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Phorbol ester binding to protein kinase C requires a cysteine-rich zinc-finger-like sequence

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ABSTRACT **Protein kinase C normally has a tandem repeat of a characteristic cysteine-rich sequence in C_1, the conserved region of the regulatory domain. These sequences resemble the DNA-binding zinc finger domain. For the γ subspecies of rat brain protein kinase C, various deletion and point mutants in this domain were constructed, and the mutated proteins were expressed in *Escherichia coli* by using the T7 expression system. Radioactive phorbol 12,13-dibutyrate binding analysis indicated that a cysteine-rich zinc-finger-like sequence was essential for protein kinase C to bind phorbol ester and that one of the two sequences was sufficient for the phorbol ester binding. Conserved region C_2, another region in the regulatory domain, was apparently needed for the enzyme to require Ca^{2+} for phorbol ester binding activity.**

Protein kinase C (PKC) has attracted much attention since it plays a pivotal role in signal transduction for activation of many cellular functions and for control of cell proliferation (1, 2). The enzyme consists of multiple subspecies that are structurally close to, but clearly distinct from, one another (3–13). Subspecies of PKC (α, βI, βII, and γ) are single polypeptides with four conserved (C_1–C_4) and five variable (V_1–V_5) regions (2, 4). The amino-terminal half of each polypeptide, containing regions C_1 and C_2, is the regulatory domain that interacts with Ca^{2+}, phospholipid, and diacylglycerol or phorbol ester; the carboxyl-terminal half, containing regions C_3 and C_4, constitutes the catalytic domain. The C_1 region has a tandem repeat characteristic of a sequence that resembles the cysteine-rich zinc-finger motif. Such a sequence motif has been identified in many DNA binding proteins that appear to be active in transcriptional regulation (14, 15). However, there is presently no indication that PKC interacts with nucleic acids, and hence no information is available as to the function of this cysteine-rich sequence. By using various deletion and point mutants of the γ subspecies of rat PKC expressed in *Escherichia coli*, the studies described herein will show that the cysteine-rich zinc-finger-like sequence is indispensable for the binding of phorbol ester to the enzyme.

MATERIALS AND METHODS

Construction of Expression Plasmids. Each mutant was constructed as shown in Fig. 1. The cDNA fragment encoding amino acids 1–373 of the γ subspecies of rat PKC was obtained from its cDNA clone of λCKRγ1 (10) by digestion with *Nco* I and *Bam*HI. The cohesive ends of this fragment were altered to *Bam*HI sites by *E. coli* DNA polymerase I (large fragment) and *Bam*HI linkers (8 bases). It was cloned into the *Bam*HI site of the T7 RNA polymerase-dependent expression vector pET3c (16, 17), forming an in-frame fusion product with the first 11 amino acids of the T7 gene *10* product. This plasmid, designated pTB965, could express 389 amino acid residues because of the addition of 16 residues derived from T7 and linkers. Plasmid pTB967 was constructed by insertion of a *Bam*HI–*Sau*3A fragment, encoding amino acids 1–173 of the C_1 region of PKC from plasmid pTB965 into the *Bam*HI site of pET3c. This plasmid encoded 207 amino acids derived from the PKC, T7, and linkers. Plasmid pTB965 was digested with *Stu* I, blunt-ended by T4 DNA polymerase, and, after ligation of 10 bases of *Bam*HI linker, digested with *Bam*HI. The DNA fragment encoding amino acids 161–373 of the C_2 region was obtained and inserted into the *Bam*HI site of pET3c, and the resultant plasmid, designated pTB968, encoded a fused protein of 229 amino acid residues. Plasmid pTB969 was constructed to express the PKC region that contained the second but not the first cysteine-rich zinc-finger-like sequence by insertion of an *Apa* I fragment encoding amino acids 93–317. The cohesive ends of *Apa* I sites were blunted by T4 DNA polymerase and altered to *Bam*HI sites with 8 bases of *Bam*HI linker. The resulting fragment was cloned into the *Bam*HI site of pET3a (17) to express 260 amino acid residues as a fused protein. Plasmid pTB971 was constructed by deletion of the *Apa* I fragment from plasmid pTB965 and encoded a fused protein of 162 amino acid residues containing the first cysteine-rich sequence.

Plasmids pTB966, pTB970, and pTB972, with point mutations, were made by the *in vitro* site-directed mutagenesis kit supplied by Amersham. The primer of 5′-CAATCTCAAGTCTCCAGCCTTT was designed to replace the two cysteine codons in the first finger-like sequence with two serine codons, and 5′-AAAAGTTCCTGTAGCGAAATG was used for the substitution of the two cysteines in the second finger-like sequence.

Expression in *E. coli*. Each expression plasmid was introduced to *E. coli* BL21(DE3)/pLysS, a strain carrying a stable integrant of the T7 gene *1* (RNA polymerase) under the control of the *lacUV5* promoter. T7 RNA polymerase was induced at an A_{600} of 0.5 unit in cultures by the addition of isopropyl β-D-thiogalactopyranoside to the final concentration of 0.5 mM. The cells were harvested after 3 hr of induction.

Immunoblot Analysis. The culture (1 ml) was centrifuged, and the cell pellet was resuspended in 300 μl of 2× $NaDodSO_4$ sample buffer [0.1 M Tris·HCl, pH 6.8/20% (vol/vol) glycerol/4% (wt/vol) $NaDodSO_4$/5% (vol/vol) 2-mercaptoethanol/0.02% bromophenol blue], and a 10-μl aliquot was electrophoresed on a $NaDodSO_4$/17% polyacrylamide gel according to the method of Laemmli (18). Proteins were stained with Coomassie brilliant blue or transferred electrophoretically to a nitrocellulose filter (19). Polyclonal antibodies, CKpC1β-a and CKpC2/V3α-a, were prepared against synthetic polypeptides, Phe-Ala-Arg-Lys-Gly-Ala-

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Abbreviations: PKC, protein kinase C; PBt_2, phorbol 12,13-dibutyrate.

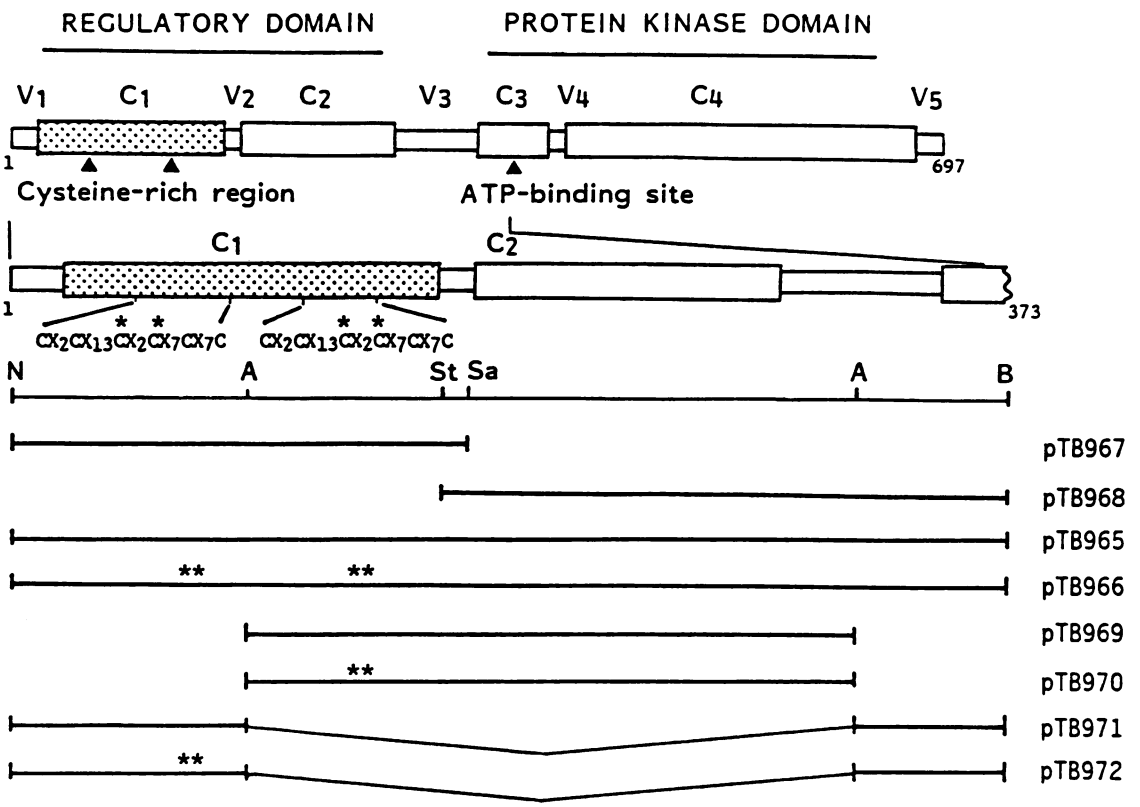

FIG. 1. Mutated PKC cDNAs. Various mutated cDNAs of the γ subspecies of rat PKC were constructed. The whole structure of the PKC molecule is shown for reference at the top. C_1–C_4 and V_1–V_5 indicate the conserved and variable regions, respectively. Figures on the left and right sides of the structure indicate the numbers of deduced amino acid residues. A tandem repeat of the characteristic cysteine-rich sequences, which resemble the cysteine-rich zinc-finger motif, is also shown, where C is cysteine and X is any amino acid. Asterisks in the finger-like sequences indicate the positions of the cysteine residues that were replaced by serine residues in each point mutant indicated. The cleavage sites of the cDNA with the restriction enzymes, which were used to construct the deletion mutants, are indicated as follows: N, *Nco* I; A, *Apa* I; St, *Stu* I; Sa, *Sau*3A; and B, *Bam*HI.

Leu-Arg-Gln-Lys-Asn-Val-His-Glu-Val-Lys-Asn-His-Lys-Phe and Leu-Leu-Asn-Gln-Glu-Gly-Glu-Tyr-Tyr-Asn-Val-Phe-Ile-Phe-Glu, respectively, which correspond to the C_1 region of amino acids 20–39 deduced from the β cDNA clone (5) and the C_2/V_3 region of amino acids 277–292 from the α cDNA clone (10), respectively. These antibodies recognize α, βI, βII, and γ subspecies (20). Immunoblot analysis was carried out by the immunoblotting system supplied by Promega Biotec using a mixture of the polyclonal antibodies, CKpC1β-a and CKpC2/V3α-a, and anti-rabbit second antibody conjugated to alkaline phosphatase (21).

Assay of Phorbol Ester Binding. After induction, the cells in 10 ml of culture were harvested, lysed by freezing and thawing in 300 μl of a buffer containing 20 mM Tris·HCl (pH 7.5), 0.25 M sucrose, 10 mM EGTA, 2 mM EDTA, 0.5% Triton X-100, and leupeptin (20 μg/ml), followed by sonication for 1 min. The homogenate was centrifuged for 20 min at 20,000 × g. The supernatant was diluted with 5 vol of buffer containing 20 mM Tris·HCl (pH 7.5) and 0.5 mM EDTA and assayed for the binding of phorbol 12,13-dibutyrate (PBt_2). The phorbol ester binding assay (22) was carried out in 300 μl of buffer containing 50 mM Tris·HCl (pH 7.5), bovine serum albumin (4 mg/ml), 10 nM [^{3}H]PBt_2 (14.3 Ci/mmol, 1 Ci = 37 GBq; Amersham), and 10 μl of crude cell extract in the presence or absence of phosphatidylserine (100 μg/ml) and 2 mM $CaCl_2$. After incubation at 30°C for 30 min, the bound [^{3}H]PBt_2 was separated from the free ligand by filtering through a Whatman GF/B glass fiber filter and washed with five 1-ml samples of ice-cold 20 mM Tris·HCl (pH 7.5). The nonspecific binding was determined under the same conditions with the addition of 10 μM unlabeled PBt_2. The specific phorbol ester binding activity was expressed as cpm per 10 μl of crude extract.

RESULTS AND DISCUSSION

When whole-cell proteins from *E. coli* harboring a cDNA construct were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis, an apparently new protein band was detected that was heavily stained with Coomassie brilliant blue (Fig. 2*a*). This protein migrated to the position corresponding to the expected molecular mass of the polypeptide encoded by the mutated cDNA. Immunoblot analysis confirmed that the new protein was recognized by specific anti-PKC antibodies (Fig. 2*b*). Similar results were obtained for all mutant proteins. The control *E. coli* extract did not contain any protein that reacted with the antibodies. The results indicate that the cDNAs were effectively transcribed by the T7 system and translated to produce the PKC-related polypeptides in the *E. coli* cells.

The next set of experiments was performed to examine the capability of the bacterial cell extracts to bind a radioactive phorbol ester. The results obtained are summarized in Table 1. The normal *E. coli* extract did not bind PBt_2. The cell extract containing the C_1 region alone, expressed from plasmid pTB967, showed PBt_2 binding activity, apparently similar to the cell extract having the whole regulatory domain expressed by plasmid pTB965. No binding activity was observed in the cell extract containing the C_2 region alone, expressed from plasmid pTB968. The results from cells expressing the fragments of PKC encoded by plasmids pTB969 and pTB971 showed that either one of the two

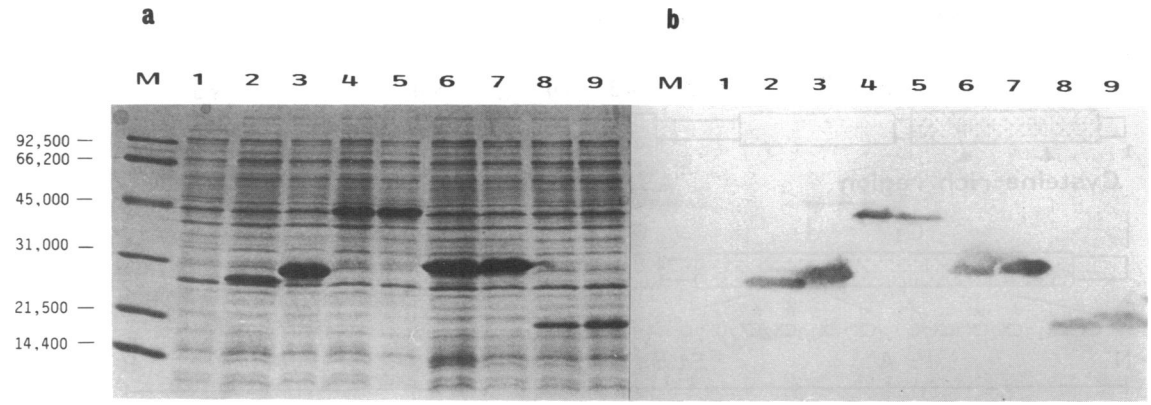

FIG. 2. Expression of various mutated cDNAs of the γ subspecies of rat PKC in *E. coli*. Each expression plasmid was introduced to *E. coli* and the bacterial cell lysates were subjected to immunoblot analysis after $NaDodSO_4$/polyacrylamide gel electrophoresis. (*a*) $NaDodSO_4$/polyacrylamide gel electrophoresis analysis. (*b*) Immunoblot analysis. Lanes: M, molecular weight standards; 1–9, lysates from the transformant with pET3c (control), pTB967, pTB968, pTB965, pTB966, pTB969, pTB970, pTB971, and pTB972, respectively.

cysteine-rich sequences in the C_1 region was sufficient for PBt_2 binding activity.

It has been reported that point mutations of the human estrogen receptor that replace two cysteine residues with histidine residues in its potential DNA-binding zinc finger prevent the receptor from activating transcription of the estrogen-inducible gene that contains the estrogen-responsive element (23). Also mutations in the zinc-finger region of the human glucocorticoid receptor abolish the affinity of the receptor for DNA (24). Thus, another series of experiments were carried out using site-directed mutagenesis of the C_1 region of PKC. The two cysteine residues in either one or both of the two zinc-finger-like sequences were replaced by a pair of serine residues as indicated, and the resulting three plasmids, pTB966, pTB970, and pTB972, were expressed in *E. coli*. None of these cell extracts showed PBt_2 binding activity (Table 1). Thus at least one of the cysteine-rich zinc-finger-like sequences is needed for binding of the tumor-promoting phorbol ester to PKC.

Although one of the two finger-like sequences appears to be sufficient for the binding of phorbol ester to the enzyme, kinetic properties of the binding vary significantly with the presence of another part of the regulatory domain. For instance, the polypeptide fragment containing the C_1 region alone (pTB967) could bind PBt_2 in the absence of added Ca^{2+} when phospholipid was present, while the polypeptide fragment containing both the C_1 and C_2 regions (pTB965) required Ca^{2+} as well as phospholipid (Fig. 3), as observed for native PKC (1). Possibly the C_2 region plays some role in the Ca^{2+} sensitivity of PKC, although this region has no obvious Ca^{2+}-binding EF hand structure (25). Consistent with this observation, three additional members of the PKC family, the δ, ε, and ζ subspecies, all of which lack the C_2 region, are insensitive to Ca^{2+} and show a significant catalytic activity in the absence of Ca^{2+} (11). The present studies do not exclude involvement of other parts of the regulatory domain in the activation of PKC, but it is clear that the cysteine-rich zinc-finger-like sequence is indispensable for binding of the tumor promoter, and presumably diacylglycerol, resulting in activation of the enzyme.

Table 1. PBt_2 binding activity in *E. coli* extracts from the transformant with expression plasmids

Sample	Specific PBt_2 binding activity, cpm
pTB965 (C_1 and C_2 regions)	9,630
pTB966 (point mutant from pT965)	40
pTB967 (C_1 region)	10,500
pTB968 (C_2 region)	0
pTB969 (second cysteine-rich sequence)	19,200
pTB970 (point mutant from pTB969)	90
pTB971 (first cysteine-rich sequence)	18,700
pTB972 (point mutant from pTB971)	0
pET3c (control)	0

Each expression plasmid was introduced to *E. coli*, and PBt_2 binding activity of the cell extracts containing the mutated PKC molecules was assayed in the presence of phosphatidylserine and $CaCl_2$.

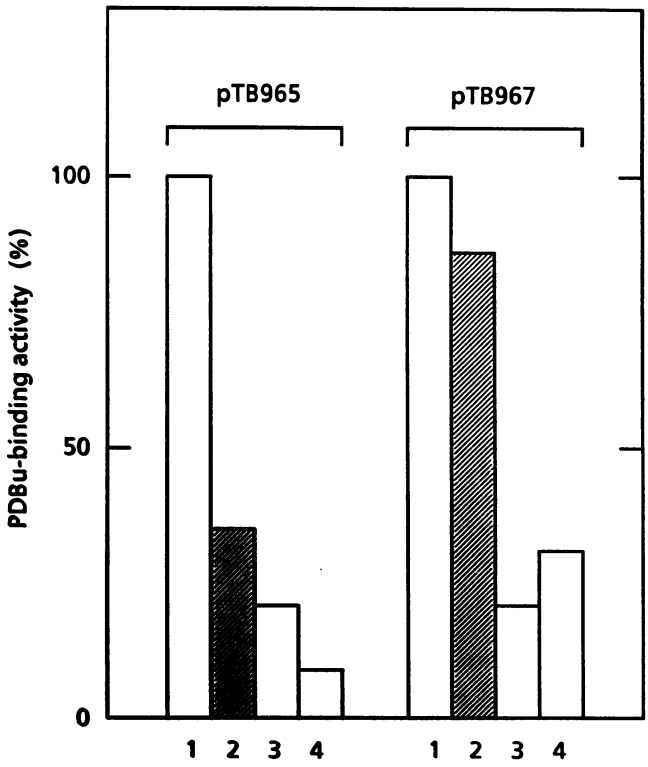

FIG. 3. Effects of Ca^{2+} and phospholipid on the phorbol ester binding activity of mutated PKC molecules. Expression plasmids pTB965 and pTB967 were introduced to *E. coli*. The PBt_2 (PDBu) binding activity in the cell extracts was assayed in the presence or absence of phosphatidylserine and $CaCl_2$. The values are the means of several independent experiments. Where indicated, EGTA (final 2 mM) was added instead of $CaCl_2$. Results were normalized to the maximal activity obtained in the presence of phosphatidylserine and $CaCl_2$. The PBt_2 binding activity in the presence of phosphatidylserine and EGTA was emphasized. Bars: 1, phosphatidylserine and $CaCl_2$; 2, phosphatidylserine and EGTA; 3, $CaCl_2$ without phosphatidylserine; and 4, EGTA without phosphatidylserine.

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