Identification of Tyrosine 187 as a Protein Kinase C-δ Phosphorylation Site*

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Protein kinase C-δ (PKC-δ) has been demonstrated to be phosphorylated on tyrosine residue(s) in many different biological systems (Li, W., Yu, J.-C., Michieli, P., Beeler, J. F., Ellmore, N., Heidaran, M. A., and Pierce, J. H. (1994) Mol. Cell. Biol. 14, 6727–6735; Li, W., Misshak, H., Yu, J.-C., Wang, L.-M., Mushinski, J. F., Heidaran, M. A., and Pierce, J. H. (1994) J. Biol. Chem. 269, 2349–2352; Denning, M. F., Dlugosz, A. A., Howett, M. A., and Yuspa, S. H. (1993) J. Biol. Chem. 268, 26079–26081). Tyrosine phosphorylation of PKC-δ has also been shown to occur in vitro when purified PKC-δ is coincubated with different tyrosine kinase sources. However, the tyrosine phosphorylation site(s) is currently unknown and the exact effect of this phosphorylation on its serine/threonine kinase activity and biological functions is still controversial. To directly investigate the potential role of PKC-δ tyrosine phosphorylation, tyrosine 187 was converted to phenylalanine (PKC-δY187F) by site-directed mutagenesis, and expression vectors containing PKC-δY187F cDNAs were transfected into both 32D myeloid progenitor cells and NIH 3T3 fibroblasts. The results showed that tyrosine 187 of PKC-δ became phosphorylated in vitro in response to 12-O-tetradecanoylphorbol-13-acetate stimulation or platelet-derived growth factor receptor activation. In vitro labeling and subsequent two-dimensional phosphopeptide analysis demonstrated that one phosphopeptide was absent in PKC-δY187F when compared to wild type PKC-δ, further substantiating that tyrosine 187 of PKC-δ is phosphorylated in vivo. Although the phosphotyrosine content of PKC-δY187F was reduced compared with PKC-δWT, the kinase activity of PKC-δY187F toward a PKC-δ substrate was not altered. Moreover, 12-O-tetradecanoylphorbol-13-acetate-mediated monocyte cytodifferentiation of 32D cells was not affected by expression of the PKC-δY187F mutant. Taken together, these results suggest that tyrosine phosphorylation of PKC-δ on 187 may not influence PKC-δ activation and known functions.

Protein kinase C-δ (PKC-δ) is a serine/threonine kinase. It belongs to a novel PKC subfamily that can be distinguished from the other subgroups by the lack of a calcium binding domain in the regulatory region (1, 2). When PKC-δ was over-expressed in the 32D myeloid progenitor line, the cells underwent monocyte differentiation in response to 12-O-tetradecanoylphorbol-13-acetate (TPA) stimulation (3). PKC-δ was also defined as an important downstream molecule in the platelet-derived growth factor receptor (PDGF-βR) signaling pathway (4, 5). PKC-δ autophosphorylation, translocation from the cytosol to the membrane and membrane-associated enzymatic activity were all increased in response to PDGF stimulation in both NIH 3T3 fibroblasts overexpressing PKC-δ and 32D cells coexpressing PDGF-βR and PKC-δ. Coexpression of PDGF-βR with PKC-δ in 32D cells allowed monocyctic differentiation of the transfectants in response to PDGF-BB stimulation (4). More recently, an ATP-binding mutant of PKC-δ was generated that inhibited wild type PKC-δ (PKC-δWT) activity in vitro (6). Expression of this mutant severely impaired Src-induced transformation of NIH 3T3 cells (13). All of these results indicate that PKC-δ plays an important role in PDGF-mediated biological responses, including monocyctic differentiation and cellular transformation.

It has been demonstrated that PKC-δ becomes phosphorylated on tyrosine in many different systems. Initially, we found that PKC-δ was phosphorylated on tyrosine in response to TPA stimulation when PKC-δ was over-expressed in either 32D hematopoietic cells or NIH 3T3 fibroblasts (7). Interestingly, an inactive ATP-binding mutant of PKC-δ-δ, designated PKC-δK376R, was found to be constitutively phosphorylated on tyrosine residue(s) (6). Constitutive tyrosine phosphorylation of PKC-δ was also observed in Ras-transformed murine keratinocytes (8). PKC-δ from freshly dispersed parotid acinar cells became tyrosine phosphorylated in response to carbachol, substance P or TPA stimulation (9). When rat basophilic leukemia cells were stimulated with an IgE receptor cross-linking agent, endogenous PKC-δ was also phosphorylated on tyrosine (10). Recently, PKC-δ was also found to be tyrosine phosphorylated in response to epidermal growth factor stimulation (12). Although tyrosine phosphorylation of PKC-δ has been demonstrated to occur in response to a variety of stimuli, the phosphorylation site(s) and effect of this phosphorylation on PKC-δ serine/threonine kinase activity are still unclear. When purified PKC-δ was coincubated with different tyrosine kinase sources, such as the PDGF-βR, Lyn, Src, and insulin receptor in vitro, it also became tyrosine phosphorylated (7, 11). Subsequent in vitro measurement of PKC-δ activity on a PKC-δ pseudosubstrate region-derived substrate demonstrated that...
Phosphorylation of Tyrosine 187 in PKC-δ

Fig. 1. Conversion of PKC-δ tyrosine 187 to phenylalanine inhibits its tyrosine phosphorylation in response to TPA stimulation in the 32D cell system. A, 32D cells and 32D/pCEV transfectants were either unstimulated or stimulated with TPA for 10 min and lysed. Equivalent amounts of proteins were subjected to SDS-PAGE and immunoblotted (Blot) with anti-HA and anti-PKR antibodies. B, equivalent amounts of proteins were subjected to SDS-PAGE and transferred proteins were immunoblotted (Blot) with anti-PKR monoclonal antibody. C, The 32D/pLTR transfectants were untreated or stimulated with TPA for 10 min and lysed. Equivalent amounts of proteins were immunoprecipitated (IP) with anti-HA and transferred proteins were immunoblotted (Blot) with anti-PKR monoclonal antibody. D, The same blot from C was reblotted with anti-PKR serum and proteins were visualized by the ECL detection system. Markers are given in kilodaltons.

PKC-δ activity was increased after PKC-δ was phosphorylated on tyrosine (7, 11). In contrast, PKC-δ activity was found to be lower in the tyrosine-phosphorylated fraction when compared with the unphosphorylated fraction in v-H-ras transformed murine keratinocytes (8).

In this report, we present evidence that PKC-δ tyrosine residue 187 is phosphorylated in response to TPA or PDGF stimulation in vivo. Our results also show that conversion of tyrosine 187 to phenylalanine does not alter PKC-δ activity in vitro and PKC-δ-mediated monocytic differentiation of 32D cells in vivo.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis, cDNA Cloning and Expression Vectors—The Bio-Rad Muta-gene Phagemid in vitro mutagenesis kit (version 2) was used to mutate tyrosine 187 of PKC-δ. The oligonucleotide 5'-CACGACGGCTTAAAATGGGCAA'A-3' was used as a mutant primer in the in vitro mutagenesis reaction where the tyrosine at amino acid 187 of murine PKC-δ was converted to phenylalanine (Y187F, underlined in the oligonucleotide sequence). The successful mutation of this site would generate a new SacI restriction site, which was used to screen all the reaction products. The mutation was confirmed by DNA sequencing. The PKC-δY187F mutant cDNA was inserted back into the pCEV-HA vector with SacI at the 5' end and EcoRI at the 3' end, generating pCEV-Y187F-HA. The generation of the pCEV-HA (three tandem repeats of hemagglutinin) vector and insertion of the PKC-δK376R mutant and wild type PKC-δ (PKC-δWT) cDNAs into this vector was described before (6). Tyrosine 187 was also converted to a phenylalanine in the PKC-δK376F mutant cDNA through the same mutagenesis strategy described above, and inserted into the pCEV-HA vector, generating a pCEV-K376F.Y187F-HA double mutant. The pLTR vector has been described before (3). To introduce PKC-δY187F into the pLTR vector, the PKC-δY187F insert in pCEV-HA vector was released by digesting with SacI at the 5' end and NdeI at the 3' end after the second HA epitope. The pLTR vector was linealized with EcoRI. Both the PKC-δY187F insert (SacI-NdeI fragment) and linealized pLTR vector were blunt-ended, ligated, and transformed using standard cloning procedures (14), generating pLTR-Y187F-HA. The same strategy was applied to generate pLTR-SWT-HA and pLTR-K376F-HA.

Transfection of 32D and NIH 3T3 Cells with the Various PKC-δ-Containing Vectors—The 32D cells were transfected by electroporation with 10 μg of each plasmid DNA and selected in the presence of genetin (750 μg/ml) or mycophenolic acid (80 μM) depending on the selectable marker present in the DNA vectors as described before (3). 32D cells and transfectants were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum and 5% WEHI-3B-conditioned medium as a source of interleukin-3. The calcium phosphate method was utilized for NIH 3T3 cell transfection (4). One μg of vector DNA containing the different PKC-δ cDNAs was used for transfection. The NIH 3T3 cells and transfectants were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum and selected for marker resistance as described above.

Immunoprecipitation and Immunoblot Analysis—Serum starvation, TPA (100 ng/ml), or PDGF-BB (100 ng/ml) stimulation, and cell lysis have been described before (4, 6, 7). For immunoprecipitation, equivalent amounts of proteins (3–5 mg/sample) were immunoprecipitated with an anti-HA monoclonal antibody (12CA5, 4 μg/sample; Boehringer Mannheim) or with anti-PKR-δ serum (5 μl/sample; R&D Antibodies). The precipitated proteins were subjected to sodium dodecyl sulfate-7.5% polyacrylamide gel electrophoresis (SDS-7.5% PAGE), and separated proteins were transferred to Immobilon membranes (Millipore). The transferred proteins were immunoblotted with a murine anti-phosphotyrosine monoclonal antibody (anti-Tyr(P), 4G10, 2 μg/ml; Upstate Biotechnology, Inc.) or with rabbit anti-PKR-δ serum (1 to 1000 dilution; R&D Antibodies). [125I]Conjugated protein A or the ECL detection system were used to visualize proteins. Protein levels were quantitated using a densitometer (Molecular Dynamics).

In Vitro PKC Activity Assay—The procedures for enrichment of PKC-δ from cell lysates by DE52 ion exchange chromatography (Bio-Rad) and the subsequent PKC activity assay using a PKC-δ pseudosubstrate region-derived peptide (MNRRSGIKQAKI; Peptide Technologies Corp.) as the substrate have been reported (4, 6). Briefly, cell lysates containing equal amounts of proteins were first enriched for PKC by one step DE52 ion exchange chromatography according to the protocol supplied by the company (Life Technologies, Inc.). Eluates (10 μl) were added on ice to 40 μl of kinase buffer containing 10 μM PKC-δ substrate peptide, 20 mM Tris-HCl, pH 7.5, 1 mM CaCl₂, 10 mM magnesium acetate, 1 μM TPA, 50 μM phosphatidylserine, 0.1 mM ATP, and 1 μCi [γ-32P]ATP. The reactions were incubated at 30 °C for 30 min, and 25 μl from each reaction was spotted onto phosphocellulose disk sheets (Life Technologies, Inc.). The sheets were washed twice with 1% phosphoric acid and twice with distilled water, and samples were counted by liquid scintillation. The nonspecific catalytic activity was measured as above except that TPA and phosphatidylserine were omitted from the reaction. The specific PKC activity was calculated by subtracting the non-
The in vivo labeling of 32D transfectants with \(^{32}\text{P}\) orthophosphate (7) and subsequent two-dimensional phosphopeptide analysis have been described previously (15). Briefly, the serum-starved 32D transfectants were labeled with \(^{32}\text{P}\) orthophosphate (1 mCi/ml; DuPont) for 3 h and then labeled with \(^{32}\text{P}\) orthophosphate for 3 h. The cells were either unstimulated or stimulated with TPA for 10 min, lysed, and separated by SDS-7.5% PAGE. The dried gel was autoradiographed for 30 min at room temperature.

### RESULTS

**Conversion of PKC-\(\delta\) Tyrosine 187 to Phenylalanine Severely Impairs Its TPA-induced Tyrosine Phosphorylation in Vivo in 32D Myeloid Progenitor Cells**—PKC-\(\delta\) was previously demonstrated to become phosphorylated on tyrosine residue(s) in response to TPA stimulation in vivo when it was overexpressed in either 32D or NIH 3T3 cells (4, 7). We have also observed that another member of the novel PKC subfamily, PKC-\(\epsilon\), is weakly phosphorylated on tyrosine residue(s) in vivo in a TPA-independent manner, while PKC-\(\alpha\) does not appear to be tyrosine phosphorylated before or after TPA stimulation (data not shown). Using “Intelligenes Suite of Molecular Biology” program, we aligned mouse PKC-\(\delta\), PKC-\(\epsilon\), and PKC-\(\alpha\) amino acid sequences. Because tyrosine 187 of PKC-\(\delta\) is the only conserved tyrosine in the corresponding PKC-\(\epsilon\) sequence and is not conserved in the PKC-\(\alpha\) sequence, we chose to mutate tyrosine 187 of PKC-\(\delta\) to phenylalanine by site-directed mutagenesis. The mutated cDNA was inserted into two expression vectors containing HA epitopes, designated pCEV-\(\delta\)Y187F-HA and pLTR-
tyrosine phosphorylation of 32D cells transfected with pCEV-δWT-HA and pCEV-δY187F-HA was subsequently analyzed by immunoprecipitation with anti-HA antibody followed by anti-Tyr(P) immunoblot analysis (Fig. 1A). Mutation of tyrosine 187 greatly reduced PKC-δ phosphotyrosine content in two 32D transfected lines when compared to that detected in two PKC-δWT transfectants. To determine protein expression levels in each transfectant, we performed direct immunoblot analysis on cell lysates utilizing a monoclonal antibody against PKC-δ. As shown in Fig. 1B, the monoclonal anti-PKC-δ antibody detected endogenous PKC-δ expressed in 32D cells and all the transfectants (lower bands, 80 kDa). Exogenously expressed PKC-δ proteins in the 32D/pCEV-δWT-HA or 32D/pCEV-δY187F-HA transfectants were detected in the 90 kDa size range due to the HA epitope sequences present in the expression vector. The PKC-δY187F levels expressed in two different lines were 86% and 53% of the level present in pCEV-δWT-HA.1 (Fig. 1B). After normalizing for protein expression levels, the PKC-δ phosphotyrosine content in 32D/pCEV-δY187F-HA.1 and 32D/pCEV-δY187F-HA.2 accounted for 23% and 17%, respectively, of that detected in pCEV-δWT-HA.1.

To further substantiate that tyrosine 187 was a PKC-δ phosphorylation site, we inserted PKC-δY187F and PKC-δWT cDNAs into the pLTR-HA expression vector and determined the PKC-δ phosphotyrosine content from each 32D transfectant utilizing this different expression vector system by immunoprecipitation with anti-HA followed by immunoblot analysis with anti-Tyr(P). As shown in Fig. 1C, mutation of tyrosine 187 reduced its phosphotyrosine content by 95% and 99% (faint tyrosine phosphorylation of pLTR-δY187F-HA.2 was visible after longer exposure of the autoradiograph, data not shown) when compared with that of PKC-δWT-HA.1. Since the pLTR-HA vector contains one less HA epitope than that present in the pCEV-HA vector, exogenously expressed PKC-δWT and PKC-δY187F proteins from the pLTR-HA vector could not be readily separated from endogenous PKC-δ by SDS-PAGE. Thus, we were not able to easily distinguish PKC-δ exogenously expressed by the pLTR vector from endogenous PKC-δ utilizing direct immunoblot analysis. Therefore, PKC-δ levels in each line were determined by reblotting the membrane from Fig. 1C with anti-PKC-δ serum (Fig. 1D), followed by normalization for transfected protein expression levels, PKC-δ phosphotyrosine content in the 32D/pLTR-δY187F-HA.1 and 32D/pLTR-δY187F-HA.2 transfectants was reduced by 97% and 99%, respectively, when compared with that of the wild type protein in the 32D/pLTR-δWT-HA.1 transfectant. Taken together, these results demonstrate that mutation of tyrosine 187 to phenylalanine severely impairs PKC-δ tyrosine phosphorylation in response to TPA stimulation.

Constitutive Tyrosine Phosphorylation of an ATP-binding Mutant of PKC-δ Is Dramatically Reduced by Conversion of Tyrosine 187 to Phenylalanine—As previously demonstrated (6), expression of PKC-δK376R in 32D cells resulted in striking constitutive phosphorylation of this ATP-binding mutant on tyrosine residue(s). To test whether PKC-δK376R was also preferentially phosphorylated on tyrosine 187, we converted tyrosine 187 to phenylalanine in the PKC-δK376R cDNA, generating PKC-δK376R.Y187F. This double mutant cDNA was subsequently inserted into the pCEV-HA vector. As shown in Fig. 2, expression of PKC-δK376R.Y187F in 32D cells greatly reduced its phosphotyrosine content compared to that of PKC-δK376R. The expression levels of PKC-δK376R and PKC-δK376R.Y187F proteins in the transfectants were very similar (data not shown).

Conversion of Tyrosine 187 of PKC-δ to Phenylalanine Potently Inhibits Its Phosphorylation in Response to TPA or PDGF-BB Stimulation in NIH 3T3 Fibroblasts—Since we demonstrated that tyrosine 187 is a PKC-δ tyrosine phosphorylation site in the 32D cell system, we asked whether the same site was phosphorylated in NIH 3T3 fibroblasts. The same expression vectors containing the various PKC-δ cDNAs were transfected into NIH 3T3 fibroblasts and analyzed for phenylalanine content. As shown in Fig. 3A, the phosphotyrosine content of PKC-δK376R in response to TPA stimulation was reduced by 80% when compared with that of the PKC-δWT in NIH 3T3 transfectants. Expression of PKC-δK376R in NIH 3T3 cells resulted in constitutive tyrosine phosphorylation, confirming our previous finding in 32D cells (6). The expression level of PKC-δY187F was found to be similar to that of PKC-δWT in NIH 3T3 cells after transfection as determined by immunoprecipitation of cell lysates with anti-PKC-δ followed by anti-HA immunoblot analysis (Fig. 3B).

PKC-δ was previously demonstrated to be a substrate of activated PDGF-Rs in vivo in 32D cells coexpressing either PDGF-αR or PDGF-βR with PKC-δWT and in NIH 3T3 cells overexpressing PKC-δWT (4). When NIH 3T3 cells overexpressing PKC-δY187F were stimulated with PDGF-BB for 10 min, the phosphotyrosine content of the mutant protein was reduced by 85% compared with that of PKC-δWT after normalizing for protein expression levels in each transfectant (Fig. 3, C and D). These data indicate that tyrosine 187 of PKC-δ is not only a TPA-mediated phosphorylation site in vivo, but is also a site that is phosphorylated by activated PDGF-Rs.

Comparison for Tryptic Phosphopeptides between PKC-δ Y187F and PKC-δ WT by Two-dimensional Phosphopeptide Analysis—To confirm that tyrosine 187 of PKC-δ is a phosphorylation site in vivo, 32D/pLTR-δY187F-HA.1 and 32D/pLTR-δWT-HA.1 transfectants were in vivo labeled with [32P]orthophosphate and either unstimulated or stimulated with TPA for 10 min. Labeled cell lysates were immunoprecipitated with the anti-HA monoclonal antibody and subjected to SDS-PAGE. As shown in Fig. 4, PKC-δWT was weakly phosphorylated prior to TPA treatment. However, TPA stimulation of the PKC-δWT transfectant resulted in a 3- to 4-fold increase in phosphotyrosine content when compared with the untreated transfectant. Thus, PKC-δWT is a substrate for TPA-mediated tyrosine phosphorylation. Conversely, PKC-δY187F was almost completely stable under the same conditions and was not stimulated by TPA.
Phosphorylation of Tyrosine 187 in PKC-\(\delta\) activity toward its substrate in vitro is not altered when tyrosine 187 is converted to phenylalanine—Having shown that tyrosine 187 of PKC-\(\delta\) is a phosphorylation site in vivo in two independent cell systems in response to different agonist stimulation, it was now possible to determine whether expression of PKC-\(\delta\)Y187F would affect PKC-\(\delta\) kinase activity in vitro. Therefore, cell lysates from the 32D parental cell line and the various 32D transfectants were enriched for PKC by DE52 ion exchange chromatography. The PKC activity assay was subsequently performed utilizing a PKC-\(\delta\) pseudosubstrate region-derived peptide as the substrate. As shown in Table I, 32D cells possessed some endogenous PKC activity. Overexpression of PKC-\(\delta\)WT caused 2.9-, 3.4-, and 4.6-fold increases in activity in lysates from 32D/pLTR-\(\delta\)WT-HA.1, 32D/pLTR-\(\delta\)WT-HA.2, and 32D/pLTR-\(\delta\)WT-HA.3, respectively, when compared with the activity detected in the parental 32D line. Expression of PKC-\(\delta\)K376R exhibited a 1.3-fold reduction in activity compared with endogenous PKC activity, confirming our previous finding that the PKC-\(\delta\) ATP-binding mutant competitively inhibits endogenous PKC activity (6). Expression of the PKC-\(\delta\)Y187F in 32D/pLTR-\(\delta\)Y187F-HA.1 and 32D/pLTR-\(\delta\)Y187F-HA.2 lines caused 4.6- and 3.9-fold increases in PKC-\(\delta\) activity compared with the activity detected in the parental 32D line. The activities in lysates from PKC-\(\delta\)Y187F mutant transfectants were not significantly altered when compared with the activities detected in the lysates from PKC-\(\delta\)WT transfectants (Table I) even after normalizing for protein expression levels (data not shown). Taken together, these results indicate that tyrosine phosphorylation of PKC-\(\delta\) on tyrosine 187 does not affect its kinase activity toward the PKC-\(\delta\) pseudosubstrate region-derived substrate in vitro.

TPA-mediated monocytic differentiation of 32D Cells Is Similar in 32D Transfectants Overexpressing either PKC-\(\delta\)WT or PKC-\(\delta\)Y187F—Overexpression of PKC-\(\delta\)WT in 32D myeloid progenitor cells has been demonstrated to be required for mediating monocytic differentiation in response to TPA stimulation (3). We analyzed whether expression of PKC-\(\delta\)Y187F in

FIG. 6. TPA-mediated monocytic differentiation of 32D cells is not affected by expression of the PKC-\(\delta\)Y187F mutant. Cells were untreated (---) or exposed to TPA (----) overnight and subjected to flow cytometry after incubation with anti-Mac-1 (A) or anti-Mac-2 (B) antibodies. The x axis represents the mean fluorescence intensity (FL1) represents fluorescence generated by FITC, and the y axis represents the relative cell number.
32D cells would affect this differentiation process as determined by expression of specific monocytic cell surface differentiation markers. Transfectants were incubated with TPA overnight and analyzed by flow cytometry utilizing FITC-conjugated anti-Mac 1 and anti-Mac 2 antibodies (Fig. 6). Overexpression of PKC-δ WT in three transfectants resulted in TPA-mediated increased expression of Mac-1 and Mac-2. As previously shown, TPA treatment of the transfectant expressing the ATP-binding mutant, PKC-δ K376R, did not exhibit any increase in expression of these two antigens (6). In contrast, overexpression of PKC-δ Y187F in two transfectants did result in TPA-induced increases in Mac-1 and Mac-2 expression (Fig. 6, A and B). TPA treatment of 32D/pLTR-γY187F-HA transfectants, like the 32D/pLTR-γWT-HA transfectants, induced strong adherence of the cells to the culture plates, and the cells also displayed features common to mature macrophages such as a decreased nucleus-to-cyttoplasm ratio, membrane ruffling, and cytoplasmic vacuolation as determined by Wright-Giemsa staining (data not shown). Thus, dramatically decreased tyrosine phosphorylation of PKC-δ did not affect PKC-δ-mediated monocytic differentiation in the 32D system.

**DISCUSSION**

In the present study, we have demonstrated that tyrosine 187 is a PKC-δ phosphorylation site. After converting tyrosine 187 to phenylalanine and expressing the mutant protein utilizing two different expression vectors in both 32D and NIH 3T3 cells, PKC-δ phosphotyrosine content was reduced by more than 75% in response to TPA or PDGF stimulation. The site was also phosphorylated in PKC-δ K376R, an ATP-binding mutant of PKC-δ in vivo. We mutated two other tyrosine residues (tyrosine 64 and 238) within the regulatory domain of PKC-δ. Moreover, mutation of those tyrosines did not impair TPA-induced tyrosine phosphorylation of PKC-δ when expressed in 32D cells.

We confirmed that tyrosine 187 is a PKC-δ phosphorylation site in vivo in response to TPA stimulation by performing two-dimensional phosphopeptide analysis. Complete digestion of PKC-δ at tyrosine 187 site by trypsin will generate a tetrapeptide containing QYG^187FK. If tyrosine 187 is the correct phosphorylation site, its conversion to phenylalanine should abolish the appearance of this tetrapeptide. The absence of phosphopeptide 6 in the PKC-δ Y187F transfectant strongly indicates that phosphopeptide 6 in PKC-δ WT encompasses tyrosine 187. The phosphorylation levels of phosphopeptides 2–5 were also reduced in PKC-δ Y187F. It is possible that they are incompletely digested products of tetrapeptide QYG^187FK that may extend to certain positions where other phosphoamino acids exist. It is also likely that these phosphopeptides contain different phosphotyrosines whose phosphorylation is dependent on phosphorylation of tyrosine 187. We still do not know why phosphopeptide 15 also reduced its intensity in PKC-δ Y187F since it was not immunoprecipitable by anti-Tyr(P)^9 (see Fig. 5). Phosphopeptide 1 was also immunoprecipitable by anti-Tyr(P) antibody. However, the phosphorylation level of this phosphopeptide was not reduced in the PKC-δ Y187F transfectant, strongly suggesting that this peptide contains another potential tyrosine phosphorylation site.

Although our results provide evidence that PKC-δ tyrosine 187 is a phosphorylation site in vivo, we were unable to detect any differences in biochemical or biological functions resulting from expression of the mutant protein. The in vitro enzymatic activity of PKC-δ Y187F toward the PKC-δ pseudosubstrate region-derived substrate was comparable to that of PKC-δ WT in PKC-enriched lysates from the various 32D transfectants (Table I). Moreover, autophosphorylation of PKC-δ Y187F was also comparable to that of PKC-δ WT (see Fig. 4). Finally, the biological function of PKC-δ as determined by its ability to mediate monocytic differentiation of 32D cells was not altered by expression of PKC-δ Y187F. Based on all of these results, we conclude that phosphorylation of PKC-δ on tyrosine 187 does not play a dramatic role in regulating known PKC-δ functions in the in vitro and in vivo systems we analyzed.

In a previous study, we demonstrated that the enzymatic activity of purified PKC-δ was increased when PKC-δ was phosphorylated on tyrosine in vitro (7). However, no decreased PKC-δ activity was observed in PKC-enriched lysates from PKC-δ Y187F transfectants (Table I). It is possible that the tyrosine residues phosphorylated in vitro may differ from those utilized in vivo. Thus, in vivo phosphorylation of sites other than 187 may have resulted in the increased kinase activity we observed. Based on the phosphopeptide pattern generated by trypsin digestion of PKC-δ Y187F, TPA stimulation may induce phosphorylation of at least one more tyrosine residue (phosphopeptide 1, see Fig. 5C). This additional phosphorylation site may function in vivo in regulating PKC-δ activity or cooperate with tyrosine 187 to regulate PKC-δ activity. We are currently attempting to map this other tyrosine phosphorylation site on PKC-δ and to conclusively define the effect of tyrosine phosphorylation on PKC-δ activity.

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