THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 272, No. 39, Issue of September 26, pp. 24550–24555, 1997
Printed in U.S.A.

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

(Received for publication, April 9, 1997, and in revised form, July 22, 1997)

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 transfectant. 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)[1] 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 (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 β 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-α (23) and βII (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, Bldg. 37, Rm. 1E24, 9000 Rockville Pike, Bethesda, MD 20892. Tel.: 301-496-1347; Fax: 301-496-8479. E-mail: Liwe@dc37a.nci.nih.gov.

¶ Recipient of the American Cancer Society Junior Faculty Award and also supported by American Chemical Society Research Grant IM-782.

[1] The abbreviations used are: PKC, protein kinase C; WT, wild type; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; HA, hemagglutinin; PDGF-βR, platelet-derived growth factor β receptor; PAGE, polyacrylamide gel electrophoresis; anti-Tyr(P), anti-phosphotyrosine; mAb, monoclonal antibody.

δ	630	N F D P **E** F L N E K P Q - L **S** F S D
a	625	N F D K F F T R G Q P V - L **T** P P D
βI	629	N F D K E F T R - Q P V E L **T** P T D
βII	628	N F D R F F T R H P P V - L **T** P P D

FIG. 1. **Serine 643 of PKC-δ is a conserved phosphorylation site in other PKC isoenzymes.** Sequence alignment is based on the previous report described by Keranen *et al.* (20). *In vivo* phosphorylation of PKC-α, -βI, and -βII at the threonine sites 638, 642, and 641, respectively, has been demonstrated (20, 23, 29). These sites correspond to serine 643 in PKC-δ. The conserved residues are shown as *bold letters*.

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 isoenzymes have been mapped (20–22). Recently, conserved threonine autophosphorylation sites on two cPKCs (PKC-α and PKC-βI) 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-βI 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 *in vitro* mutagenesis kit (version 2) was used for the site-directed mutagenesis. The oligonucleotide 5′-GAATGAGAAACCTCAGCTTGCATTCAG-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 *Bsm*I restriction site that was used to screen all the reaction products. The mutation was confirmed by DNA sequencing. The PKC-δS643A mutant cDNA was subcloned into pCEV-HA (three hemagglutinin epitope repeats, *neo* selection) and pLTR (two HA epitope repeats, *gpt* selection) vectors, generating pCEV-δS643A-HA and pLTR-δS643A-HA, respectively. The generation of these two vectors and subcloning of PKC-δWT cDNA into these vectors have been previously described (6, 30). The 32D cells were transfected with different cDNA expression vectors using the electroporation procedure described previously (5). 32D cells and transfectants were cultured in RPMI 1640 medium with 10% fetal calf serum and 5% WEHI-3B conditioned medium as a source of murine interleukin-3.

Immunoprecipitation, Immunoblot Analysis, and Subcellular Fractionation—These procedures have been described previously (6, 10, 13, 30). 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 mg 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 before (6, 13).

In Vitro PKC-δ Autophosphorylation Assay—The *in vitro* autophosphorylation assay utilizing anti-HA antibody for immunoprecipitation was performed by following a previously described protocol (6). Briefly, cell lysates were immunoprecipitated with anti-HA antibody as described above. Washed immunoprecipitates were incubated on ice for 30 min with 50 μl of autophosphorylation buffer that contained 20 mM Tris-HCl, pH 7.5, 5 mM magnesium acetate, 50 μg/ml phosphatidylserine (Sigma), 100 ng/ml TPA, 10 μg/ml leupeptin, 1 mM Na_3VO_4, 1 μM ATP (Boehringer Mannheim), and 5 μCi of [γ-^{32}P]ATP (3000 Ci/mmol, Amersham Corp.). The reaction was stopped by washing twice with Triton X-100 containing lysis buffer, and denatured proteins were separated by SDS-PAGE. The dried gel was autoradiographed.

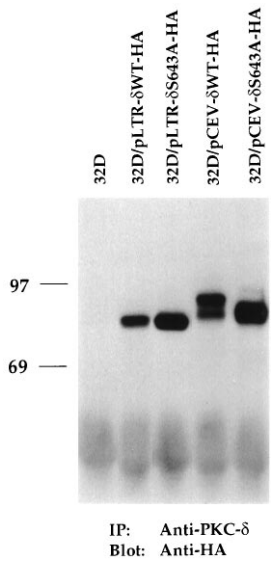


FIG. 2. **The PKC-δS643A mutant protein is expressed in the various 32D transfectants.** Equal amounts of cell lysates (4 mg per sample) from 32D cells and transfectants were immunoprecipitated (*IP*) with anti-PKC-δ serum. Washed immunoprecipitates were subjected to SDS-PAGE, and proteins transferred to an Immobilon membrane were immunoblotted (*Blot*) with anti-HA mAb.

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 [^{32}P]orthophosphate (1 mCi/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 mAb, and immunoprecipitates were resolved by SDS-PAGE. Radiolabeled 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 Assays—DE52 ion exchange chromatography to enrich PKC from the cell lysates and the subsequent measurement of PKC activity utilizing PKC-δ pseudosubstrate region-derived peptide as a substrate have been described previously (6, 13, 30). Direct measurement of PKC-δ activity on PKC-δ substrate utilizing anti-HA immunoprecipitates as the kinase sources was also employed. Briefly, the equal amounts of protein (6 mg per sample) from the various PKC-δ transfectants were immunoprecipitated with anti-HA antibody (4 μg per sample). Washed immunoprecipitates were incubated at room temperature with 40 μl of reaction buffer that contained 10 μM PKC-δ substrate derived from PKC-δ pseudosubstrate region (6), 20 mM Tris-HCl, pH 7.5, 1 mM $CaCl_2$, 10 μM magnesium acetate, 1 μM TPA, 50 μg/ml phosphatidylserine (Sigma), 30 μM ATP, and 30 μCi of [γ-^{32}P]ATP for 20 min. The reaction tube was centrifuged, and 20 μl of the supernatant was spotted on phosphocellulase disk sheets (Life Technologies, Inc.). The sheets were washed twice with 1% phosphoric acid and twice with distilled water, and samples were analyzed by liquid scintillation. The nonspecific catalytic activity was measured in the same reaction buffer except that TPA and phosphatidylserine were omitted from the reaction. The specific PKC-δ activity was calculated by subtracting the nonspecific catalytic activity from the total catalytic activity and expressed as counts per min (cpm).

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-FcγII/III receptor (anti-FcγII/IIIR, Pharmigen) as described previously (6, 30). The cells were subjected to flow cytometry using a Becton-Dickinson FACScan.

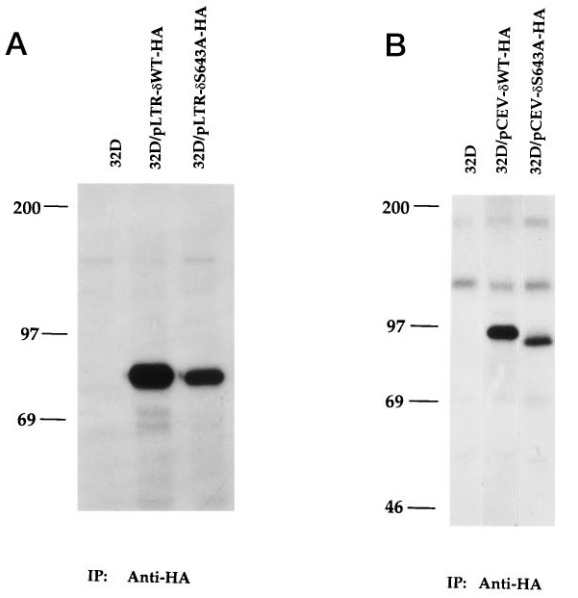


FIG. 3. **Autophosphorylation of PKC-δS643A *in vitro* is dramatically reduced in comparison to PKC-δWT.** *A*, 32D cells and pLTR-HA transfectants were serum-starved for 2 h, and equal amounts of cell lysates were immunoprecipitated (*IP*) with anti-HA antibody. Washed immunoprecipitates were subjected to an *in vitro* autophosphorylation assay (see "Experimental Procedures"). Radiolabeled proteins were resolved by SDS-PAGE and autoradiographed. *B*, the experiment was performed in a similar manner to that in *A* except that the transfectants generated with the pCEV-HA vector were utilized for the autophosphorylation assay.

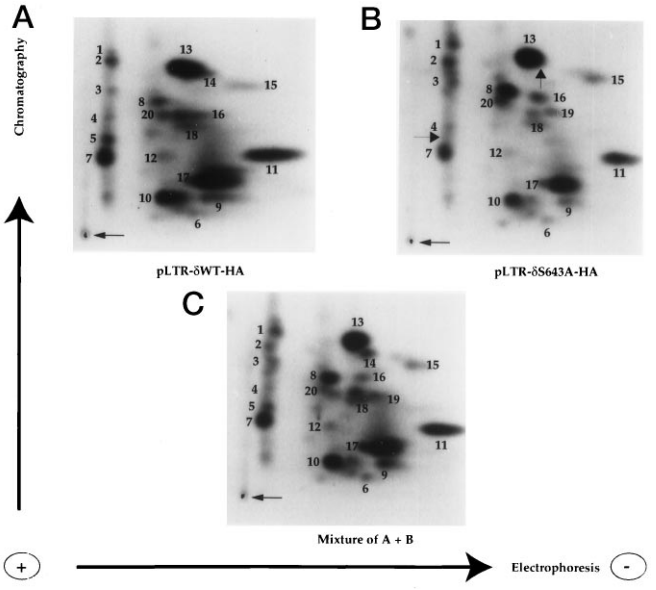


FIG. 4. **Two-dimensional phosphopeptide analysis of PKC-δS643A.** The radiolabeled PKC-δWT-HA and PKC-δS643A-HA proteins from TPA-stimulated transfectants were immunoprecipitated with anti-HA, excised after SDS-PAGE, and subjected to phosphopeptide analysis as described under "Experimental Procedures." The directions for electrophoresis and chromatography are marked by *long arrows*. The individual phosphopeptides are designated by *numbers*. After trypsin digestion, 2000 cpm from each sample were subjected to two-dimensional phosphopeptide analysis (*A* and *B*). The mixture in *C* was generated by including 1000 cpm of the sample in *A* plus 1000 cpm of the sample in *B*. The points of origin in each panel are marked by *arrows*. Two missing phosphopeptides (5 and 14) in PKC-δS643A-HA (*B*) are also marked by *arrows*.

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-δS643A-HA, or into pLTR-HA vector (2 × HA repeats), generating pLTR-δS643A-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. 3*A*, 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. 3*B*). 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. 4*A*, 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-

tide 5 was missing in PKC-δS643A-HA (compare peptide 5 in Fig. 4, *A* and *C*). Since the PKC-δWT-HA sample migrated slightly slower than the others in chromatography, only a tail of peptide 14 can be observed (Fig. 4*A*). This peptide was not detected in PKC-δS643A-HA sample (Fig. 4*B*). Therefore, whether the intensity of peptide 14 detected in the mixture experiment was reduced (Fig. 4*C*) is difficult to judge. In addition, the intensity of peptide 11 was greatly reduced in the PKC-δS643A-HA sample when compared with PKC-δWT-HA, and intermediate intensity was observed in the mixture experiment (Fig. 4*C*). On the other hand, phosphopeptide 19 may be absent in PKC-δWT-HA. Taken together, the results of two-dimensional phosphopeptide analysis clearly indicate that the absence or reduction in intensity of phosphopeptides 5, 14, and 11 may account for the reduced autophosphorylation of PKC-δS643A *in vitro* (see Fig. 3).

Enzymatic Activity of PKC-δS643A Mutant Is Greatly Decreased in Comparison to That of PKC-δWT—The enzymatic activity of PKC-δS643A expressed in pLTR-HA system was measured utilizing two separate procedures. In the first assay, the activities were measured utilizing anti-HA immunoprecipitates as the kinase sources. This method has been recently used in other PKC studies to measure PKC activity (8, 31). As shown in Table I, the immunoprecipitates derived from pLTR-δWT-HA and pLTR-δS643A-HA mutant transfectants displayed similar nonspecific catalytic activities when they were incubated with the PKC-δ pseudosubstrate region-derived peptide in the absence of TPA and phosphatidylserine, two important cofactors required for specific PKC activation *in vitro*. However, the specific PKC-δ catalytic activity of pLTR-δS643A-HA mutant was reduced by 54% when compared with that of pLTR-δWT-HA protein.

In another PKC activity assay, DE52 ion exchange chromatography was utilized to enrich PKC-δ proteins before performing the kinase assay (6, 30). pLTR-δWT-HA overexpression resulted in a 14-fold increase in the enzymatic activity compared with that of endogenous PKC-δ (Table II). The increased activity observed in the pLTR-δWT-HA transfectant correlated with the levels of overexpressed PKC-δ protein (data not shown). Expression of pLTR-δS643A-HA reduced its specific catalytic activity by 39% compared with that of pLTR-δWT-HA. By normalizing the protein expression level of pLTR-δS643A-HA in comparison to that of pLTR-δWT-HA, a 78–84% reduction in pLTR-δS643A-HA enzymatic activity was calculated from the results of these two assays (see Fig. 2). In summary, these results indicate that PKC-δ serine 643 is not only important for autophosphorylation but also for transphosphorylation of its *in vitro* substrate.

The PKC-δS643A Mutant Protein Is Not Thermal Labile—Recent work on the PKC-αT638A mutant suggested that mutation of threonine 638 rendered the enzyme very sensitive to heat treatment (23). Thus, we were interested in determining whether there were any changes in the heat sensitivity of PKC-δS643A mutant in comparison to PKC-δWT. As shown in Fig. 5, pLTR-δWT-HA, pLTR-δS643A-HA, and endogenous PKC activities remained very stable even after a 30-min period of preincubation at 25 °C. Surprisingly, both pLTR-δWT-HA and pLTR-δS643A-HA mutant activities were slightly increased after the preincubation period. This result suggests that phosphorylation of PKC-δ on serine 643 does not affect the heat stability of the enzyme, even though the enzymatic activity and autophosphorylation of PKC-δS643A are greatly reduced in comparison to PKC-δWT.

Localization, Translocation, and Tyrosine Phosphorylation of PKC-δ Are Not Altered When Serine 643 Autophosphorylation Is Abolished—PKC-δ normally resides in the cytosol (S100) of the cell. In response to stimulation by TPA, a portion translocates to the membrane fraction (P100) (13). Our previous data demonstrated that PKC-δ was tyrosine-phosphorylated *in vivo* in response to TPA stimulation, and tyrosine-phosphorylated PKC-δ could be detected only in the membrane fraction (6, 10, 13, 30). Thus, we investigated whether mutation of serine 643 would affect localization, translocation, or tyrosine phosphorylation of the enzyme. As shown in Fig. 6*A*, the pLTR-δS643A-HA mutant as well as pLTR-δWT-HA proteins resided in the cytosol in resting cells after cell fractionation and immunoprecipitation with anti-HA mAb followed by anti-PKC-δ immunoblot analysis. Stimulation with TPA for 10 min caused translocation of a similar portion of both pLTR-δWT-HA and pLTR-δS643A-HA mutant proteins to the membrane fraction (Fig. 6*A, lanes 4* and *6*). Reblotting the membrane with anti-Tyr(P) mAb showed that both pLTR-δWT-HA and pLTR-δS643A-HA mutant proteins were tyrosine-phosphorylated in TPA-stimulated samples (Fig. 6*B, lanes 4* and *6*). As previously demonstrated (6, 13), tyrosine phosphorylation was observed only in the membrane fraction. Taken together, the results indicate that autophosphorylation of PKC-δ on serine 643 does not affect localization, translocation, or tyrosine phosphorylation of the enzyme.

TPA-induced Monocytic Differentiation of 32D Cells Medi-

TABLE I

The PKC-δS643A mutant protein expressed in 32D cells possesses reduced enzymatic activity as measured by utilizing anti-HA immunoprecipitates as PKC-δ kinase sources

The method for measuring PKC-δ activity by using anti-HA immunoprecipitates as PKC-δ sources was described under "Experimental Procedures." Only one sample from each lysate was utilized for anti-HA immunoprecipitation and the subsequent activity assay. Thus, no standard deviation was available. PKC-δ specific activity was obtained by subtracting nonspecific activity from total catalytic activity. The activity is presented as cpm.

Cell lines	Total catalytic activity	Nonspecific activity	PKC-δ activity
32D/pLTR-δWT-HA	1,401,383	246,742	1,154,641
32D/pLTR-δS643A-HA	747,810	214,401	533,409 (54%)[a]

[a] The % inhibition of enzymatic activity was determined by subtracting the PKC-δ activity of the pLTR-δS643A-HA transfectant from the pLTR-δWT-HA transfectant and dividing the difference by the activity of the pLTR-δWT-HA transfectant.

TABLE II

The PKC-δS643A mutant protein expressed in 32D cells possesses reduced enzymatic activity as measured by utilizing DE52 column eluates as PKC-δ kinase sources

The method for PKC enrichment by DE52 ion exchange chromatography and the subsequent activity assay has been described previously (6, 30). PKC-δ specific activity was obtained by subtracting nonspecific activity from total catalytic activity. The results represent the mean value of three individual samples. The activity is presented as cpm.

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-δS643A-HA	79,106 ± 2,786	11,708 ± 332	67,398 (39%)[a]

[a] The % inhibition of enzymatic activity was determined by subtracting the PKC-δ activity of the pLTR-δS643A-HA transfectant from the pLTR-δWT-HA transfectant and dividing the difference by the activity of the pLTR-δWT-HA transfectant.

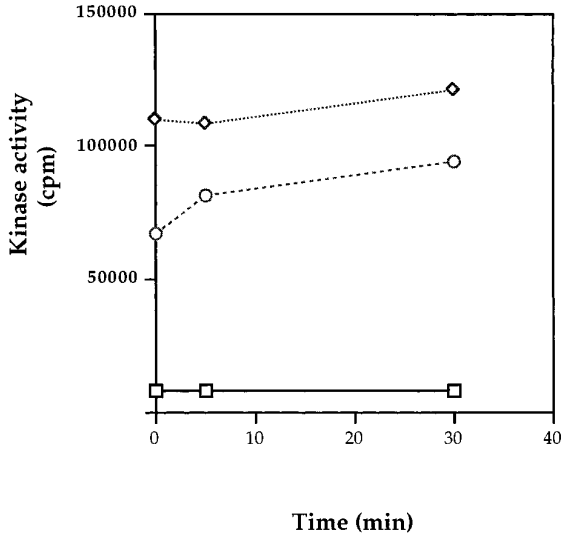
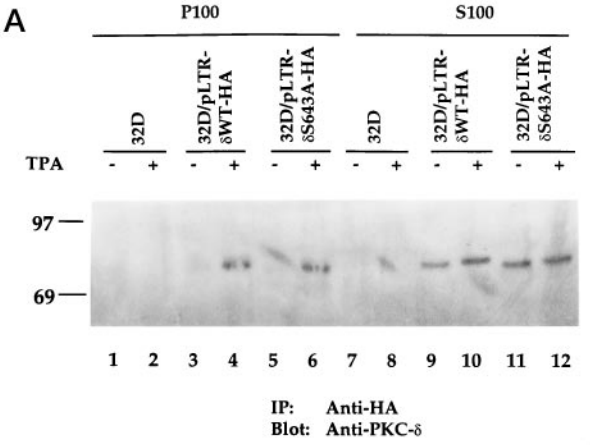


FIG. 5. **PKC-δS643A mutant protein is thermal stable.** Equal amounts of the various cell lysates were enriched for PKC-δ using DE52 chromatography. Eluates were incubated in a 25 °C water bath for various periods and assayed for PKC-δ activity using the PKC-δ pseudosubstrate region-derived peptide 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.



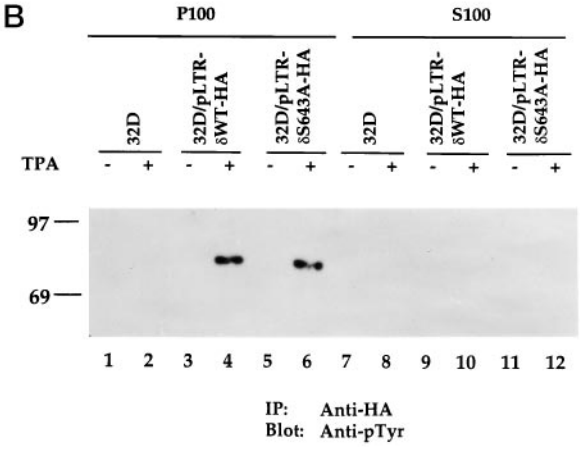


FIG. 6. **Localization, translocation, and tyrosine phosphorylation of the PKC-δS643A mutant protein are not altered in comparison to PKC-δWT.** *A*, 32D cells and the transfectants were serum-starved for 2 h and stimulated with TPA for 10 min. The membrane fraction (*P100*) was separated from cytosolic fraction (*S100*) according to previously established methods (13). Equal amounts of proteins were immunoprecipitated (*IP*) with anti-HA mAb and resolved by SDS-PAGE. Transferred proteins were immunoblotted (*Blot*) with anti-PKC-δ serum. *B*, the same Immobilon membrane utilized in *A* was reblotted (*Blot*) with anti-Tyr(P) mAb.

ated by the PKC-δS643A Mutant Transfectant in Comparison to the PKC-δWT Transfectant Is Impaired—TPA treatment of 32D cells overexpressing PKC-δWT was able to mediate monocytic differentiation, as judged by changes in morphology, cell adhesion, nonspecific esterase staining, and cell surface differentiation marker expression (5, 6). Since mutation of PKC-δ on serine 643 reduced its autophosphorylation and its enzymatic activity, we tested whether PKC-δ-mediated monocytic differentiation would be affected. Treatment of the pLTR-δS643A-HA mutant 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. 7*A*) and FcγII/IIIR (Fig. 7*B*). 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 comparison 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 [^{32}P]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 [^{32}P]orthophosphate.[2] 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.

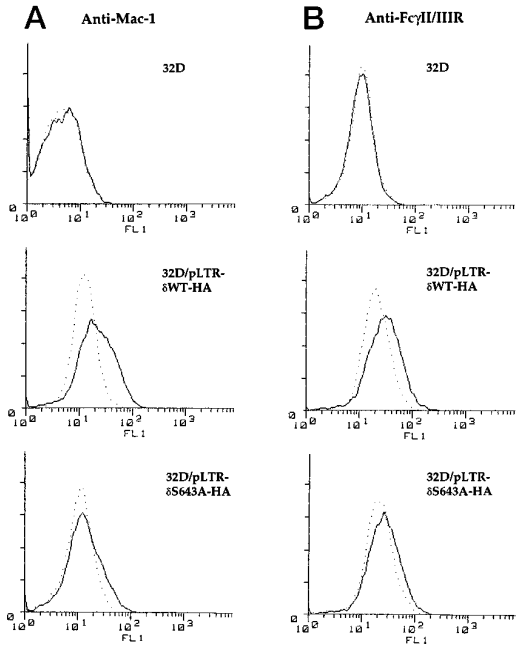


FIG. 7. **Monocytic differentiation mediated by PKC-δS643A mutant expression in 32D cells in response to TPA treatment is impaired.** Cells were either untreated (···) or exposed to TPA (—) overnight and subjected to flow cytometry after incubation with anti-Mac-1 (*A*) or anti-FcγII/IIIR (*B*) antibodies conjugated with fluorescein isothiocyanate. The *x* axis represents the mean fluorescence intensity of fluorescein isothiocyanate and *y* axis represents relative cell number.

must contribute to PKC-δ autophosphorylation.[2] Moreover, the present results provide evidence that autophosphorylation of the PKC-δS643A mutant is not completely abolished (see Fig. 3). PKC-δS643A mutant could be labeled *in vivo* to a similar extent as PKC-δWT (see Fig. 4). Based on recent mapping and site-directed mutagenesis results involving PKC-α at serine 657 (24) and PKC-βII at serine 660 (20), we predict that the corresponding serine 662 of PKC-δ may be an additional autophosphorylation site.

Generation of a serine 643 to alanine mutant of PKC-δ did not affect the translocation of PKC-δ from the cytosol to the membrane in response to TPA stimulation, nor did it affect its tyrosine phosphorylation *in vivo*. These data indicate that site-directed mutagenesis did not alter the general conformation of the molecule. This is also suggested by the similar two-dimensional phosphopeptide pattern observed for both PKC-δWT and PKC-δS643A (see Fig. 4, *A* and *B*). Translocation of PKC from the cytosol to the membrane is dependent on the binding of phorbol ester or endogenously produced diacylglycerol to the regulatory domain of PKC (1). Tyrosine phosphorylation of PKC-δ has also been mapped at the N terminus of PKC-δ (30). Therefore, it was not surprising that mutation of serine 643 did not affect these events since this mutation resides in the C terminus of the molecule. Although phosphorylation has been implicated to be important for PKC localization, expression of PKC-δS643A did not alter the localization of the molecule. This can be best explained by the finding that *in vitro* autophosphorylation was 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.

Acknowledgment—We are grateful to Nelson Ellmore for excellent technical assistance.

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