Identification of Serine 643 of Protein Kinase C-δ as an Important Autophosphorylation Site for Its Enzymatic Activity*

Weiqun Li, Jiachang Zhang, Donald P. Bottaro, Wei Li, and Jacalyn H. Pierce

From the Laboratory of Cellular and Molecular Biology, NCI, National Institutes of Health, Bethesda, Maryland 20892 and the \$Ben May Institute for Cancer Research and Department of Pharmacological and Physiological Sciences, the University of Chicago, Chicago, Illinois 60637

To investigate the role of serine/threonine autophosphorylation of protein kinase C-δ (PKC-δ), we mutated serine 643 of PKC-δ to an alanine residue (PKC-δS643A). Two different expression vectors containing PKC-δS643A mutant cDNAs were transfected and expressed in 32D myeloid progenitor cells. In vitro autophosphorylation assays demonstrated 65–83% reduction in autophosphorylation of PKC-δS643A in comparison to wild type PKC-δ (PKC-δWT). The enzymatic activity of PKC-δS643A mutant as measured by phosphorylating the PKC-δ pseudosubstrate region-derived substrate was also reduced more than 70% in comparison to that of PKC-δWT. In vivo labeling and subsequent two-dimensional phosphopeptide analysis demonstrated that at least one phosphopeptide was absent in PKC-δS643A when compared with PKC-δWT, further substantiating that serine 643 is phosphorylated in vivo. Localization and 12-O-tetradecanoylphorbol-13-acetate-dependent translocation and tyrosine phosphorylation of PKC-δS643A were not altered in comparison to PKC-δWT, indicating that mutagenesis did not affect the structural integrity of the mutant protein. 12-O-Tetradecanoylphorbol-13-acetate-mediated monocytic differentiation of 32D cells overexpressing PKC-δS643A mutant protein was impaired in comparison to that of PKC-δWT transfected. Taken together, our results demonstrate that serine 643 of PKC-δ is a major autophosphorylation site, and phosphorylation of this site plays an important role in controlling its enzymatic activity and biological function.

Protein kinase C (PKC) is composed of a family of serine/threonine kinases. To date, 11 different PKC isoenzymes have been identified that are divided into three different subgroups, conventional PKCs (cPKCs), novel PKCs (nPKCs), and atypical PKCs (aPKCs) (1–3). PKCs have been defined as important signaling molecules in cell growth, differentiation, secretion of hormones and neurotransmitters, and cellular transformation (2). PKC-δ belongs to nPKC subgroup and is ubiquitously expressed in many tissues and cell lines (4).

We have focused our efforts on understanding the role of PKC-δ in various signaling transduction pathways. Overexpression of wild type of PKC-δ (PKC-δWT) in 32D myeloid progenitor cells led to monocytic differentiation in response to 12-O-tetradecanoylphorbol-13-acetate (TPA) treatment (5), suggesting a causal role for PKC-δ in hematopoietic cell differentiation. An ATP binding mutant of PKC-δ (PKC-δK376R) was generated by site-directed mutagenesis and was demonstrated to lack autophosphorylation capacity in vitro completely (6). Moreover, the PKC-δK376R mutant competitively inhibited PKC-δWT phosphorylation of an exogenous substrate in vitro. Recently, our group and several others (7–12) observed tyrosine phosphorylation of PKC-δ in vivo in response to its activation by various agonists. PKC-δ was also demonstrated to be an important substrate in the platelet-derived growth factor receptor (PDGF-βR) pathway (13). It was phosphorylated by the activated PDGF-βR in vivo and in vitro on tyrosine residue(s) (10, 13). The relevance of PKC-δ in mediating c-sis PDGF-B transformation of NIH 3T3 cells was recently elucidated (14). In this study, expression of the PKC-δK376R mutant led to dramatic inhibition of c-sis-induced NIH 3T3 cell transformation. These results demonstrate that PKC-δ plays a physiological role in a signaling pathway leading to malignant transformation of fibroblasts induced by sis oncogene.

Serine/threonine phosphorylation of PKC in vivo was first observed approximately 10 years ago (15–19). Several in vivo phosphorylation sites have been mapped utilizing different methods (20–22). Based on studies performed on cPKCs (20, 23–27), it is generally believed that PKC is first synthesized as an immature precursor protein that does not show any catalytic activity. Phosphorylation of PKC on the “activation loop,” which corresponds to threonines 497 and 500 of PKC-δII (26), respectively, by an unidentified PKC kinase then renders PKC catalytic domain competent. However, transphosphorylation of PKC on its activation loop does not alter the mobility of the protein as observed by SDS-polyacrylamide gel electrophoresis (PAGE). Subsequent autophosphorylation on threonine 641 of PKC-βII results in the first upward shift of the mobility of the protein. This event is followed by a second autophosphorylation on serine 660 of PKC-βII which further shifts the protein to the mature 80-kDa form. Generation of diacylglycerol through different mechanisms recruits PKC to the membrane where the pseudosubstrate region-mediated autoinhibition of the catalytic domain is released. The enzyme is then able to phosphorylate substrates and transmit the downstream signals. How the mature enzyme returns to the cytosol after activation remains unclear. This may be regulated by serine/threonine phosphatase activity (1).

Autophosphorylation of PKC has been observed both in vivo and in vitro (15–19). It is thought that autophosphorylation of

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† To whom correspondence should be addressed: Laboratory of Cellular and Molecular Biology, NCI, National Institutes of Health, Bethesda, MD 20892. Tel.: 301-496-1347; Fax: 301-496-8479. E-mail: Liwe@dc37a.nci.nih.gov.
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PKC enhances its binding to phorbol ester and reduces the $K_m$ for its substrates in vitro (16, 18). Several in vivo autophosphorylation sites for different PKC isoforms have been mapped (20–22). Recently, conserved threonine autophosphorylation sites on two cPKCs (PKC-α and PKC-βl) were characterized by site-directed mutagenesis (23, 28, 29). Mutation of threonine 638 to alanine in the PKC-α molecule did not dramatically affect its enzymatic activity (23). In striking contrast, mutation of this conserved site (threonine 642 to alanine) in PKC-βl completely abolished its enzymatic activity and in vivo phosphorylation (29). Since PKC-δ belongs to the nPKC subfamily and a serine residue rather than a threonine residue exists at this conserved position (see Fig. 1), we have attempted to elucidate whether PKC-δ is phosphorylated on this conserved site and, if so, whether this phosphorylation would influence PKC-δ function. Our results indicate that serine 643 is a major PKC-δ autophosphorylation site, and phosphorylation of this site significantly affects its enzymatic activity.

**EXPERIMENTAL PROCEDURES**

**Construction of a Serine to Alanine Mutant of Murine PKC-δ, cDNA Expression Vectors, and Cell Lines**—The Bio-Rad Muta-gene Phagemid Expression Vectors, and Cell Lines—The Bio-Rad Muta-gene Phagemid Expression Vectors, and Cell Lines—

**Construction of a Serine to Alanine Mutant of Murine PKC-δ**—The Bio-Rad Muta-gene Phagemid Expression Vectors, and Cell Lines—

The oligonucleotide 5′-GAAAGAAATCAGCTTG-3′ was used as a mutant primer in the in vitro mutagenesis reaction where the serine residue at amino acid 643 of murine PKC-δ was changed to alanine (underlined in the sequence). The successful mutation of this site generated a new BamHI restriction site that was used to screen all the reaction products. The mutation was confirmed by DNA sequencing.

**PKC Activity Assay**—PKC activity utilizing anti-HA immunoprecipitation of PKC-δ and PKC-βl from 32D cells and transfected cells was assayed by thin layer electrophoresis, pH 8.9, followed by ascending chromatography, pH 1.9. Dried plates were autoradiographed for 1 week.

**In Vivo Labeling and Two-dimensional Phosphopeptide Analysis**—Both in vivo labeling and subsequent two-dimensional phosphopeptide analysis have been described previously (30). Briefly, serum-starved 32D transfectants were labeled with [32P]orthophosphate (1 μCi/ml; NEN Life Science Products) for 3 h and were stimulated with TPA (100 ng/ml) for 10 min. Cell lysates were immunoprecipitated with anti-HA antibody (4 μg per sample), boehringer manheim) and immunoprecipitates were resolved by SDS-PAGE. Radiolaabeled PKC-δWT-HA and PKC-δS643A-HA bands were excised from the gel and exhaustively digested with trypsin (tosylphenylalanyl chloromethyl ketone-treated). The resulting phosphopeptides were resolved by thin layer electrophoresis, pH 8.9, followed by ascending chromatography, pH 1.9. Dried plates were autoradiographed for 1 week.

**In Vitro PKC-δ Activity Assay**—The method for the subcellular fractionation has been described previously (6, 13). Briefly, the 32D transfectants were serum-starved for 2 h and left untreated or stimulated with 100 ng/ml TPA (Sigma) for 10 min. The cell pellets were lysed in Triton X-100 containing lysis buffer (13) and clarified by centrifugation. For immunoprecipitation, equal amounts of proteins (1–5 μg per sample) were incubated with polyclonal anti-PKC-δ serum (5 μl per sample, Calbiochem) together with 40 μl of protein G-coupled Sepharose (Pharmacia Biotech, Inc.) or with anti-HA monoclonal antibody (mAb; 4 μg per sample, Boehringer Mannheim) together with 25 μl of protein A-Sepharose beads (Pierce). Anti-phosphotyrosine (anti-Tyr(P), 2 μg/ml, Upstate Biotechnology) and anti-PKC-δ (1:1000) were utilized for immunoblot analysis. The enhanced chemiluminescence system (Amersham Corp.) was used to visualize proteins, and the densities of the bands from SDS-PAGE and autoradiography were quantified by using a densitometer (Molecular Dynamics). The method for the subcellular fractionation has been described previously (6, 13).

**Flow Cytometry**—32D cells or 32D transfectants were untreated or exposed to TPA (100 ng/ml) overnight. Cells were incubated with fluorescein isothiocyanate-conjugated anti-Mac-1 (CalTag) or anti-FcγRI/II receptor (anti-FcγRI/II, Pharmigen) as described previously (6, 13). The cells were subjected to flow cytometry using a Becton-Dickinson FACScan.
**RESULTS**

**Mutation of PKC-δ Serine 643 and Expression of This Mutant in 32D Cells**—In an attempt to define which amino acids within PKC-δ are autophosphorylation sites and determine whether mutation of one of these sites would affect PKC-δ enzymatic activity, we chose to mutate serine 643 to alanine by site-directed mutagenesis. This putative autophosphorylation site is conserved in other PKC sequences, including PKC-α, PKC-βI, and PKC-βII (Fig. 1). In vivo phosphopeptide mapping or site-directed mutagenesis of the corresponding threonine sites within PKC-α, PKC-βI, and PKC-βII revealed that these residues were all phosphorylated in vivo (20–23, 28, 29). The mutant cDNA, designated PKC-δS643A, was inserted into the pCEV-HA (3 × HA repeats) vector, generating pCEV-S643A-HA, or into pLTR-HA vector (2 × HA repeats), generating pLTR-S643A-HA. PKC-δWT cDNA was previously inserted into these same vectors and designated pCEV-δWT-HA and pLTR-δWT-HA, respectively (30).

32D cells were transfected with expression vectors containing the various cDNA constructs, and drug-resistant 32D transfectants were subjected to immunoprecipitation and immunoblot analysis to detect PKC-δS643A and PKC-δWT expression. As shown in Fig. 2, immunoprecipitation with anti-PKC-δ serum followed by immunoblot analysis with the anti-HA mAb detected both pLTR-δWT-HA and pCEV-δWT-HA proteins with mobilities of 80 and 90 kDa, respectively. The mobilities of PKC-δWT proteins expressed in these two vectors were identical to those reported in our previous study (30). Endogenous PKC-δ expression in 32D cells was not detected, since the anti-HA mAb was utilized for immunoprecipitation. The levels of PKC-δS643A expression in cells transfected with pLTR-HA and pCEV-HA vectors were 2.8- and 1.8-fold higher than those of PKC-δWT in the corresponding vectors, respectively (Fig. 2).

**Autophosphorylation of the PKC-δS643A Mutant Is Reduced in Comparison to That of PKC-δWT**—We performed in vitro autophosphorylation assays utilizing the anti-HA mAb for immunoprecipitation. As shown in Fig. 3A, autophosphorylation of pLTR-δS643A-HA protein was reduced by 54% when compared with that of the pLTR-δWT-HA molecule. Autophosphorylation of the pCEV-δS643A-HA protein was decreased by 37% when compared with that of pCEV-δWT-HA (Fig. 3B). Autophosphorylation of endogenous PKC-δ from parental 32D cells was not detected since the anti-HA mAb would not recognize endogenous PKC-δ. By normalizing protein expression levels of PKC-δS643A in comparison to those of PKC-δWT in the various transfectants (see Fig. 2), an 83% reduction in pLTR-δS643A-HA autophosphorylation and a 65% reduction in pCEV-δS643A-HA autophosphorylation were observed. These results strongly suggest that serine 643 of PKC-δ is a major autophosphorylation site, and mutation of this site dramatically reduces autophosphorylation.

**Comparison of Tryptic Phosphopeptides Generated from PKC-δS643A and PKC-δWT by Two-dimensional Phosphopeptide Analysis**—To confirm that serine 643 is an in vivo phosphorylation site, two-dimensional tryptic phosphopeptide analysis was performed. As shown in Fig. 4A, tryptic digestion of in vivo labeled PKC-δWT-HA from the TPA-treated transfectant resulted in the detection of approximately 20 distinct phosphopeptides. The phosphopeptide pattern generated from PKC-δWT-HA is consistent to that generated in a previous study (30), assuring that this assay is very reproducible. Although most of PKC-δWT-HA phosphopeptides were also detected from tryptic digestion of in vivo labeled PKC-δS643A-HA after TPA treatment of 32D/pLTR-δS643A-HA transfectant, two phosphopeptides (peptides 5 and 14) were absent from PKC-δS643A-HA sample. The reduced intensity of peptide 5 in a mixture experiment, where equal amounts of PKC-δWT-HA and PKC-δS643A-HA samples were mixed before performing two-dimensional phosphopeptide analysis, confirmed that pep-
Table I

| Cell lines       | Total catalytic activity | Nonspecific activity | PKC-δ activity |
|------------------|--------------------------|----------------------|----------------|
| 32D/pLTR-S643A-HA| 1,401,383                | 246,742              | 1,154,641      |
| 32D/pLTR-S643A-HA| 747,810                  | 214,401              | 533,409 (54%)  |

* The % inhibition of enzymatic activity was determined by subtracting the PKC-δ activity of the pLTR-S643A-HA transfecant from the pLTR-δWT-HA transfectant and dividing the difference by the activity of the pLTR-δWT-HA transfectant.

Table II

| Eluates of DE52 column | Total catalytic activity | Nonspecific activity | PKC-δ activity |
|-------------------------|--------------------------|----------------------|----------------|
| 32D                     | 14,060 ± 503             | 6,438 ± 432          | 7,622          |
| 32D/pLTR-δWT-HA         | 130,123 ± 8,326          | 20,051 ± 264         | 110,072        |
| 32D/pLTR-S643A-HA       | 73,106 ± 3,786           | 11,708 ± 332         | 61,398 (39%)   |

* The % inhibition of enzymatic activity was determined by subtracting the PKC-δ activity of the pLTR-S643A-HA transfecant from the pLTR-δWT-HA transfectant and dividing the difference by the activity of the pLTR-δWT-HA transfectant.
amounts of the various cell lysates were enriched for PKC-
entiation would be affected. Treatment of the pLTR-
32D cells overexpressing PKC-
derived peptide as a substrate (6). The PKC-δ specific activity was calculated by subtracting the nonspecific catalytic activity from the total catalytic activity as described in Table II. The variation between the three samples utilized to calculate the mean value of the total catalytic and nonspecific activity was less than 5% of the mean value. The lines with diamonds, circles, and squares represent PKC-specific activity from 32D/pLTR-δWT-HA, 32D/pLTR-δS643A-HA, and the parental 32D line, respectively.

As seen in Fig. 7, stimulation of pLTR-δWT transfectant (data not shown). Flow cytometric analysis was used to detect cell surface differentiation marker expression. As seen in Fig. 7, stimulation of pLTR-δWT-HA transfectant with TPA overnight resulted in reduced cell adhesion and less morphological changes indicative of the macrophage phenotype as analyzed by Wright-Giemsa staining when compared with the pLTR-δWT-HA transfectant (data not shown). Flow cytometric analysis was utilized to detect cell surface differentiation marker expression. As seen in Fig. 7, stimulation of pLTR-δWT-HA transfectant with TPA overnight resulted in increased expression of Mac-1 (Fig. 7A) and FcγRII/III (Fig. 7B). TPA treatment of the pLTR-δS643A-HA mutant transfectant resulted in reduced increases in marker expression in comparison to the pLTR-δWT-HA transfectant (Fig. 7, A and B). However, the TPA-induced increase in marker expression observed for the pLTR-δS643A-HA mutant transfectant was still greater than that for the parental 32D cells, indicating that the remaining kinase activity provided by the pLTR-δS643A-HA mutant was able to partially mediate the differentiation process. These results suggest that serine autophosphorylation on amino acid 643 plays an important role in PKC-δ-mediated monocytic differentiation of 32D myeloid progenitor cells.

**DISCUSSION**

In the present study, we have demonstrated that serine 643 of PKC-δ is a major autophosphorylation site in vitro and autophosphorylation of PKC-δ on this site is required for its full enzymatic activity. TPA-induced monocytic differentiation of 32D cells overexpressing PKC-δS643A is reduced in comparision to the PKC-δWT transfectant, suggesting that the mutant protein is less efficient at activating key substrate(s) which affect the differentiation process. The effects of site-directed mutagenesis of PKC-α and PKC-βI at similarly conserved sites were recently reported (23, 29). Although no in vitro autophosphorylation data were presented in either study, transphosphorylation of the histone substrate in vitro by PKC-αT638A mutant was reduced by 26% (23). In contrast, the PKC-βIT642A mutant completely abolished in vivo phosphorylation and enzymatic activity (29). Whether mutagenesis of PKC-βI affected the general conformation of the protein remains to be determined. This was suggested by the inability to label in vivo the PKC-βIT642A mutant protein with [32P]orthophosphate. Although an ATP binding mutant of PKC-δ (PKC-δK376R) generated in our laboratory was completely devoid of autophosphorylation capacity (6), it could still be labeled in vivo by [32P]orthophosphate. Two-dimensional phosphopeptide mapping of the PKC-δK376R mutant revealed that at least two autophosphorylation sites were absent when compared with PKC-δWT, indicating that other sites in addition to serine 643

2 W. Li, unpublished observations.
Autophosphorylation of Serine 643 in PKC-δ is diminished by only 65–83% in the mutant (see Fig. 3). Thus, alternative autophosphorylation sites may compensate and allow the mutant protein to normally regulate localization through phosphorylation and dephosphorylation dynamics.

In summary, our results demonstrate that serine 643 is a major autophosphorylation site of PKC-δ. Autophosphorylation of PKC-δ on this site is indispensable for its full enzymatic activity but is not required or sufficient for determining the localization, translocation, or tyrosine phosphorylation of PKC-δ. Mapping the remaining autophosphorylation site(s) within PKC-δ should make it feasible to determine the complete role of autophosphorylation and its effects on the various aspects of PKC-δ function.

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