Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 6130–6134, July 1993
Biochemistry

Characterization of site-specific mutants altered at protein kinase C β_1 isozyme autophosphorylation sites

JI ZHANG, LYNN WANG, JOANNE PETRIN, W. ROBERT BISHOP*, AND RICHARD W. BOND

Molecular Pharmacology Section, Schering–Plough Research Institute, Kenilworth, NJ 07033-0539

Communicated by Mary Jane Osborn, March 19, 1993

ABSTRACT **The autophosphorylation sites of the β_2 isozyme of protein kinase C (PKC) were recently identified as Ser-16/Thr-17 near the NH_2 terminus, Thr-314/Thr-324 in the hinge region, and Thr-634/Thr-641 near the COOH terminus [Flint, A. J., Paladini, R. D. & Koshland, D. E. (1990) *Science* 294, 408–411]. To define the role of autophosphorylation we constructed three site-directed mutants of PKC β_1 isozyme in which each pair of phosphorylatable residues is changed to alanine. Wild-type PKC β_1 and the mutant proteins were transiently overexpressed in COS cells, resulting in at least a 20-fold increase in [^{3}H]phorbol 12,13-dibutyrate binding compared with control transfectants. Enzyme assays of PKC partially purified from transfected cells indicated at least a 5-fold increase in PKC activity upon expression of the wild-type protein or the NH_2-terminal and hinge mutants. In contrast, no increased activity was detected upon expression of the COOH-terminal mutant. Immunoblot analysis using a β isoform-specific antibody showed that wild-type, NH_2-terminal mutant, and hinge mutant proteins are similarly distributed between the Triton-soluble and insoluble fractions. In contrast, the COOH-terminal mutant protein is largely Triton-insoluble. Immunoblot analysis also indicated that this mutant is resistant to down-regulation upon chronic exposure of cells to phorbol ester. Moreover, RNA blot analysis showed that overexpression of wild-type PKC but not of the COOH-terminal mutant enhances phorbol ester induction of c-FOS and c-JUN mRNA. Our results indicate that (*i*) alteration in the NH_2-terminal and hinge autophosphorylation sites has no effect on PKC function by the criteria examined and (*ii*) the COOH-terminal autophosphorylation sites are critical for PKC function and possibly subcellular localization in COS cells.**

Protein kinase C (PKC) is a family of serine/threonine protein kinases that require diacylglycerol and phosphatidylserine for catalytic activity (1, 2). Activation of PKC plays a crucial role in the transduction of extracellular signals leading to a variety of cellular responses—such as proliferation and differentiation, secretion of hormones and neurotransmitters, and gene expression (3–5). The importance of PKC in multistage carcinogenesis was demonstrated by the finding that the tumor-promoting phorbol esters bind to and activate PKC both *in vitro* and *in vivo* (3).

Each member of the PKC family is composed of a lipid-binding regulatory domain in the NH_2-terminal region and an ATP-binding catalytic domain in the COOH-terminal portion of the protein (1, 2). *In vitro* PKC phosphorylates many protein substrates (3); however, the relevant *in vivo* substrates are just beginning to be defined (6). In addition to transphosphorylation reactions, PKC autophosphorylates both *in vitro* and *in vivo* with both the regulatory and catalytic domains becoming phosphorylated (7–9).

The biological role of PKC autophosphorylation has not been determined. Several studies have indicated that autophosphorylation increases the affinity of PKC for the tumor-promoting phorbol esters, enhances its Ca^{2+} sensitivity, and decreases its K_m for histone H1 *in vitro* (8, 9). A potential regulatory role of PKC autophosphorylation was also suggested by the observation that PKC activation and down-regulation by phorbol ester correlated with its autophosphorylation state (10–12). Therefore, autophosphorylation may be important for PKC activation and for PKC-mediated signal transduction.

Recently, the autophosphorylation sites of the β_2 isozyme of PKC were identified by Koshland and coworkers (13). These correspond to Ser-16/Thr-17 near the NH_2 terminus, Thr-314/Thr-324 in the hinge region, and Thr-634/Thr-641 near the COOH terminus. In the work reported here the function of each pair of autophosphorylation sites was examined using site-directed mutagenesis and transient expression in COS cells. Our results indicate that mutation in the NH_2-terminal or hinge sites has no detectable effect on PKC β_1 function. However, the COOH-terminal autophosphorylation sites appear critical for PKC β_1 function and possibly for its subcellular localization in COS cells.

MATERIALS AND METHODS

Construction of PKC β_1 Expression Vector and Site-Directed Mutagenesis. The vector pMV7-PKC (14) containing the rat PKC β_1 cDNA was obtained from P. Kirschmeier (Schering–Plough). The PKC portion was amplified by 12 rounds of PCR using *Pfu* DNA polymerase (Stratagene) and oligonucleotides containing *Sal* I and *Bam*HI sites: (5′ oligonucleotide = GATCGTCGACAGCCCGCGGTCCC; 3′ oligonucleotide = GATCGGATCCTTACACACAGGCTCAG). The amplified DNA was cut with *Sal* I and *Bam*HI and ligated into the *Xho* I/*Bam*HI-digested vector pSRS containing the SRα promoter (ref. 15; ATCC 68234) to produce pKCRS-X. Mutagenesis was done by using the Mutagene kit from Bio-Rad following their protocol, except that T7 DNA polymerase was used for the extension reaction. The double-stranded DNA produced was used to transform the *Escherichia coli* strain MV1190 (*dut*$^+$*ung*$^+$), single colonies were picked, and their plasmid DNA was sequenced to confirm the mutations. The antisense oligonucleotides used were as follows: M6 = GAAGCGCACCGCGGCCTCCTCGCCC; M7 = GGAGCCTTGGCACCTTGGCCAA and TTTGGATATAGCGTTCGCTGTC; M9 = GTGGGAGCCAGTTCCACAGGCTGCCTGGCGAACTCTTT (altered bases are underlined). After the mutations were produced in pKCTZ the PKC cDNAs were cut out with *Sal* I and *Bam*HI and ligated into *Xho* I/*Bam*HI-digested pSRS to produce pKCM6RS-X, pKCM7RS-X, and pKCM9RS-X.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

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

*To whom reprint requests should be addressed at: Schering–Plough Research Institute, 2015 Galloping Hill Road, K-15-4 (4700), Kenilworth, NJ 07033-0539.

Cell Culture and Transfection. COS cells were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO)/10% fetal bovine serum (GIBCO). Cells were harvested at 50–80% confluency and concentrated to 4×10^7 cells per ml in DMEM for transfection. Cells (10^7) were transfected with 25 μg of plasmid DNA by electroporation with a Gene Pulser apparatus (Bio-Rad) at settings of 960 μF and 170 V. After electroporation, cells were diluted with DMEM/10% fetal bovine serum and seeded into 100-mm plates at 2×10^6 cells per plate.

[^{3}H]Phorbol 12,13-Dibutyrate (PBt_2) Binding. After electroporation, 10^6 cells were seeded into each 35-mm well of a 6-well plate, grown for 40–48 hr and then washed twice with cold phosphate-buffered saline (PBS). [^{3}H]PBt_2 binding to whole cells was measured essentially as described by Housey *et al.* (14). Each well was incubated with 2 ml of serum-free DMEM containing 1 μCi of [^{3}H]PBt_2 (20.0 Ci/mmol; New England Nuclear; 1 Ci = 37 GBq) for 1 hr at 37°C. After being washed twice with cold PBS, cells were lysed in 1% SDS and 10 mM dithiothreitol at 37°C for 2 hr, and cell-associated radioactivity was measured. Nonspecific [^{3}H]PBt_2 binding was determined in the presence of a 1000-fold excess of unlabeled PBt_2.

Preparation of Cellular Fractions. Cell extracts were prepared by a described method (14) with minor modifications. Briefly, 2.5×10^6 cells were seeded into a 100-mm dish after electroporation and cultured for 40–48 hr. Three dishes of cells were washed twice with cold PBS, scraped, and homogenized (15 strokes in a Dounce homogenizer) in 3 ml of buffer (20 mM Tris·HCl, pH 7.5/5 mM EGTA/5 mM EDTA/15 mM 2-mercaptoethanol/soybean trypsin inhibitor at 10 μg/ml/leupeptin at 10 μg/ml/phenylmethylsulfonyl fluoride at 40 μg/ml/0.1% Triton X-100) and centrifuged at 2000 × *g* for 15 min at 4°C. The Triton-soluble supernatant was collected and fractionated by DEAE-Sephacel chromatography (see below). The Triton-insoluble pellet was solubilized in preboiling buffer A (20 mM Tris·HCl, pH 7.5/10 mM 2-mercaptoethanol/0.5 mM EGTA/0.5 mM EDTA/leupeptin at 10 μg/ml/phenylmethylsulfonyl fluoride at 40 μg/ml) containing 1% SDS, followed by a 30-sec sonication. For whole-cell extracts, COS cells in one 100-mm dish were solubilized with 1 ml of preboiling buffer A/1% SDS, scraped, and sonicated for 30 sec. Protein concentrations were determined by using the bicinchoninic acid method (Pierce).

Immunoblotting Analysis. Protein fractions were precipitated with 15% trichloroacetic acid and dissolved by boiling in SDS sample buffer [62.5 mM Tris·HCl, pH 6.8/1% (wt/vol) SDS/0.0025% bromphenol blue/2.5% 2-mercaptoethanol/10% glycerol] containing 5 M urea. Proteins were resolved by electrophoresis on 10% polyacrylamide/SDS minigels (Novex, San Diego) and electrophoretically transferred to nitrocellulose membranes. Membranes were blocked by overnight incubation at 4°C in PBS/5% dry milk/0.1% Tween-20. Membranes were then incubated for 2 hr at room temperature in 5 ml of PBS/1% fetal bovine serum/0.1% Tween-20/5 μg of a murine monoclonal antibody specific for the β isoform of rat PKC (MC-2a from Seikagaku, Rockville, MD). Antibody was detected by incubation for 1 hr in 5 ml of PBS containing 0.1% Tween and 2.5 μCi of affinity-purified ^{125}I-labeled protein A (30 mCi/mg; Amersham) followed by autoradiography. In some cases immunoreactive bands were excised and quantitated by counting γ emissions.

Measurement of PKC Activity *in Vitro*. Triton-soluble fractions were loaded on 0.5 ml of DEAE-Sephacel columns (Pharmacia), washed with buffer A/30 mM NaCl, and PKC was eluted with buffer A/500 mM NaCl. *In vitro* kinase assays were done in a reaction mixture (250 μl) containing 20 μl of DEAE eluate, 20 mM Tris·HCl (pH 7.5), 10 mM $MgCl_2$, 200 μM $CaCl_2$, phosphatidylserine at 80 μg/ml (Avanti Polar Lipids), histone III-S at 2 mg/ml (Sigma), and 70 μM [γ-^{32}P]ATP (2.5×10^6 cpm/nmol). After incubation at room temperature for 15 min, reactions were terminated by adding 1 ml of ice-cold 15% trichloroacetic acid and 1 ml of bovine serum albumin at 0.5 mg/ml. After incubation on ice for at least 10 min, samples were collected on Whatman GF/C filters using a Brandel cell harvester, and precipitable radioactivity was measured by liquid scintillation spectrometry. To assess protein kinase activity not due to PKC, reactions were done in which 1 mM Tris, pH 7.5, was added in place of phosphatidylserine and $CaCl_2$. The difference between kinase activity with or without cofactors was used to determine PKC enzyme activity.

Total RNA Isolation and RNA Blotting. After electroporation 2×10^6 COS cells were seeded into 100-mm plates and grown for 40–48 hr in 10 ml of DMEM/10% fetal bovine serum. COS cells were treated with 150 nM phorbol 12-myristate-13-acetate (PMA in 0.05% dimethyl sulfoxide) for various times, and total RNA was prepared by using a single-step guanidinium method (16). Total RNA was electrophoresed through 1.2% agarose-formaldehyde gels and transferred to Hybond-N nylon membranes (Amersham) (17). Probes were labeled with [α^{32}P]dCTP (6000 Ci/mmol; New England Nuclear) by using prime-IT random primer kit according to the manufacturer's instructions (Stratagene). A 1200-bp *Pst* I fragment of PKC β_1 cDNA was used as PKC β_1 probe (14). A 747-bp *Acc* I fragment (+2080 to +2827) containing the exon 4 region of the human c-FOS gene was used as c-FOS probe (18). A 1040-bp *Pst* I fragment containing human c-JUN DNA sequence (+599 to +1639) was used as c-JUN probe (19). Membranes were first prehybridized in a solution containing 6× standard saline citrate/0.5% SDS/5× Denhardt's solution/denatured salmon sperm DNA at 100 μg/ml at 64°C for 4 hr and then hybridized in the same solution with the appropriate ^{32}P-labeled DNA probe overnight at 60°C (17). Membranes were then washed and subjected to autoradiography at −80°C.

RESULTS

PKC β_1 and β_2 isozymes are derived from a single RNA transcript by alternative splicing and differ from each other only slightly at the COOH terminus (1); therefore, they are likely to share either the same (Ser-16/Thr-17 and Thr-314/Thr-324) or very similar (Thr-634/Thr-641 for β_2 and Thr-635/Thr-642 for β_1) autophosphorylation sites (13, 14). We have examined the role of these sites in the function of PKC β_1 using site-directed mutagenesis to change the phosphorylatable residues to alanine (Fig. 1). Three site-directed mutants of PKC β_1 isozyme were constructed and are referred to as M6 (altered at Ser-16/Thr-17 near the NH_2 terminus), M7 (altered at Thr-314/Thr-324 in the hinge region), and M9 (altered at Thr-635/Thr-642 near the COOH terminus).

For biochemical and functional characterization of the mutant proteins, they were transiently overexpressed by transfection of COS cells with plasmids encoding either wild-type or mutant PKC β_1 isozyme. To determine the PKC β_1 expression level, mRNA was analyzed by RNA blotting with a ^{32}P-labeled PKC β_1 probe. COS cells transfected with plasmids encoding wild-type or mutant PKC contained a prominent mRNA species homologous to this probe, whereas cells transfected with control plasmid showed no endogenous PKC β_1 mRNA (Fig. 2*A*). All PKC transfectants expressed similar and very high levels of PKC β_1 mRNA (detectable by 10-min exposure). To monitor expression of PKC β_1 derivatives at the protein level, [^{3}H]PBt_2 binding assays were done on intact transfected COS cells. Expression of wild-type PKC β_1 and all mutant proteins caused at least a 20-fold increase in [^{3}H]PBt_2 binding compared with that of control transfectants (Fig. 2*B*). This result further confirmed that expression

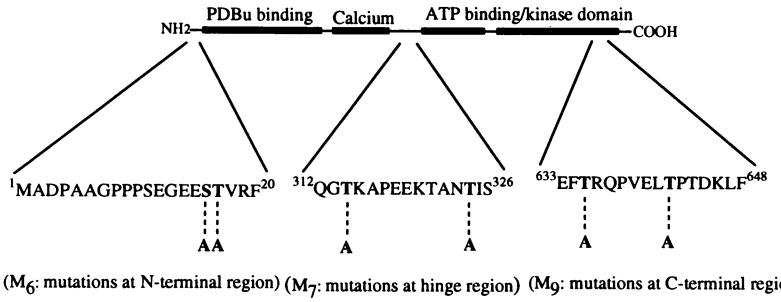

FIG. 1. Schematic illustration of PKC β_1 mutants altered at the autophosphorylation sites. The primary sequence surrounding each autophosphorylation site in PKC β_1 isozyme is shown. The phosphorylated residues are in boldface type and were changed to alanine in the indicated mutant constructs. This figure was adapted from Flint *et al.* (13). PDBu, PBt_2.

of wild-type and mutant PKC β_1 from these plasmids is strong and comparable. In addition, alteration of the autophosphorylation sites apparently has no effect on the ability of PKC β_1 to bind phorbol esters.

To examine the subcellular distribution and kinase activity of PKC β_1, transfected cells were lysed in buffer/0.1% Triton X-100/chelators and separated into Triton-soluble and Triton-insoluble fractions by centrifugation. Triton-soluble fractions were further fractionated on DEAE-Sephacel columns. The presence of overexpressed wild-type and mutant PKC β_1 in both fractions was shown by immunoblot analysis using a β-isoform-specific monoclonal antibody (Fig. 3 *A* and *B*). A band corresponding to 80-kDa rat PKC β_1 was detected in both fractions from cells expressing wild-type or mutant protein. This band was absent from cells transfected by control plasmid.

Interestingly, the wild-type, NH_2-terminal (M6) and hinge (M7) mutant proteins showed a similar distribution between the Triton-soluble and insoluble fractions, whereas the COOH-terminal mutant protein (M9) was less efficiently recovered in the Triton-soluble fraction. The experiment of Fig. 3*A* is one in which the recovery of soluble M_9 was high, and this material was used for subsequent determination of enzyme activity (see below). Decreased recovery of soluble M_9 is not from loss of mutant PKC during chromatography because no immunoreactive protein was recovered in the DEAE loading or wash fractions. In addition, decreased recovery of soluble M_9 was also seen when soluble protein was analyzed before chromatography (data not shown). For example, in a typical experiment the percent of immunoreactive protein in the soluble fraction was 40% for wild type, 49% for M_7, 36% for M_6, and only 14% for M_9 mutant protein. Therefore, COOH-terminal autophosphorylation may influence subcellular localization of PKC β_1 isozyme.

To test the function of these mutant PKC β_1 proteins directly, *in vitro* histone kinase assays were done on the DEAE-purified Triton-soluble fractions shown in Fig. 3*A*. There is at least a 5-fold increase in PKC activity in the fractions containing wild-type protein or the NH_2-terminal or hinge mutant proteins compared with control transfectants (Table 1). In contrast, no increased activity was detected in this fraction from cells expressing the COOH-terminal (M9) mutant. Although recovery of the M9 protein from the DEAE-Sepharose column was less than that of the other proteins, immunoreactive protein was consistently recovered in this fraction (Fig. 3*A*) but in every instance failed to show increased histone kinase activity compared with controls. In fact, we always observed a slight decrease in PKC activity from this mutant compared with COS control cells (Table 1). Therefore, these results suggest that the COOH-terminal autophosphorylation sites are critical for PKC β_1 function in COS cells.

To explore the role of these autophosphorylation sites in down-regulation of PKC β_1 upon chronic treatment of cells with high doses of phorbol ester, whole-cell extracts were prepared from transfected COS cells after treatment with 300 nM PMA for 24 hr. Immunoblot analysis (Fig. 4) indicated that after phorbol ester treatment steady-state levels of the COOH-terminal mutant protein remained high compared with the wild-type and other mutant proteins. These results further indicate that alteration in the NH_2-terminal and hinge autophosphorylation sites has no effect on PKC β_1 isozyme function but that the COOH-terminal mutation alters the sensitivity of PKC β_1 to down-regulation as well as its subcellular distribution and apparent enzymatic activity.

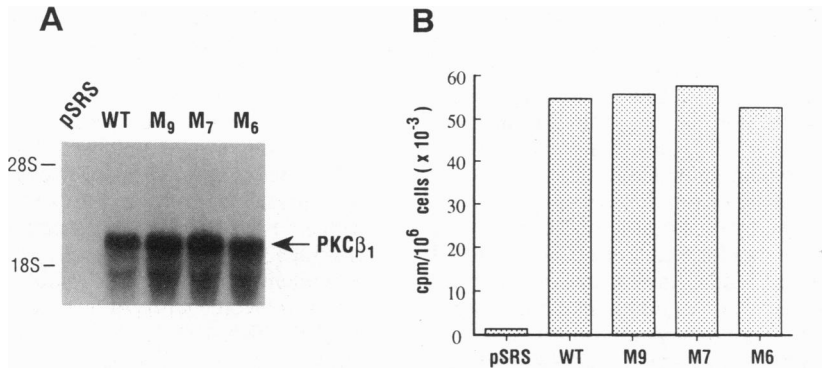

FIG. 2. Overexpression of wild-type and mutant PKC β_1 isozyme in COS cells. (*A*) RNA blot analysis. Thirty micrograms of total RNA was subjected to electrophoresis and probed with a ^{32}P-labeled *Pst* I fragment of rat PKC β_1 cDNA. The autoradiograph was obtained by a 10-min exposure time. (*B*) [^3H]PBt_2 binding assay. COS cells were electroporated with control plasmid (pSRS), or pSRS containing cDNA encoding wild-type PKC β_1(WT), the COOH-terminal mutant PKC β_1 (M9), the hinge mutant PKC β_1 (M7), or the NH_2-terminal mutant PKC β_1 (M6). [^3H]PBt_2 binding activities of cells was measured 48 hr after electroporation, as described. These results are typical of at least five separate experiments.

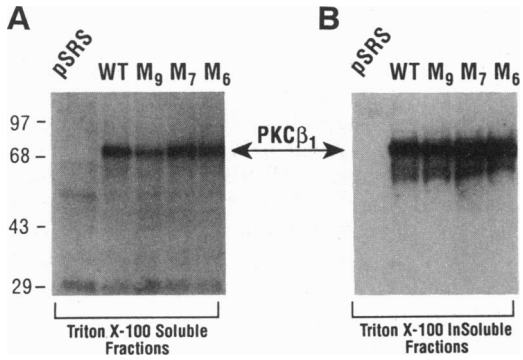

FIG. 3. Subcellular localization of wild-type and mutant PKC β_1 isozyme in COS cells. One hundred and fifty micrograms of protein of DEAE-purified Triton-soluble fractions (*A*) and 50 μg of protein from Triton-insoluble fractions (*B*) were subjected to SDS/PAGE, and the wild-type and mutant PKC β_1 isozyme were detected by immunoblot analysis with a PKC β-specific monoclonal antibody, as described. pSRS, WT, M9, M7, and M6 are as described in the legend for Fig. 2. Similar results were seen in at least three independent experiments.

To further study the functional role of the COOH-terminal autophosphorylation sites in PKC β_1 cellular signaling, we performed Northern blot analysis with ^{32}P-labeled DNA probes to measure expression of c-FOS and c-JUN protooncogenes in PMA-stimulated transfected COS cells. c-FOS and c-JUN are phorbol ester-inducible genes belonging to the AP-1 transcription factor family (5). c-FOS and c-JUN are both inducibly expressed in COS cells after PMA treatment (Fig. 5). At 30 min after PMA addition, the expression of both c-FOS and c-JUN mRNA was very similar in control COS cells and in cells overexpressing either wild-type or mutant PKC β_1 isozyme (Fig. 5 *A* and *B*). However, at 60 min after PMA addition overexpression of wild-type PKC β_1, but not of the COOH-terminal mutant, increased phorbol ester-induced expression of both c-FOS and c-JUN mRNA to levels 2- to 3-fold above those in control transfectants (Fig. 5 *A* and *B*). The similar levels of 28S and 18S ribosomal RNA (Fig. 5*C*) indicate that approximately equal amounts of total RNA were used. Again, this result indicates the importance of the COOH-terminal autophosphorylation sites for PKC β_1 isozyme function in COS cells.

DISCUSSION

Previous studies in other laboratories have suggested a correlation between PKC activation and its autophosphorylation (10–12). In this study, the functional role of autophosphorylation of rat PKC β_1 isozyme was examined by site-directed mutagenesis and biochemical analysis. Our results indicate that (*i*) alteration in the NH_2-terminal and hinge autophosphorylation sites has no effect on PKC β_1 isozyme function by the criteria examined and (*ii*) the COOH-terminal autophosphorylation sites are critical for PKC β_1 function (histone kinase activity and protooncogene expression), down-regulation, and possibly subcellular localization.

Table 1. PKC enzyme activity from transfected COS cells

PKC construct	Enzyme activity: PKC activity, cpm/μg	Enzyme activity: Fold increase
pSRS (control)	336	(1.0)
PKC β_1 (WT)	1794	5.3
M_9 (COOH terminus)	262	0.8
M_7 (hinge)	2895	8.6
M_6 (NH_2 terminus)	2726	8.1

PKC was partially purified from Triton X-100 soluble fractions by DEAE-Sephacel chromatography, and PKC assays were done as described. Similar results were obtained with three different preparations. WT, wild type.

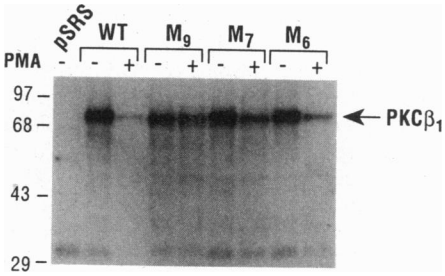

FIG. 4. Down-regulation of wild-type and mutant PKC β_1. COS cells were transfected with plasmids encoding wild-type or mutant PKC β_1 and then cultured for 24 hr. Subsequently, cells were grown for another 24 hr in the presence (+) or absence (−) of 300 nM PMA. Whole-cell extracts were prepared, and 50 μg of protein was subjected to SDS/PAGE followed by immunoblotting, as described. pSRS, WT, M9, M7, and M6 are as described in the legend for Fig. 2. Similar results were seen in at least three independent experiments.

Upon expression of wild-type and mutant forms of PKC β_1 isozyme in COS cells most of the protein is recovered in a Triton-insoluble fraction. Similar results were reported with other forms of PKC expressed in this cell line (19, 20). PKC

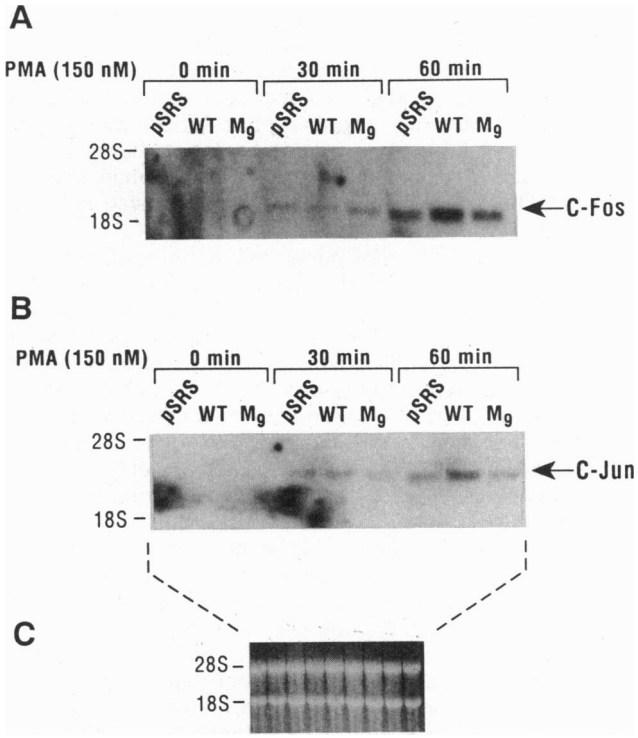

FIG. 5. Effect of wild-type and mutant PKC β_1 overexpression on phorbol ester-induced synthesis of c-FOS and c-JUN mRNA. COS cells overexpressing either wild type (WT) or the COOH-terminal mutant (M9) of PKC β_1 isozyme were incubated with 150 nM PMA, and total RNA was extracted at the times indicated (0, 30, 60 min). Thirty micrograms of total RNA was analyzed by Northern blot analysis using a ^{32}P-labeled *Acc* I fragment of human c-FOS gene (*A*) or a ^{32}P-labeled *Pst* I fragment of human c-JUN gene (*B*). (*C*) The amount of total RNA loaded per lane was assessed by UV illumination of the ethidium bromide-stained gel. c-FOS and c-JUN mRNA were detected by 3-day and 6-hr autoradiographic exposure times, respectively. Similar results were seen in at least three independent experiments.

recovered in this fraction may represent aggregated, denatured protein (20); however, this PKC appears functional on the basis of several observations. (*i*) Overexpression of wild-type PKC (or of PKC altered at the NH_2 terminus or hinge) caused a much greater increase (20-fold) in cellular [^{3}H]PBt_2 binding activity compared with a 5- to 8-fold increase in histone kinase activity recovered from the Triton-soluble fraction. This result suggests that the detergent-insoluble form of PKC β_1 isozyme functions as a phorbol ester receptor. In addition, chronic treatment of COS cells with phorbol ester down-regulates almost all the overexpressed wild-type PKC β_1 isozyme, indicating that Triton-insoluble PKC is still subject to normal regulatory mechanisms.

The M_9 mutant form of PKC β_1 altered in the COOH-terminal autophosphorylation sites is even less well solubilized by detergent than the wild type. The reason for this is not yet understood. The autophosphorylation of sites near the COOH terminus of PKC β_1 isozyme could play a regulatory role in its intracellular localization. It is thought that Triton-insoluble material is largely of cytoskeletal and/or nuclear origin, and an association of PKC with the detergent-insoluble cytoskeleton has been reported (21–23). In addition, several cytoskeletal proteins are either *in vitro* or *in vivo* substrates for PKC, including a predominant cellular PKC substrate, the myristoylated alanine-rich C-kinase substrate (MARCKS) protein (4, 6).

The apparent resistance of the COOH-terminal mutant to down-regulation by chronic phorbol ester treatment agrees with the observation of Ohno *et al.* (11) that inactivation of PKC α kinase activity by mutagenesis of the ATP-binding site leads to resistance to down-regulation. Why the results described in other recent reports (24, 25) disagree with the conclusion of Ohno *et al.* is unclear; however, the mutants used in our study provide a direct means to examine the relationship between autophosphorylation and down-regulation. It should be pointed out that the studies described here measure steady-state levels of PKC protein that are influenced by the rate of *de novo* synthesis as well as by the rate of proteolytic degradation.

The role of the COOH-terminal PKC autophosphorylation sites in PKC-mediated signal transduction is further indicated by the finding that overexpression of wild-type PKC β_1 isozyme but not the COOH-terminal mutant results in an increase of phorbol ester-inducible c-FOS and c-JUN mRNA synthesis. The increase in phorbol ester-inducible gene expression seen upon overexpression of the wild-type PKC resembles that seen by others upon PKC overexpression in various cell lines (20, 26). The lack of activity of the COOH-terminal mutant supports the lack of *in vitro* kinase activity of this protein.

PKC autophosphorylation is thought to occur by an intramolecular reaction (9, 27). Autophosphorylation of multiple residues located in widely separated domains suggests a great deal of protein flexibility. The present study has revealed a critical role for autophosphorylation of the COOH-terminal site(s) in PKC β_1 isozyme function. Importantly, comparison of amino acid sequences among PKC subspecies indicates that the NH_2 terminus and hinge autophosphorylation sites are unique to the β isoforms (13, 28). In contrast, the COOH-terminal autophosphorylation sites and surrounding amino acid sequence are conserved in the α, β_1, β_2, γ, ε, and ζ isozymes (13, 28), suggesting an essential and common role in PKC function, consistent with our findings.

The detailed mechanism by which autophosphorylation may control PKC activity remains to be elucidated. One possible mechanism is that COOH-terminal autophosphorylation triggers a conformational change in PKC subsequent to binding of the PKC activators, diacylglycerol, and PtdSer. Such a conformational change may be important in relieving inhibition of PKC by the NH_2-terminal pseudosubstrate prototope (2). Favoring this hypothesis, the putative substrate (and pseudosubstrate) binding site is thought to be located near the COOH-terminal autophosphorylation sites (29). Furthermore, this model is supported by the observation that the catalytic fragment of PKC retains histone kinase activity, even though it can no longer autophosphorylate (9), suggesting that removal of the regulatory domain eliminates the requirement for COOH-terminal autophosphorylation. Alternatively, this autophosphorylation may be critical for enzyme–substrate recognition or for interaction of PKC with regulatory factors.

We thank Drs. Linda McMahan and Stanley Lin of Schering-Plough Research Institute for providing us with human c-JUN and c-FOS genomic plasmids, respectively. We also thank Dr. James J.-K. Pai for many helpful contributions during our initial studies directed toward mapping the autophosphorylation sites and Dr. Jerome Schwartz for supporting this work.

1. Kikkawa, U., Kishimoto, A. & Nishizuka, Y. (1989) *Annu. Rev. Biochem.* **58,** 31–44.
2. Bell, R. M. & Burns, D. J. (1991) *J. Biol. Chem.* **266,** 4661–4664.
3. Nishizuka, Y. (1984) *Nature (London)* **308,** 693–698.
4. Nishizuka, Y. (1986) *Science* **233,** 305–311.
5. Karin, M. & Smeal, T. (1992) *Trends Biol. Sci.* **17,** 418–422.
6. Aderem, A. (1992) *Cell* **71,** 713–716.
7. Kikkawa, U., Takai, Y., Minakuchi, R., Inohara, S. & Nishizuka, Y. (1982) *J. Biol. Chem.* **257,** 13341–13348.
8. Huang, K.-P., Chan, K.-F. J., Singh, T. J., Nakabayashi, H. & Huang, F. L. (1986) *J. Biol. Chem.* **261,** 12134–12140.
9. Mochly-Rosen, D. & Koshland, D. E. (1987) *J. Biol. Chem.* **262,** 2291–2297.
10. Mitchell, F. E., Marais, R. M. & Parker, P. J. (1989) *Biochem. J.* **261,** 131–136.
11. Ohno, S., Konno, Y., Akita, Y., Yano, A. & Suzuki, K. (1990) *J. Biol. Chem.* **265,** 6296–6300.
12. Molina, C. A. & Ashendel, C. L. (1991) *Cancer Res.* **51,** 4624–4630.
13. Flint, A. J., Paladini, R. D. & Koshland, D. E. (1990) *Science* **249,** 408–411.
14. Housey, G. M., Johnson, M. D., Hsiao, W. L. W., O'Brian, C. A., Murphy, J. P., Kirschmeier, P. & Weinstein, I. B. (1988) *Cell* **52,** 343–354.
15. Takebe, Y., Seiki, M., Fujisawa, J.-I., Hoy, P., Yokota, K., Arai, K.-I., Yoshida, M. & Arai, N. (1988) *Mol. Cell. Biol.* **8,** 466–472.
16. Chomczynski, P. & Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159.
17. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), 2nd Ed.
18. Straaten, F. V., Muller, R., Curran, T., Beveren, C. V. & Verma, I. M. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 3183–3187.
19. Muramatsu, M.-A., Kaibuchi, K. & Arai, K.-I. (1992) *FEBS Lett.* **311,** 75–79.
20. Pears, C. J., Kour, G., House, C., Kemp, B. E. & Parker, P. J. (1990) *Eur. J. Biochem.* **194,** 89–94.
21. Jaken, S., Leach, K. & Klauck, T. (1989) *J. Cell Biol.* **109,** 697–704.
22. Wolf, S. & Sahyoun, N. (1986) *J. Biol. Chem.* **261,** 13327–13332.
23. Spudich, A., Meyer, T. & Stryer, L. (1992) *Cell Motil. Cytoskel.* **22,** 250–256.
24. Pears, C. & Parker, P. J. (1991) *FEBS Lett.* **284,** 120–122.
25. Freisewinkel, I., Riethmacher, D. & Stabel, S. (1991) *FEBS Lett.* **280,** 262–266.
26. Wartmann, M., Jans, D. A., Parker, P. J., Nagamine, Y., Hemmings, B. A., Jaken, S., Eppenberger, U. & Fabbro, D. (1991) *Cell Regul.* **2,** 491–502.
27. Newton, A. C. & Koshland, D. E. (1987) *J. Biol. Chem.* **262,** 10185–10188.
28. Ono, Y., Fujii, T., Ogita, K., Kikkawa, U., Igarashi, K. & Nishizuka, Y. (1988) *J. Biol. Chem.* **263,** 6927–6932.
29. House, C., Robinson, P. J. & Kemp, B. E. (1989) *FEBS Lett.* **249,** 243–247.