascade that is induced by overexpressing this gene in fibroblasts (5).

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