Protein kinase C (PKC) inhibits proliferation and decreases expression of the differentiation marker glutamine synthetase (GS) in C6 glioma cells. Here, we report that distinct, specific tyrosine residues on PKC\(\delta\) are involved in these two responses. Transfection of cells with PKC\(\delta\) mutated at tyrosine 155 to phenylalanine caused enhanced proliferation in response to 12-phorbol 12-myristate 13-acetate, whereas GS expression resembled that for the PKC\(\delta\) wild-type transfectant. Conversely, transfection with PKC\(\delta\) mutated at tyrosine 187 to phenylalanine resulted in increased expression of GS, whereas the rate of proliferation resembled that of the PKC\(\delta\) wild-type transfectant. The tyrosine phosphorylation of PKC\(\delta\) and the decrease in GS expression induced by platelet-derived growth factor (PDGF) were abolished by the Src kinase inhibitors PP1 and PP2. In response to PDGF, Fyn associated with PKC\(\delta\) via tyrosine 187. Finally, overexpression of dominant negative Fyn abrogated the decrease in GS expression and reduced the tyrosine phosphorylation of PKC\(\delta\) induced by PDGF. We conclude that the tyrosine phosphorylation of PKC\(\delta\) and its association with tyrosine kinases may be an important point of divergence in PKC signaling.

Protein kinase C (PKC)\(^1\) comprises a family of phospholipid-dependent serine-threonine kinases that play important roles in signal transduction of various physiological stimuli, including growth factors, hormones, and neurotransmitters (1–3). Activation of PKC leads to the phosphorylation of proteins that are involved in the regulation of cell growth, differentiation, and apoptosis (4–7). PKC consists of at least 11 isoforms showing diversity in their structures, cellular distributions, and biological functions (8). The members of the classical PKCs \(\alpha\), \(\beta_1\), \(\beta_2\), and \(\gamma\) bind phorbol esters and are \(\text{Ca}^{2+}\) dependent. The novel PKCs \(\delta\), \(\varepsilon\), \(\eta\), and \(\theta\) do not depend on \(\text{Ca}^{2+}\) but bind phorbol esters. The third subfamily includes the atypical PKCs (PKC\(\zeta\) and PKC\(\lambda\)), which do not bind either \(\text{Ca}^{2+}\) or phorbol esters, and PKC\(\mu\), which exhibits unique characteristics (9). All PKC isoforms can be divided into an N-terminal regulatory domain and a C-terminal catalytic domain with serine-threonine kinase activity (10, 11). Both domains contain conserved (C) regions of extended sequence homology and variable (V) regions. In the classical PKC isoforms the regulatory domain contains a \(\text{Ca}^{2+}\) binding domain, and in both the classical and novel PKC isoforms it contains a pair of highly conserved zinc fingers (C1 domains) that bind phorbol esters and a pseudosubstrate region (12–14). PKC chimeras have been used to study the role of the regulatory and catalytic domains of different PKC isoforms.

PKC\(\delta\) is a widely expressed member of the novel PKCs (15). This isoform has been associated with the proliferation of various cells in a cell type-specific manner. For example, PKC\(\delta\) inhibited the proliferation of smooth muscle cells (16) and glial cells (17) and caused cell arrest at the G2/M phase of the cell cycle in Chinese hamster ovary cells (18). In contrast, in breast cancer cells PKC\(\delta\) has been shown to increase tumorigenicity (19). PKC\(\delta\) has also been reported to play a role in cell differentiation. Thus, PKC\(\delta\) has been shown to undergo translocation and activation during differentiation of keratinocytes (20) and overexpression of this isoform induced squamous (21) and myeloid cell differentiation (22).

Recent studies suggest that PKC\(\delta\) associates with different tyrosine kinases and that this association can induce the tyrosine phosphorylation of PKC\(\delta\) itself and can affect the activity of both the tyrosine kinases and PKC (15). PKC\(\delta\) has been shown to be tyrosine phosphorylated in response to various stimuli such as PMA, EGF, PDGF (23–25), ligands for the IgE receptor (26, 27), ATP, and \(\text{H}_2\text{O}_2\) (28). The phosphorylation site(s) and the role of tyrosine phosphorylation of PKC\(\delta\) in its activity and in its function are just beginning to be understood. Two specific sites in the regulatory domain, tyrosines 187 and 52, have been reported so far to be phosphorylated in response to PDGF and FceRI, respectively (29), and tyrosine 311 has been shown to be phosphorylated by Src (30). In contrast, tyrosine residues in the catalytic domain of PKC\(\delta\) have been reported to be phosphorylated in response to \(\text{H}_2\text{O}_2\) (28).

In a recent study (23), we found that in C6 glioma cells tyrosine phosphorylation of PKC\(\delta\) in the regulatory domain mediated the inhibitory effect of this isoform on the expression of the astrocytic marker, glutamine synthetase (GS). In the present study, we found that tyrosine phosphorylation of PKC\(\delta\) also plays a role in the inhibitory effect of PKC\(\delta\) on cell proliferation and have identified different tyrosine residues that are involved in the selective effects of PKC\(\delta\) on cell proliferation and GS expression.

**EXPERIMENTAL PROCEDURES**

**Materials**—PDGF and an affinity-purified polyclonal anti-PKC\(\delta\) antibody against a polypeptide corresponding to amino acids 726–737 of...
PKCε were purchased from Life Technologies, Inc. Monoclonal anti-PKC and anti-GS antibodies were obtained from Transduction Laboratories (Lexington, KY). Polyclonal anti-PKC antibodies and anti-Src,-Fyn, and - Lyn antibodies were from Santa Cruz (Santa Cruz, CA). FMA was from Alexis Co. (San Diego, CA). Leupeptin, aprotinin, phenylmethylsulfonyl fluoride, and sodium vanadate were obtained from Sigma.

Generation of PKC Chimeras—The PKC chimeras were generated by exchanging the regulatory and catalytic domains of PKCα, -δ, and -ε as described by Acs et al. (31). PKCα/δ6 refers to the chimera with the PKCα regulatory domain and the PKCδ catalytic domain, and PKCα/δ6 refers to the reciprocal chimera. The PKC δ CNAs were subcloned into the metallothionein promoter-driven eukaryotic expression vector (MTH). The vector sequence encodes a C-terminal PKCε-derived 12-amino acid tag (εMTH) that is added to the expressed proteins (44). The expression of these chimeras and their activities in C6 cells were described recently (23).

Site-directed Mutagenesis of PKCα—Mouse PKCα was cloned into the pGEM-T vector (Promega, Madison, WI) as described previously (23). This plasmid served as our "master" vector for the site-directed mutagenesis, using the Transformer Site-Directed Mutagenesis Kit from CLONTECH (Palo Alto, CA). Conversion of tyrosine residues at sites 52, 64, 155, 187, and 565 into phenylalanine was performed as described previously (23). PKCδ and the PKCα mutants were subcloned into the metallothionein promoter-driven eukaryotic expression vector (εMTH).

Construction of PKC6-GFP Fusion Protein—DNAAs encoding the murine PKC6 and the various PKCα mutants were fused into the N-terminal-enhanced GFP vector pEGFP-N1 (CLONTECH, Palo Alto, CA). The original pEGFP-N1 vector was modified by the insertion of an MluI site in the plasmid polylinker. The restriction site was created by ligating a phosphorlated linker containing the MluI site into pEGFP-N1 digested with SmaI. The construct was verified by sequencing. The clones containing the GFP-PKC6- or GFP-fused to the different PKCδ mutants were constructed by the excision of PKCδ or the specific mutants from MTH-PKCα plasmids by digestion with XhoI and MluI. The inserts were then ligated into the modified GFP vector using the same restriction sites. DNA sequencing of the GFP-PKC constructs confirmed the intended reading frame.

C6 Glial Cultures and Cell Transfection—C6 cells of late passages (50–60), that exhibit an astrocitary phenotype, were used in this study. Cells of these passages showed somewhat smaller response to overexpression of PKCδ as compared with the C6 cells of passage 30, which exhibit progenitor properties (17, 23). For the current studies we chose cells of passages 50, because we wanted to focus on the effects observed on the expression of GS and not on general aspects of cell differentiation. Cells (1 × 10⁶ cells/ml) were seeded on tissue culture dishes (10 cm) and were grown in medium consisting of Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal calf serum, 2 mM glutamine, penicillin (50 units/ml), and streptomycin (0.05 mg/ml). The cells were transfected either with the empty vectors, the different PKC δ expression vectors, or a Fyn dominant negative mutant in pSG5 (kindly provided by Alan P. Saltiel) using LipofectAMINE (Life Technologies, Inc.) as described previously (23). Experiments were routinely carried out on a clone of the transfected cells, but all the results were confirmed on one pool and two additional individual clones.

For overexpression of the GFP-PKC6 fusion proteins, C6 cells were seeded onto 40-mm round glass coverslips at a density of 5 × 10⁴ cells/coverslip. Twenty-four hours later, cells were transfected with the different GFP-PKC6 constructs using LipofectAMINE Plus reagent according to the manufacturer's instructions. All experiments were performed 48 h post-transfection.

Preparation of Cell Homogenates—Cells were washed and resuspended in serum-free medium. The plates were placed on ice, scraped with a rubber policeman, and centrifuged at 1,400 rpm for 10 min. The supernatants were aspirated, and the cell pellets were resuspended in 100 μl of lysis buffer (25 mM Tris-HCl, pH 7.4, 50 mM NaCl, 5% sodium deoxycholate, 2% Nonidet P-40, 0.2% SDS, 1 mM phenylmethylsulfonyl fluoride, 50 μg/ml aprotinin, 50 μg/ml leupeptin, 0.5 mM Na3VO4) on ice for 15 min. The cell lysates were centrifuged for 15 min at 14,000 rpm in an Eppendorf microcentrifuge, supernatants were removed buffer was added, and the samples were preabsorbed with 25 μl of protein A/G-Sepharose (50%) for 10 min, and immunoprecipitation was performed using 4 μg/ml antibody for 1 h at 4 °C and then incubated with 30 μl of A/G-Sepharose for an additional hour. Following washes, the pellets were resuspended in 25 μl of SDS sample buffer and boiled for 5 min. The entire supernatants were subjected to Western blotting. Membranes were incubated with horse-radish peroxidase-conjugated secondary antibodies for 1 h at room temperature. The membranes were washed and visualized by the ECL system.

Cell Proliferation Assay—Cells overexpressing the wild-type PKCδ or the PKC6 mutants were seeded in triplicate and incubated in the absence or presence of ZnCl2 (20 μM) for 24 h followed by treatment with PMA (30 nM) for an additional 48 h. Cells were pulsed with 0.5 μCi of [3H]thymidine for the last 6 h and then harvested. The incorporation of [3H]thymidine was determined in a Beckman Scintillation counter.

Confocal Microscopy—Confocal fluorescent images were collected with a Bio-Rad MRC 1024 confocal scan head (Bio-Rad) mounted on a Nikon microscope with a 60× planapochromat lens. Excitation at 488 nm was generated by a krypton-argon gas laser with a 522/32 emission filter for green fluorescence. For kinetics of GFP-PKC6 translocation in living cells, cells plated on a 40-mm-round coverslip were enclosed in a Bioptechs Focht Chamber System (Bioptechs, Butler, PA). The chamber was inverted and attached to the microscope stage with a custom stage adapter; a temperature controller set at 37 °C was connected, and medium was perfused through the chamber with a Lambda microfusion pump. Sequential images of the same cell were collected at various time points using LaserSharp Software.

Statistical Analysis—The results are presented as the means ± S.E. All data were analyzed using a paired Student's t test to determine the level of difference between the treatments.

RESULTS

The Regulatory Domain of PKCδ and Its Tyrosine Phosphorylation Mediate the Decrease in Cell Proliferation Induced by PMA—In a recent study we demonstrated that the regulatory domain of PKCδ mediated its inhibitory effect on the expression of the astrocitary marker GS (23). To characterize the effect of PKCδ on C6 cell proliferation we first examined the relative contributions of the regulatory and catalytic domains of this isoform. For these studies, we used chimeras between the regulatory and catalytic domains of PKCα, -δ, and -ε, combined at the highly conserved hinge region. The expression and activity of C6 cells overexpressing the different chimeras were already described previously (23).

Cells were transfected for 24 h with ZnCl2, followed by PMA treatment (20 nM) for an additional 48 h. This concentration of PMA induced prolonged activation of PKCδ without marked down-regulation. Cells overexpressing PKCδ exhibited a lower rate of cell proliferation than control cells. In contrast, cells overexpressing PKCδ/α and δ/ε exhibited a higher rate of cell proliferation than controls both in the presence and absence of PMA. Similar to cells expressing PKCδ/δ, cells overexpressing the chimeras containing the regulatory domain of PKCδ, namely δ/ε and δ/ε, also showed a reduced level of cell proliferation, whereas chimeras expressing chimeras containing the catalytic domain of PKCδ together with the regulatory domain of PKCα, -δ, or -ε exhibited a higher cell proliferation similar to that observed expressing cells expressing PKCδ/α or PKCδ/ε (Fig. 1A). Untreated cells overexpressing PKCδ/δ or chimeras containing the regulatory domain of PKCδ also showed decreased cell proliferation as compared with control vector cells, but the magnitude of this decrease was much lower than that observed in PMA-treated chimeras (Fig. 1A).
We reported that the expression of the PKC\(\delta\)5 mutant (in which tyrosines 52, 64, 155, and 187, and 565 were mutated to phenylalanine) in C6 cells abolished the decrease in the expression of the astrocytic marker GS induced by PMA or PDGF. Expression of the PKC\(\delta\)5 mutant also resulted in a lower tyrosine phosphorylation of PKC\(\delta\) in response to these treatments (23). Using cells expressing PKC\(\delta\)5 we found that in response to PMA (20 nM) these cells displayed enhanced proliferation as compared with vector control cells, contrasting with cells overexpressing PKC\(\delta\) WT which exhibited a significantly lower tyrosine phosphorylation of PKC\(\delta\) and this effect was abolished in the PKC\(\delta\)5 mutant (in which tyrosines 52, 155, and 187 are being phosphorylated in response to PMA). In contrast, PDGF-stimulated cells overexpressing PKC\(\delta\)5 mutant also resulted in a lower tyrosine phosphorylation of PKC\(\delta\) expressed in the presence of 1 \(\mu\)M PMA and 100 \(\mu\)g/ml phosphatidylinositol. The values for the PKC5 and the PKC\(\delta\)5 mutants are expressed as percent of control (empty vector cells) (B). The results represent one of three separate experiments which gave similar results.

**Tyrosine Phosphorylation of the PKC\(\delta\) Mutants by PMA and PDGF—**The degree of tyrosine phosphorylation of the various PKC\(\delta\) mutants was examined in response to PMA and PDGF. As illustrated in Fig. 3, a small basal level of tyrosine phosphorylation was observed in untreated cells. PMA and PDGF induced marked tyrosine phosphorylation of PKC\(\delta\) WT but, as already described, no significant degree of enhanced tyrosine phosphorylation of PKC85 \((p > 0.07, n = 5)\). The degree of tyrosine phosphorylation of PKC85Y155F and PKC85Y187F in response to PMA was intermediate between that of PKC\(\delta\) WT and that of the PKC\(\delta\)5 mutant, suggesting that both tyrosines 155 and 187 are being phosphorylated in response to PMA. In contrast, cells overexpressing PKC\(\delta\)5F2 exhibited a level of tyrosine phosphorylation similar to that of PKC\(\delta\) WT, suggesting that this tyrosine may not be phosphorylated in response to PMA. As already described, PDGF also induced tyrosine phosphorylation of PKC\(\delta\) and this effect was abolished in the PKC\(\delta\)5 cells. We found that treatment of cells overexpressing PKC85Y187F with PDGF resulted in a very low tyrosine phosphorylation of PKC\(\delta\). In contrast, PDGF-stimulated cells overexpressing PKC85Y155F or PKC85Y52F exhibited similar levels of tyrosine phosphorylation to those observed in cells overexpressing PKC\(\delta\) WT.

**Tyrosine 155 Mediates the Inhibitory Effect of PKC\(\delta\) on Cell Proliferation, whereas Tyrosine 187 Mediates the Inhibitory Effect of PKC\(\delta\) on GS Expression—**We then examined the degree of cell proliferation and GS expression in cells overexpressing the different PKC mutants. We found that, in re-
expression of GS in response to PDGF. Cells were pretreated
ated an increased expression of GS in response to stimulation
ained in cells overexpressing PKC
PMA induced translocation of the PKC
sent one of three separate experiments which gave similar results.
PKC
additional 48 h. As described previously, cells overexpressing
creased levels of GS compared with vector control cells. Cells
PKC
C6 cells transfected with PKC
obtained in cells treated with 20 nM PMA (data not shown).
Tyrosine Phosphorylation of PKCδ
Translocation and Degradation of PKCδ and the PKCδ Mu-
Translocation and Degradation of PKCδ and the PKCδ Mu-
tants—One possible explanation for the differential effect of the
ants on cell proliferation and GS expression is transloca-
tion to different cellular compartments following stimulation.
We therefore examined the translocation of the different PKCδ
mutants in response to PMA and PDGF. For these experiments we
used GFP-tagged PKCδ WT or the different PKCδ mutants.
Cells were transiently transfected with the specific GFP-PKCδ
mutants, and the response of the cells to PMA or PDGF was
monitored over a period of 30 min. Stimulation of the cells with
100 nM PMA induced initial translocation of PKCδ to the
plasma membrane followed by some translocation of PKCδ to
the perinuclear membrane (Fig. 5). PDGF also induced translo-
cation of PKCδ, but the magnitude of this translocation was
much lower than that induced by PMA. PDGF induced some
membranous translocation of PKCδ and distribution of PKCδ
around the perinuclear membrane (Fig. 5). A similar pattern of
translocation was observed for PKCδ and the PKCδ mutants in
response to PMA (Fig. 6) and PDGF (data not shown). Thus,
PMA induced translocation of the PKCδ mutants to both the
plasma membrane and the perinuclear membrane (Fig. 6). The
kinetics of the translocation as well as the response to lower
concentrations of PMA were likewise similar in all of the mu-
tants (data not shown). PMA or PDGF did not induce any
changes in cells overexpressing GFP protein alone (data not shown).

Recent studies suggested that tyrosine phosphorylation of
PKCδ may play a role in its degradation (30). Since the inhibi-
tory effects of PMA on GS expression and cell proliferation
require a prolonged exposure of the cells to PMA, we examined
whether the various PKCδ mutants exhibit different degrees of
degradation as compared with PKCδ WT. For these experi-
ments, cells overexpressing PKCδ WT or the PKCδ mutants were
further support that the tyrosine-phosphorylated form of PKC
was the predominant form. Furthermore, the decrease in GS
expression by PDGF appeared to involve phosphorylation of
GS expression (Fig. 7A). We also examined the expression of
GS in the different PKCδ mutants (data not shown).

Phosphorylation of PKCδ by PDGF Is Inhibited by PP1 and
To examine the role of Src-related kinases in the tyrosine
phosphorylation of PKCδ, we employed the Src kinase inhibitors
PP1 and PP2. Pretreatment of the cells with either PP1 or
PP2 abolished the tyrosine phosphorylation of PKCδ in re-
response to PDGF (Fig. 7A). PP1 and PP2 also abrogated the
inhibitory effect of PKCδ on GS expression (Fig. 7B), providing
further support that the tyrosine-phosphorylated form of PKC
is involved in the inhibition of GS expression. Since PP1 has
also been reported to inhibit the kinase activity of the PDGFRβ
at a similar concentration range (30), our results suggest that
either Src-related kinases or the PDGFRβ are involved in the
phosphorylation of PKCδ in response to PDGF.

Association of PKCδ with Src-related Kinases—To examine
the association of PKCδ with Src-related kinases we performed
co-immunoprecipitation of PKCδ with Src, Fyn, and Lyn. We
found that in unstimulated C6 cells PKCδ was constitutively
associated with p60Src (Fig. 8A). Stimulation of the cells with
either PMA or PDGF for 1–60 min did not induce further
association of PKCδ with Src (data not shown). In contrast,
PDGF induced association of Fyn with PKCδ and, to a lesser
extent, association of Lyn (Fig. 8A). To further characterize the
association of Fyn with PKCδ, we performed a kinetic study
and found that PDGF induced association of Fyn following 1
min of treatment and that this association was decreased after
30 min (Fig. 8B).

Since the decrease in GS expression by PDGF appeared to
involve phosphorylation of tyrosine 187 in PKCδ, we examined
the role of this tyrosine residue in the association of Fyn and
PKCδ. Stimulation of cells overexpressing PKCδY155F with
PDGF (Fig. 8B) or PMA (data not shown) did not lead to
association of the mutated PKCδ with Fyn as determined by
co-immunoprecipitation, indicating the tyrosine 187 is essen-
tial for the association. In contrast, the mutant PKCδY155F
associated with Fyn similarly to PKCδ WT (data not shown),
suggesting the tyrosine 155 does not play a role in the associ-
ation of PKCδ and Fyn.

Fyn Is Involved in the Inhibitory Effect of PKCδ on GS
Expression—Since Fyn associates via tyrosine 187 with PKCδ in
response to PDGF treatment, we wanted to examine the role
of Fyn in the tyrosine phosphorylation of PKCδ and in the inhi-
bitory effect of PKCδ on GS expression. We stably transfect-
a Fyn dominant negative mutant in C6 cells and examined their
response to PDGF. Cells overexpressing empty vector exhibited
an increase in the tyrosine phosphorylation of PKCδ following
10 min of PDGF treatment. In contrast, a decrease in the
degree of the tyrosine phosphorylation of PKCδ was observed in
cells overexpressing the Fyn dominant negative mutant (Fig.
9A). We also examined the expression of GS in the different
cells. PDGF decreased the expression of GS in cells overex-
pressing control vector. In contrast, there was no significant decrease in the expression of GS in response to PDGF in cells overexpressing the Fyn dominant negative mutant (Fig. 9B). Interestingly, the inhibitory effect of the Fyn dominant negative on the decrease in GS expression induced by PDGF was more marked than the decrease in tyrosine phosphorylation of PKCδ, suggesting that Fyn is not the only tyrosine kinase phosphorylating PKCδ in response to PDGF. Similar results with the Fyn dominant negative mutant were obtained in cells treated with 20 nM PMA (data not shown). In contrast, Fyn dominant negative did not abolish the decrease in cell proliferation induced by PMA, suggesting that the effect of Fyn dominant negative was specific to GS expression (data not shown).

**DISCUSSION**

In this study we explored the mechanisms involved in the inhibitory effects of PKCδ on C6 cell proliferation and GS expression. We found that the regulatory domain of PKCδ is responsible for the effects of this isoform on C6 cell proliferation. These results are similar to those we recently described regarding the regulation of GS expression by PKCδ (23). Chimeras have been used to delineate the contributions of individual PKC domains to the specific functions of different PKC isoforms in a number of systems. Both the catalytic and the regulatory domains of PKC may determine isoform-specific functions depending on the specific system. For example, the catalytic domain of PKCβ was found to confer isoform-specific function in the differentiation of erythroleukemia cells (14), and the catalytic domain of PKCδ in reciprocal δ- and ε-chimeras mediated PMA-induced macrophage differentiation of mouse promyelocytes (32). In contrast, the regulatory domain of PKCε enhanced cell growth and induced colonies in soft agar in NIH 3T3 cells (33). Recently, it has been reported that the regulatory domain of PKCδ overexpressed by itself inhibited mammary tumor cell metastases (34).

PKCδ has been shown to become tyrosine-phosphorylated in the regulatory domain in response to PMA and PDGF in C6 cells (23). Tyrosine phosphorylation of PKCδ has been reported in response to EGF stimulation in keratinocytes (24), in response to carbachol, substance P and PMA stimulation in parotid acinar cells (35), in response to H2O2 in CHO-K1 cells (28), and in response to activation of the IgE receptor in RBL-2H3 cells (26, 27). Constitutive tyrosine phosphorylation was reported in Ras-transformed mouse keratinocytes (36). Our results suggest that Src-related kinases are involved in the...
tyrosine phosphorylation of PKCδ in response to PDGF, since the Src kinase inhibitors PP1 and PP2 reduced significantly the phosphorylation induced by PDGF. Our results using cells overexpressing a Fyn dominant negative mutant further suggest that Fyn contributes to PKCδ tyrosine phosphorylation in response to PDGF. Since PP1 has also been reported to inhibit the kinase activity of PDGFRβ (3), we cannot exclude at this point that this receptor directly phosphorylates PKCδ upon PDGF binding. Indeed, Li et al. (22) reported that the PDGF receptor phosphorylated PKCδ in vitro.

The effect of tyrosine phosphorylation on the activity of PKCδ or on its function differs, depending on the specific system. Thus, tyrosine phosphorylation of PKCδ has been reported to reduce its activity in Ras-transformed cells and in response to activation of the EGF receptor (24, 36). In contrast, tyrosine phosphorylation of PKCδ by Fyn increased the kinase activity (37). Recently it was suggested that the tyrosine phosphorylation of PKCδ in response to engagement of the IgE receptor leads to altered substrate specificity (26). A previous report has shown that mutation of PKCδ at tyrosine 187 did not change kinase activity (25). In this study we did not further explore the kinase activity of the different PKC mutants using different substrates, since the nature of the endogenous substrates involved in the effects of PKCδ on cell proliferation and GS expression have not yet been identified.

Our results using the PKCδ5 mutant suggest that tyrosine phosphorylation of PKCδ plays a role in the inhibitory effect exerted by this isoform on cell proliferation. These results are similar to our recent findings, which showed a role for tyrosine phosphorylation of PKCδ in the inhibitory effect of this isoform on the expression of the astrocytic marker GS (23). Thus, the PKCδ5 mutant appears to act in an opposite way to PKCδ in the effect of this isoform on both GS expression and cell proliferation. Since tyrosine phosphorylation of PKCδ in response to PMA occurs only in the regulatory domain of this isoform in the C6 cells (23), our results are consistent with the importance of the regulatory domain of PKCδ in the inhibitory effect of this isoform on different cellular functions.

Although tyrosine phosphorylation in the regulatory domain of PKCδ mediates the inhibitory effects of this isoform on both GS expression and cell proliferation, it appears that different tyrosine residues are involved in the different effects. Thus, tyrosine 155 is implicated as a phosphorylation site that is involved in the inhibitory effect of PKCδ on cell proliferation but not on GS expression, whereas tyrosine 187 appears to be involved in the inhibitory effect of PKCδ on GS expression. There have been a number of reports regarding the phosphorylation of specific tyrosines on PKCδ. For example, tyrosine

FIG. 6. Cellular localization of PKCδ and the PKCδ mutants in PMA-treated C6 cells. Cells were transiently transfected with GFP-PKCδ or the different GFP-PKCδ mutants. Following 48 h, cells were treated with PMA (100 nm), and sequential confocal images were taken every 30 s for a period of 30 min. The figures present images taken at time 0 (−PMA) and 30 min (+PMA) after treatment. Cells shown are representative of four independent experiments.

FIG. 7. PP1 and PP2 inhibit the PDGF-induced tyrosine phosphorylation of PKCδ and the decrease in GS expression. C6 cells were treated with PP1 or PP2 for 1 h following by stimulation with PDGF for an additional 15 min (A). Cells were then harvested and immunoprecipitation of PKCδ was performed as described under “Experimental Procedures.” Following SDS-PAGE, membranes were stained with anti-phosphotyrosine antibody (anti-Tyr(P)) or with anti-PKCδ antibody. C6 cells were pretreated with PP1 or PP2 for 1 h and then with PDGF for 48 h (B). GS expression was determined using Western blot analysis. The results represent one of three separate experiments that gave similar results.

FIG. 8. Association of Src-related kinases with PKCδ. C6 cells were treated with PDGF for 15 min. Immunoprecipitation of PKCδ was performed as described under “Experimental Procedures,” and the membranes were stained with anti-Src, anti-Fyn, or anti-Lyn (A). Cells overexpressing PKCδWT or PKCδY187F were treated with PDGF for different periods of time. PKCδ or PKCδY187F was immunoprecipitated using anti-PKCδ antibody, and the association of Fyn with PKCδ was measured (B). The results represent one of three separate experiments that gave similar results.
phosphorylation in the catalytic domain of PKCδ was described in response to treatment with H2O2 (28). Tyrosine 311 was reported to be phosphorylated in response to Src (30), and engagement of the FcεRI resulted in tyrosine phosphorylation of tyrosine 52 in the regulatory domain (27, 29). Interestingly, tyrosine 52 did not appear to be phosphorylated in response to either PMA or PDGF in the C6 cells. Our results of PDGF-induced phosphorylation of tyrosine 187 in PKCδ are consistent with the results of Li et al. (25), who reported that PMA and PDGF-induced phosphorylation of PKCδ on tyrosine 187 in NIH 3T3 cells. The authors concluded that this phosphorylation was not important for the monocytic differentiation of 32D cells by PMA. Thus, the monocytic differentiation of 32D cells resembles C6 cell proliferation and contrasts with GS expression in its control by tyrosine phosphorylation of PKCδ.

One of the factors that could explain the different effects of the PKCδ mutants is a distinct pattern of translocation. Translocation of PKCδ to specific cellular compartments could lead to different effects due to the phosphorylation of different substrates and to the association of PKCδ with specific proteins present in these locations. One of the important factors that can determine the localization of PKC following its activation is association with RACKs (receptors for activated protein kinase Cs) (38). It is currently not clear to what extent tyrosine kinases can act as RACKs and affect the translocation of PKCδ isoforms. In a recent study, Ron et al. (39) suggested that Fyn, which is associated with PKCδ, might act as a RACK of this PKCδ isoform. We found that PMA induced initial translocation of PKCδ to the plasma membrane followed by translocation to the perinuclear membrane. This pattern of translocation is similar to the translocation of PKCδ reported in CHO-K1 cells (40). PDGF also induced membranal translocation of PKCδ, although to a lesser extent, with some accumulation around the perinuclear membrane. Stimulation of cells overexpressing PKCδ WT or the different PKCδ mutants with PMA or PDGF resulted in a similar pattern of translocation. Thus, the differential effects of the different PKCδ mutants on cell proliferation and GS expression are probably not due to their different translocation following activation. At this point, however, we cannot exclude the possibility that the PKCδ mutants undergo translocation to different membranal subdomains, causing their association with distinct signaling molecules.

PKCδ has been reported to associate with different tyrosine kinases such as Src (27, 30, 41, 42), Lyn (27), and c-Abl (43) in either a phosphorylation-dependent or -independent manner. The ability of PKCδ to be tyrosine phosphorylated on more than one tyrosine suggests that PKCδ can associate with different tyrosine kinases. We found that PKCδ associated with Src in a phosphorylation-independent manner and with Fyn following stimulation of PDGF via tyrosine 187. Specifically, the effect of PKCδ on cell proliferation and GS expression in C6 cells may be mediated by different downstream PKC substrates or different tyrosine kinases that are associated with PKCδ. Indeed, our results suggest that Fyn is involved in the inhibitory effect of PDGF on GS expression, since overexpression of the Fyn dominant negative mutant abrogated the inhibitory effect of PDGF. It has been reported that the differential interaction of PKCδ with tyrosine kinases may lead to changes in the activity and substrate recognition of PKCδ and to changes in the activity of the associated tyrosine kinases (26, 27). Thus, the differential phosphorylation of specific tyrosine residues may generate diversity in the effects of PKCδ and positions this isoform as an important component in a complex bi-directional interaction between serine-threonine and tyrosine kinase signaling. The identities of the tyrosine kinases that are associated with PKCδ via tyrosine 155 and are involved in the inhibitory effect of PKCδ on cell proliferation are currently under investigation.

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