PKCδ Regulates PKCα Activity In a Syndecan-4 Dependent Manner

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Running title: PKC-δ-dependent regulation of PKC-α activity
SUMMARY

The phosphorylation state of Ser\textsuperscript{183} in the cytoplasmic tail of syndecan-4 determines the binding affinity of the cytoplasmic tail to phosphatidylinositol 4,5-bisphosphate (PIP\textsubscript{2}), the capacity of the tail to multimerize, and its ability to activate protein kinase C (PKC) \(\alpha\). We sought to identify the kinase responsible for this phosphorylation, and to determine its downstream effects on PKC-\(\alpha\) activity and on endothelial cell function. Among several PKC isoenzymes tested, only PKC\(\alpha\) and \(\delta\) were able to specifically phosphorylate Ser\textsuperscript{183} in vitro. However, studies in cultured endothelial cells showed that the phosphorylation level of syndecan-4 was significantly reduced in endothelial cells expressing a dominant negative (DN) PKC\(\delta\) but not a DN PKC\(\alpha\) mutant. Syndecan-4/PIP\textsubscript{2}-dependent PKC\(\alpha\) activity was significantly increased in PKC\(\delta\) DN cells, while PKC\(\delta\) overexpression was accompanied by decreased PKC\(\alpha\) activity. PKC\(\delta\)-overexpressing cells exhibited a significantly lower proliferation rate and an impaired tube formation in response to FGF2, which were mirrored by similar observations in PKC\(\alpha\) DN endothelial cells. These findings suggest that PKC\(\delta\) is the kinase responsible for syndecan-4 phosphorylation, which, in turn, attenuates the cellular response to FGF2 by reducing PKC\(\alpha\) activity. The reduced PKC\(\alpha\) activity then leads to impaired endothelial cell function. We conclude that PKC\(\delta\) regulates PKC\(\alpha\) activity in a syndecan-4 dependent manner.
INTRODUCTION

The protein kinase C (PKC) family of enzymes is one of the most extensively studied group of proteins involved in intracellular signal transduction. However, to date little information is available regarding specific regulation of function and activity of individual PKC isoforms (1-4). Recently, syndecan-4 has been shown to be able to activate PKCα in the presence of phosphatidylinositol-4,5-bisphosphate (PIP2) and in the absence of Ca^{2+} (5-7). Syndecan-4 is a member of the syndecan gene family, a group of heparan sulfate-carrying core proteins present in the plasma cell membrane (8). While sharing the ability of other syndecans to interact with heparin-binding proteins including fibroblast growth factors (FGFs), vascular endothelial growth factors, and numerous other partners, syndecan-4 has been specifically implicating in FGF2 signaling (9) and in regulation of cell cytoskeleton, focal adhesions and migration (10,11).

FGF2-dependent activation of syndecan-4 signaling requires oligomerization of its cytoplasmic tails (12) that in turn depends on the phosphorylation state of Ser^{183} which regulates PIP2 binding to the syndecan-4 tail (6). In previous studies we have demonstrated that phosphorylation of Ser^{183} is carried out by a novel PKC (13). This chain of events, therefore, raises the possibility that one PKC isoform controls the activity of another isoform via the regulation of syndecan-4 phosphorylation. The present study was designed to explore that possibility. We find that PKCδ is the PKC isoform responsible for syndecan-4 phosphorylation and that alterations in PKCδ activity result in biologically meaningful alterations in PKCα function.
EXPERIMENTAL PROCEDURES

Materials

PIP2, phosphatidylserine (PS), and diolein were purchased from Sigma. Recombinant PKCs were synthesized and prepared as described (14). PKCβ1 optimal substrate peptide (FKLKRKGSFKKFA) was purchased from Genemed Synthesis. A 28-amino acid-long syndecan-4 cytoplasmic tail peptide RMKKKDEGYSYDLGKKPIYKAPTNEFYA (WT) and RMKKKDEGAYDLGKKPIYKAPTNEFYA (SA) were synthesized by Genemed Synthesis. Syndecan-4 ectoplasmic and cytoplasmic antisera were a gift from Dr. N.W. Shworak (Dartmouth Medical School). c-Myc antibody was purchased from Santa-Cruz Biotechnology. PKCα, PKCδ, PKCη, PKCθ and PKCζ antibodies were purchased from Santa Cruz Biotechnology and Transduction Laboratories.

Construction of RFPEC-derived cell lines

The dominant negative (DN) PKCα construct (15,16) was a generous gift from Dr. Dan Rosson (Lankenau Medical Research Center) and dominant negative PKCε construct was gift from Dr. I. Bernard Weinstein (Colombia University). These expression plasmids were generated by replacing the conserved lysine in the ATP binding domain with arginine. PKCδ cDNA containing a c-Myc tag sequence was subcloned into pRc/CMV vector (Invitrogen) between HindIII and XbaI sites. Dominant negative PKCδ constructs were created by in vitro mutagenesis replacing the conserved lysine in the ATP binding domain in position 376 with tryptophan. These constructs were stably transfected into rat fat pad endothelial cells (RFPEC) using Lipofectamine Plus and the protocol provided by the manufacturer (Life Technologies). Following neomycin (Geneticin, Life
Technologies) selection, a number of clones were isolated and expanded. Expression of constructs was verified by Western blotting. At least two different clones were used for each construct-related experiment.

**Western blot analysis**

Cells were lysed with RIPA buffer (50 mM HEPES pH 7.4, 150 mM NaCl, 5 mM EGTA, 5 mM EDTA, 50 mM NaF, 20 mM NaPyrophosphate, 1 % Triton X-100, 1 mM PMSF, 1 mM Na₃VO₄), separated on a 10 % SDS-polyacrylamide gel and transferred to an Immobilon-P PVDF membrane (Millipore). The blots were probed with appropriate antibodies as described (5).

**PKC in vitro assays**

*In vitro* PKC assays were carried out as described previously (5,14). The reaction mixture (30 µl) contained 50 µM ATP and 5 µCi of [γ⁻³²P]ATP (New England Nuclear), 1 mM dithiothreitol, 5 mM MgCl₂, 25 mM Tris-HCl (pH 7.5), 20 µM PS, 10 µM diolein, and 0.2 mM CaCl₂ (for PKCα assay), 0.5 mM EGTA (for PKCδ,ε,η,θ, and -ζ assays) and PKCβ1 optimal peptide substrate (100 µM) (for PKCα,δ,ε,θ, and -ζ assays), PKCε peptide substrate (100 µM) (Calbiochem) (for PKCη assay) or syndecan-4 cytoplasmic tail peptide (50 µM) in 25 mM Tris HCl. In syndecan-4-associated PKCα assays, the reaction mixture was supplemented with either 50 µM PIP₂ with 0.5 mM EGTA or 20 µM PS and 10 µM diolein with 0.2 mM CaCl₂ as above. Reactions were started by addition of PKC or addition of reaction mixture to the immunoprecipitates and incubated...
at 30°C for 10 min. The reaction was stopped by spotting onto P81 phosphocellulose paper or by boiling in Laemmli buffer.

**Syndecan-4 phosphorylation stoichiometry**

Confluent RFPEC were incubated for 24 hr in methionine-, sulfate-, and phosphate-free MEM prepared from MEM SELECT-AMINE kit (Life Technologies) with 1% bovine serum albumin (Life Technologies), and radiolabeled for 2 hr with 2 mCi/ml $^{35}$S-methionin (EasyTag Express, New England Nuclear) and 1 mCi/ml $^{32}$P-orthophosphoric acid (New England Nuclear). Syndecan-4 was immunoprecipitated, gel-resolved, and its the ratio between its $^{32}$P and $^{35}$S incorporation was measured by scintillation counting as described previously (13).

**Proliferation assay**

2,000 cells were plated in 96 well tissue culture plates and incubated overnight in 10% FBS-M199 medium. After that, cells were starved with 0.5% FBS for 24 hr, and then treated with 0, 5, or 25 ng/ml FGF2. For measurement of proliferation, 20 µl of CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega) was added to the wells and incubated for 2 hr. Absorbance at 490 nm was measured using 96 well plate reader both before FGF2 application and 72 hr later.

**Matrigel assay**

Matrigel (Beckton Dickinson) plates were prepared by adding 0.5 ml of thawed Matrigel to 12 well tissue culture plate. The gel was allowed to solidify for 1 hour at 37°C.
100,000 cells were plated in each well with 25 ng/ml FGF-2 in 0.5% FBS. Cells were imaged after 24 hr incubation at 37°C in a humidified chamber with 5% CO₂. Analysis of Matrigel results was carried out as previously described (9).
RESULTS AND DISCUSSION

To define the PKC isoform responsible for phosphorylation of Ser\textsuperscript{183} in the syndecan-4 cytoplasmic domain, we assayed the ability of all PKC isoform expressed in RFPEC cells to phosphorylate it \textit{in vitro}. To determine the PKC isozyme preferentially phosphorylating Ser\textsuperscript{183} over other potentially phosphorylatable residues in the syndecan-4 tail, we synthesized a peptide corresponding to the syndecan-4 cytoplasmic domain with Ser\textsuperscript{183} replaced by Ala (SA peptide). While all PKC isoforms phosphorylated both the wild type and mutant peptides with similar efficiencies, only PKC\textgreek{a} and PKC\textgreek{d} preferentially phosphorylated the Ser\textsuperscript{183} site (i.e. preferential phosphorylation of the wild type peptide compared to the SA peptide, Fig. 1A). Since our previously published PKC inhibitor studies (13) strongly argue against PKC\textgreek{a} as a biologically relevant PKC isoform phosphorylating syndecan-4, we compared the extent of syndecan-4 cytoplasmic domain phosphorylation in growth arrested wild type RFPEC cells and in an RFPEC-derived cell line stably expressing dominant negative PKC\textgreek{d}, \textgreek{e} or \textgreek{a} constructs (Fig. 1B). While PKC\textgreek{d} dominant negative expression resulted in a 2.5-fold reduction in the stoichiometry of syndecan-4 cytoplasmic tail phosphorylation, the expression of another non-calcium-dependent PKC (PKC\textgreek{e}) or a calcium-dependent PKC\textgreek{a}, had no effect on syndecan-4 phosphorylation stoichiometry. At the same time, overexpression of PKC\textgreek{d} increased the extent of Ser\textsuperscript{183} site phosphorylation (Fig. 1B). Taken together with previously published observations (13), these results identify PKC\textgreek{d} as the PKC isoenzyme responsible for syndecan-4 phosphorylation.

Since syndecan-4 phosphorylation affects its ability to activate PKC\textgreek{a} in a PIP\textsubscript{2}-dependent manner, we reasoned that PKC\textgreek{d} may inhibit PKC\textgreek{a} activity by
phosphorylating the cytoplasmic tail of syndecan-4. To examine the role of PKCδ in syndecan-4-dependent regulation of PKCα activity, we studied RFPEC-derived cell lines expressing PKCδ and PKCα dominant negative constructs as well as a wild type PKCδ construct. Since expression of dominant negative isoform-specific PKC constructs can potentially influence activities of PKCs other than the intended target (17), we assayed the activity of all PKC isoforms present in RFPEC cells expressing PKCδ or PKCα dominant negative constructs. In both cases the dominant negative construct expression resulted in a significant inhibition of the intended target (Fig. 2A) while not affecting activities of other present PKCs (Fig. 2B). At the same time, overexpression of PKCδ resulted in a significant increase in its activity in cells (Fig. 2C).

Since the extent of syndecan-4 phosphorylation can affect PKCα activity, we measured Ca²⁺ and PIP₂-activated PKCα activity in wild type RFPEC and in clones expressing wild type or dominant negative PKCδ. While the expression of both constructs had no effect on Ca²⁺-activated activity, overexpression of PKCδ significantly decreased syndecan-4/PIP₂-activated PKC-α activity, while expression of PKCδ dominant negative significantly increased it (Fig. 3).

To study whether these changes in PIP₂-activated PKC-α activity translate into functionally relevant changes in cell behavior, we measured the ability of FGF2 to induce proliferation of wild type RFPEC or RFPEC expressing either PKCα and PKCδ dominant negative constructs or unmodified PKCδ. In accord with previously published results (18), expression of PKCα dominant negative construct significantly inhibited cell growth. PKCδ overexpressing cells also had a significantly lower proliferation rate
compared to vector-transfected RFPEC cells (Fig. 4A). In fact, the proliferation rate of PKCδ overexpressors was close to that of cells expressing PKCα dominant negative construct. At the same time, cells expressing PKCδ dominant negative construct demonstrated enhanced proliferation compared to vector-transfected cells. One interesting finding is a high rate of growth of cells expressing the PKCδ dominant negative construct even in the absence of FGF2, presumably because FGF2 activated syndecan-4 phosphatase, in the absence of syndecan-4 phosphorylation, was no longer needed to activate PKCα (Fig. 4A). Similar results were obtained in an in vitro Matrigel angiogenesis assay, with PKCδ overexpressors and cells expressing PKCα dominant negative construct demonstrating reduced vascular structure formation compared to vector-transfected RFPEC cells (Fig. 4B).

To further link the effect of PKCδ on PKCα activity to changes in syndecan-4 Ser^{183} site phosphorylation, we determined this site’s stoichiometry in RFPEC-derived cell lines expressing PKCδ construct. As expected, PKCδ overexpression increased baseline syndecan-4 phosphorylation (0.28±0.05 vs. 0.19±0.03 mol phosphate/mol protein, p<0.05; PKCδ o/e vs. wild type syndecan-4). At the same time, FGF2 treatment had no appreciable effect on syndecan-4 phosphorylation in PKCδ overexpressing cells (without FGF2: 0.28±0.05; with FGF2: 0.27±0.06, mol phosphate/mol protein).

The results of this study show that PKCδ is the kinase responsible for syndecan-4 cytoplasmic domain phosphorylation and that it regulates PKCα activity via this mechanism. Several observations support this conclusions. The conclusion that PKCδ is the PKC isoenzyme responsible for Ser^{183} phosphorylation in the syndecan-4 cytoplasmic domain is supported by its ability to preferential phosphorylate the Ser^{183} site in vitro.
While PKCα also preferentially phosphorylated this site in vitro, only PKCδ had this activity in vivo as demonstrated by a decrease in the extent of Ser\textsuperscript{183} phosphorylation \textit{in vivo} in cells expressing a PKCδ DN construct, and an increase in cells overexpressing PKCδ. At the same, expression of PKCα dominant negative construct had no effect on syndecan-4 phosphorylation in vivo. It is not clear why the PKCα effect on syndecan-4 phosphorylation is different in vitro vs. in vivo settings. PKC-α does not directly interact with syndecan-4, but rather binds to the syndecans-4-PIP2 complex (6). It is quite possible that when such complex is formed, as would be the case in vivo, the \textsuperscript{183}Ser site is no longer accessible to PKCα.

The modulation of the extent of syndecan-4 phosphorylation, achieved by expression of either PKCδ or PKCδ dominant negative constructs, affected its ability to activate PKCα in the PIP$_2$-dependent manner. Interestingly, the Ca$^{2+}$-dependent PKC-α activity in cells expressing the wild type PKCδ or the PKCδ dominant-negative construct, was not affected. These alterations in PKCδ or its dominant negative construct expression (and corresponding changes in syndecan-4 Ser\textsuperscript{183} phosphorylation) resulted in significant changes in cellular function as demonstrated by the proliferation and the \textit{in vitro} Matrigel angiogenesis assays.

The changes in endothelial cell function induced by PKCδ overexpression in these experiments- inhibition of endothelial cell growth and angiogenesis, are consistent with prior publications including growth inhibition in smooth muscle cells (19), fibroblasts (20) and capillary endothelial cells (21,22). Furthermore, the similarity of the functional effects between PKCα overexpression and the expression of a PKCδ dominant negative construct, accompanied by increased PKCα activity, is in agreement with the previously
reported positive effects of PKCα and inhibitory effects of PKCδ on endothelial cell migration (21). It is also interesting to note that VEGF-induced increase in endothelial cell migration and proliferation is accompanied by a decrease in PKCδ activity (23).

The indirect modulation of the activity of one PKC isoform by another by means of regulation of the syndecan-4 phosphorylation state, represents a novel mechanism of modulation of PKC activity. It is also interesting to note that changes in PIP2- but not Ca2+-dependent PKCα activation correlated with changes in cell function, suggesting that syndecan-4/PIP2-dependent regulation of PKCα activity reflects its physiological function.
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FIGURE LEGENDS

Figure 1. Syndecan-4 cytoplasmic tail is phosphorylated by PKCδ.

A. Phosphorylation of syndecan-4 cytoplasmic tail peptides by PKC isoenzymes.

Phosphorylation levels of RMKKKDEGSYDLGKKPIYKKAPTNEFYA (WT) and RMKKKDEGAYDLGKKPIYKKAPTNEFYA (SA) peptides was measured \textit{in vitro} with 6 different recombinant PKC isoenzymes (0.002-0.005 units per sample). The graph shows the ratio between the phosphorylation levels of wild type (WT) and mutated (SA) peptides (mean ± SD, n=3; * indicates here and elsewhere p<0.05).

B. \textit{In vivo} phosphorylation stoichiometries of the syndecan-4 cytoplasmic tail Ser\textsuperscript{183} site in wild type, PKC\textgreek{a}, PKCδ dominant negative (PKCδ DN) and PKC\textgreek{e} dominant negative (PKC\textgreek{e}DN) RFPEC-derived cell lines (mol phosphate/mol protein, mean ± SD, n=4). * indicates p<0.05 vs. wild type RFPEC.

Figure 2. Expression and activities of PKC constructs in RFPEC

A. \textit{In vitro} PKC\textgreek{a} and PKCδ activity assays in RFPEC expressing PKC\textgreek{a} and PKCδ dominant negative constructs. Total cell lysates from vector transfected, PKC\textgreek{a} DN and PKCδ DN RFPEC were immunoprecipitated with, respectively, anti-PKC\textgreek{a} and PKCδ antibodies and \textit{in vitro} PKC activity assays were performed as described in Experimental Procedures. PKC isozyme activities were measured in triplicate in each experiment. Graphs summarize the results of 3 independent experiments (mean ± SD). * indicates p<0.05 vs. vector transfected cells.
Insets: PKC isozymes levels in total cell lysates (top) and immunoprecipitates (bottom). Immunoblots were done with same isozyme antibodies as used for IP.

B, *In vitro* PKC\(\eta\), -\(\theta\), and -\(\zeta\) activity assays in PKC\(\alpha\) and PKC\(\delta\) dominant negative (DN) RFPEC. Total cell lysate from vector-, PKC\(\alpha\) DN- and PKC\(\delta\) DN-transfected RFPEC were immunoprecipitated with PKC\(\eta\), -\(\theta\), and -\(\zeta\) antibodies and *in vitro* PKC assays were performed. PKC isozyme activities were measured in triplicate in each experiment.

Graphs summarize the results of 3 independent experiments (mean \(\pm\) SD). * indicates p<0.05 vs control vector transfected cells.

Insets: PKC isozymes levels in total cell lysates (top) and immunoprecipitates (bottom). Immunoblots were done with same isozyme antibodies as used for IP.

C, *In vitro* PKC\(\delta\) assay in PKC\(\delta\) overexpressing (o/e) RFPEC. Total cell lysate from vector-transfected and PKC\(\delta\) o/e RFPEC were immunoprecipitated with anti-c-Myc antibody and *in vitro* PKC assay was performed as described (mean \(\pm\) SD, n=3). * indicates p<0.05.

Inset: Expression of PKC\(\delta\) construct detected by the anti-c-Myc tag antibody.

**Figure 3. Syndecan-4-associated PKC\(\alpha\) activities**

Wild type, PKC\(\delta\) DN and PKC\(\delta\) o/e RFPEC were starved in 0.5% FBS for 24 hrs, and then incubated for another 20 min with 25ng/ml FGF-2. Total cell lysates were immunoprecipitated with antiserum to the ectoplasmic domain of syndecan-4, and then divided into 2 equal fractions. *In vitro* PKC assays were performed in 2 conditions; in the presence of phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)) or Ca\(^{2+}\), phosphatidylserine (PS) and diolein (DAG). Data are mean \(\pm\) SD, n=4 for all experiments. * indicates p<0.05.
Inset: Syndecan-4-associated PKCα levels in wild type, PKCδ DN, and PKCδ overexpressing RFPEC. Total cell lysates were immunoprecipitated using anti-syndecan-4 ectoplasmic antibody and PKCα was detected with a specific antibody.

**Figure 4. Effects of the overexpression of WT PKCδ, PKCδ DN, and PKCα DN constructs on endothelial cell function**

**A.** Proliferation assays with RFPEC. Vector-transfected, PKCδ o/e, PKCδ DN, and PKCα DN RFPEC were starved in 0.5% FBS for 24 hrs, and then treated with either 0, 5, or 25 ng/ml FGF2 for 72 hrs. The results are presented as fold-increase in cell number after the 72 hr incubation, relative to the initial cell number (mean ± SD, n=4; each cell line was assayed in duplicate).

**B.** Matrigel assays. Vector-transfected, PKCα DN, PKCδ DN, and PKCδ o/e RFPEC were plated on Matrigel with 25 ng/ml FGF2 in 0.5% FBS. Cells were imaged after 24 hr incubation. Each cell line was assayed in duplicate.
Figure 1A

Ser\textsuperscript{\text{183}} Phosphorylation (WT/SA ratio)

PKC isozyme

α  δ  ε  η  θ  ζ
Figure 1B

Syndecan-4 Phosphorylation Stoichiometry
mol phosphate / mol protein

WT  PKCα DN  PKCδ DN  PKCε DN
Figure 2A

**PKCα Western**

- Total cell lysate
- PKCα-IP

**Cell Type:** Vector PKCα DN PKCδ DN

**PKCδ Western**

- Total cell lysate
- PKCδ-IP

**Cell Type:** Vector PKCα DN PKCδ DN

**PKCα assay**

- Cell Type: Vector PKCα DN PKCδ DN

**PKCδ assay**

- Cell Type: Vector PKCα DN PKCδ DN

* indicates a significant difference.
Figure 2B

PKC$\eta$ assay

PKC$\eta$ Western

Total cell lysate

PKC$\eta$ IP

Cell Type: Vector PKC$\alpha$ DN PKC$\delta$DN

PKC$\theta$ assay

PKC$\theta$ Western

Total cell lysate

PKC$\theta$ IP

Cell Type: Vector PKC$\alpha$ DN PKC$\delta$DN

PKC$\zeta$ assay

PKC$\zeta$ Western

Total cell lysate

PKC$\zeta$ IP

Cell Type: Vector PKC$\alpha$ DN PKC$\delta$DN

PKC activity (% Vector Control)

Vector PKC$\alpha$ DN PKC$\delta$DN

Vector PKC$\alpha$ DN PKC$\delta$DN

Vector PKC$\alpha$ DN PKC$\delta$DN
Figure 2C

Vector PKC δ o/e

PKCδ activity (AU)

Vector PKC δ o/e

0 5000 10000 15000 20000 25000

*
Figure 3

**PIP2 assay**

**Ca^{2+}/ PS, DAG assay**

|            | WT  | PKCδ DN | PKCδ o/e |
|------------|-----|---------|----------|
| PKCα activity (% WT) |     |         |          |
| WT         |  0  |         |          |
| PKCδ DN    | 150 |         |          |
| PKCδ o/e   |  0  |         |          |

*Significant difference compared to WT.*
Figure 4A

Cell Number (Fold Increase) vs. FGF2 (ng/ml)

- Vector
- PKCα DN
- PKCδ o/e
- PKCδ DN

* indicates statistical significance.
Figure 4B

Vector

PKCα DN

PKCδ o/e

PKCδ DN
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