Phosphorylation of the Cytoplasmic Tail of Syndecan-4 Regulates Activation of Protein Kinase Ca

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Syndecans are transmembrane proteoglycans capable of carrying both heparan and chondroitin sulfate chains. The cytoplasmic tail of syndecan-4 was recently reported to undergo in vivo phosphorylation on Ser183 in the membrane-proximal part of the tail (Horowitz, A., and Simons, M. (1998) J. Biol. Chem. 273, 10914–10918). However, the functional consequences of this event remain unknown. The cytoplasmic tail of syndecan-4 is known to undergo multimerization and to activate protein kinase Ca (PKCa), with both events depending on the presence of the commonly occurring phospholipid phosphatidylinositol 4,5-bisphosphate (PIP2). In the present investigation we found that phosphorylation of Ser183 produced a 10-fold reduction in the ability of syndecan-4 to activate PKCa, without affecting its ability to bind the PKC. Because Ser183 is adjacent to positively charged lysine groups that resemble PIP2-binding regions in several other proteins, phosphorylation of this serine may affect the binding affinity of the syndecan-4 cytoplasmic tail to PIP2. We found that the Ser183-phosphorylated cytoplasmic tail of syndecan-4 has indeed a significantly lower affinity to PIP2 compared with the nonphosphorylated tail. Furthermore, Ser183 phosphorylation abolished PIP2-dependent oligomerization of syndecan-4 cytoplasmic tails. We conclude that Ser183 phosphorylation regulates syndecan-4-dependent activation of PKCs by reducing the affinity to PIP2 and inhibiting the oligomerization of syndecan-4 cytoplasmic tails. These results further support the role of syndecan-4 in signal transduction in endothelial cells.

EXPERIMENTAL PROCEDURES

Materials—PIP2, phosphatidylserine (PS), and diolein were purchased from Sigma. Recombinant PKCa and PKCδ were synthesized and prepared as described (9). PKCβ1 minimal substrate peptide (FKKKK05FKKFA) was purchased from Tufts University Medical School (Boston, MA). A 28-amino acid-long syndecan-4 cytoplasmic tail peptide (S4c) (RMKKDEGSYDLGKKPIYKKAPTNEFYA) was synthesized by Genemed Synthesis (South San Francisco, CA). A similar peptide with a phosphorylated Ser (S4c-P) was synthesized by the Biopolymers Laboratory, Harvard Medical School (Boston, MA).

PI3K Binding Assay—PIP2 (from Sigma, dissolved at 2 mg/ml in 20 parts CHCl3, 9 parts MeOH, 1 part H2O, 0.1 part 1 N HCl) was dried under N2 and sonicated for 5 min following the method described in Ref. 10. The samples were layered on 30-kDa molecular mass cut-off cellulose filters (Ultrafree-MC, Millipore, Bedford, MA) and spun at 2000 × g for 1 min following the method described in Ref. 10. The samples (40 μl of each in Laemmli sample buffer, 2% SDS, 10% glycerol, 0.5% β-mercaptoethanol, 0.004% bromophenol blue, 50 μm Tris-HCl, pH 6.8) were resolved by SDS-PAGE on 16.5% Tris-Tricine gels (Bio-Rad). Gels were stained with Coomassie Brilliant Blue G-250 (Bio-Rad), and

1,1-bis(hydroxymethyl)ethylglycine; RFFEC, Rat fat pad capillary endothelial cell(a).
images of the stained bands were digitized (DeskScan II on ScanJet 4c, Hewlett Packard) and quantitated by densitometry (ImageQuant, Molecular Dynamics, Sunnyvale, CA).

**Size Exclusion Chromatography—**Syndecan-4 cytoplasmic tail peptides S4c or S4c-P (300 μg) were incubated with PIP2 (350 μM, prepared as above) in 0.5 ml 50 mM HEPES (pH 7.3), 150 mM NaCl on ice for 30 min. Samples were applied at 4 °C to a Sephadex G-50 (Amersham Pharmacia Biotech) 30 × 1.6 cm column equilibrated with the incubation buffer, and the absorbency of the flow-through was measured at 280 nm.

**Immunoprecipitation—**Rat fat pad capillary endothelial cells (RFPEC, gift of Dr. R. D. Rosenberg, MIT (11)) were grown to confluence in M199 medium supplemented with 10% fetal bovine serum (Life Technologies, Inc.) at 37 °C in a 5% CO2 humidified atmosphere. The cells were harvested by trypsinization, lyzed, and subjected to immunoprecipitation with a cytoplasmic tail-specific antisera (gift of Dr. N. W. Shwora, MIT (12)) as described (7).

**Electrophoresis, Transfer, and Immunoblotting—**Immunoprecipitated syndecan-4 cytoplasmic tail was resuspended in Laemmli sample buffer and resolved by SDS-PAGE on a 4–20% Tris-glycine gel (Bio-Rad) and transferred for 2 h at 250 mA in 150 mM glycine, 20 mM Tris-HCl, and 20% methanol to a polyvinyldene fluoride (PVDF) membrane (Immobilon-P, Millipore). The membranes were immunoblotted as described (7) using polyclonal antibodies to PKCα or to PKCδ (both at 2 μg/ml, purchased from Santa Cruz Biotechnology, Santa Cruz, CA).

**Syndecan-4 Cytoplasmic Tail Peptide-PKC Binding Assay—**Cytoplasmic tail peptides S4c or S4c-P (10 μM) were incubated on ice for 30 min either in the presence or absence of PIP2 (20 μM, prepared as above) with recombinant PKCα (4 μM) in 0.5 ml of the same buffer used in the IP binding assay. The cytoplasmic tail peptide was immunoprecipitated, and the samples were resolved by SDS-PAGE, transferred, and immunoblotted as described above.

**PKC in Vitro Assays—**Samples (30 μl) consisted of PKCβ1 optimal substrate peptide (100 μM) either with or without syndecan-4 cytoplasmic tail peptides S4c or S4c-P (both at 50 μM) in 25 mM Tris-HCl (pH 7.4), 5 mM MgCl2, 1 mM dithiothreitol, 50 μM ATP, and 5 μCi of [γ-32P]ATP (NEN Life Science Products). In some assays the buffer was supplemented with either PIP2 (50 μM) or PS (4 μg/ml), diolein (6.2 μg/ml), and 0.2 mM CaCl2. In PKCα assays the buffer was supplemented with PS and diolein as above and with 0.5 mM EGTA. Upon addition of either PKCα (120 ng/ml) or PKCδ (430 ng/ml), samples were incubated at 30 °C for 10 min, and reactions were stopped by boiling in Laemmli sample buffer for 4 min. The samples were resolved on 18.5% Tris-Tricine gels (Bio-Rad), transferred to PVDF membranes, and detected as described (7).

**RESULTS**

**Effect of Syndecan-4 Cytoplasmic Tail Phosphorylation on Activation of PKCα—**Syndecan-4 cytoplasmic tail has been shown to activate a mixture of Ca2+-dependent PKCs and of recombinant PKCα in the presence of PIP2 (4, 5). To assess the effect of Ser183 phosphorylation on syndecan-4-dependent PKC activation, we studied the ability of the S4c and S4c-P peptides to activate recombinant PKCα using the PKCβ1 optimal substrate peptide (9) in an in vitro assay. When the assays were carried out with the standard cPKC cofactors PS, diacylglycerol, and calcium, the presence of neither the S4c nor the S4c-P peptides had any additional effect on the catalytic activity of PKCα (Fig. 1). That was also the case in PKCα assays where no cofactors were added. However, in the presence of PIP2 together with the S4c peptide, the catalytic activity of PKCα toward the PKCβ1 peptide was approximately 10-fold larger than in assays with PIP2 alone. On the other hand, when the S4c-P peptide was added instead of S4c, the phosphorylation level of the substrate was similar to that obtained with PIP2 alone. Unlike PKCα, the S4c peptide did not activate PKCδ under the same conditions (data not shown). The activity of PKCα in the presence of the S4c peptide and PIP2 was 72 ± 10% (± S.D., n = 3) of its activity in the presence of the S4c peptide, PS, diacylglycerol, and calcium.

**Binding of PKCα to the Cytoplasmic Tail of Syndecan-4—** The ability of the unphosphorylated but not the phosphorylated cytoplasmic tail of syndecan-4 to activate PKCα in vitro may relate to a reduced PKCα affinity upon phosphorylation of the cytoplasmic tail. Previous studies have demonstrated the ability of the cytoplasmic tail of syndecan-4 to bind PKCα (5), and the identity of the bound PKC isozyme in vivo was narrowed down to a group of four (α, β, βII, γ, and δ (5)). Although PKCα was shown to bind to the cytoplasmic tail of syndecan-4 in vitro (5), the cytoplasmic tail could also bind to and be a substrate of PKCδ (7). To determine the ability of syndecan-4 to bind PKCα or δ in vivo, RFPEC lysates were immunoprecipitated with an antisem to the cytoplasmic tail, and the immunoprecipitants were probed with antibodies specific either to the α or δ PKC isozymes. The presence of PKCα but not of PKCδ was detected in the immunoprecipitants (Fig. 2, A and B).

To analyze the effect of syndecan-4 cytoplasmic tail phosphorylation on its ability to bind PKCα, we carried out in vitro assays with recombinant PKCα and the S4c and S4c-P peptides. Incubation of PKCα with either peptide produced, however, similar degrees of binding both in the presence and absence of PIP2 (Fig. 2C). It follows, therefore, that PKCα binding is not affected by the Ser183 phosphorylation in the syndecan-4 cytoplasmic tail and thus cannot explain the effect of syndecan-4 phosphorylation on the activity of the enzyme.

**Effect of Phosphorylation on PIP2 Binding to the Cytoplasmic Tail of Syndecan-4—** Both the oligomerization and PKCα activation capacities of the cytoplasmic tail of syndecan-4 were found to depend on the presence of PIP2 (4, 8). It was of interest, therefore, to determine whether the phosphorylation of Ser183 in the cytoplasmic tail of syndecan-4 affects the affinity of the tail to PIP2. To this end, we compared the in vitro binding between PIP2 micelles and S4c or S4c-P peptides using a filtration assay. The filter retains the PIP2 micelle-bound peptide, whereas the unbound peptide passes through it. The binding affinity of the S4c peptide to PIP2, as determined by band densitometry of the SDS-PAGE-resolved filter flow-through samples, was significantly higher than that of the S4c-P peptide. At a peptide:PIP2 molar ratio of 2:1, 50% of the S4c peptide that passed through the filter in the absence of PIP2 was retained versus none of the S4c-P peptide (Fig. 3). Practically all the applied S4c peptide was retained by the filter at a peptide:PIP2 molar ratio of 1:2, whereas as much as 50% of

**FIG. 1. Activation of PKCα by syndecan-4 cytoplasmic tail peptides.** Densitometry histograms of autoradiographic images of PKCβ1 optimal substrate peptide resolved on 16.5% Tris-Tricine gels and transferred to PVDF membranes (n = 3; error bars denote S.D.). The substrate was phosphorylated in vitro by recombinant PKCα (120 ng/ml) in the presence of PS (4 μg/ml), diolein (6.2 μg/ml), and 0.2 mM calcium (white bars), without co-factors (hatched bars), and in the presence of 50 μM PIP2 (black bars). Assays were performed under each condition in the absence (Cont) and in the presence of 50 μM nonphosphorylated (S4c) or phosphorylated (S4c-P) syndecan-4 cytoplasmic tail peptides. Duct, autoradiographic images of PKCβ1 optimal substrate peptide phosphorylated in the presence of 50 μM PIP2.
the S4c-P peptide still passed through the filter under the same conditions. The apparent dissociation constants ($K_d$) calculated from the results shown in Fig. 3 were 2.4 $\mu$M for the nonphosphorylated peptide (S4c), versus 232 $\mu$M for the phosphorylated one (S4c-P). Thus, Ser$^{183}$ phosphorylation results in significant reduction in the ability of PIP$_2$ to bind to the cytoplasmic tail of syndecan-4.

Effect of Phosphorylation on Syndecan-4 Cytoplasmic Tail Oligomerization—Previous studies have demonstrated that the cytoplasmic tail of syndecan-4 undergoes oligomerization in the presence of PIP$_2$ (4, 8); furthermore this oligomerization appeared necessary for PKCa activation. The reduced affinity between the cytoplasmic tail and PIP$_2$ caused by phosphorylation could conceivably be accompanied by changes in the oligomerization properties of syndecan-4. To compare the oligomerization of the S4c peptide to that of the phosphorylated peptide S4c-P, both were incubated either in the presence or absence of PIP$_2$, as described under “Experimental Procedures” and passed through a size exclusion column. Both peptides eluted as a single peak when incubated in the absence of PIP$_2$ (Fig. 4, A and B). When incubated in the presence of PIP$_2$, the S4c peptide eluted as two peaks, one of an approximate molecular mass of 7 kDa (Fig. 4C), and another heavier peak of a molecular mass greater than 17 kDa (the molecular mass of the heaviest molecular mass standard used in this experiment). The S4c-P peptide, on the other hand, eluted as a single peak of the same approximate molecular mass as the first peak of the S4c peptide (Fig. 4D). These results indicate that the cytoplasmic tail of syndecan-4 loses its capacity to form oligomers upon phosphorylation of Ser$^{183}$. Based on the position of the first peaks of the S4c and the S4c-P peptides, it appears that both the S4c (as previously reported (4)) and the S4c-P peptides formed dimers, similar to the behavior observed in the PIP$_2$-binding experiment (Fig. 3). The broader peaks observed with both peptides when incubated in the presence of PIP$_2$, compared with the sharper ones obtained in the absence of PIP$_2$, reflect a wider spread in molecular mass, probably resulting from the range of PIP$_2$ binding to the peptides.

**DISCUSSION**

This study presents three distinct findings concerning the role of the syndecan-4 core protein in signal transduction: (a) Phosphorylation of a single serine residue (Ser$^{183}$) located in the membrane-proximal part of the cytoplasmic tail of syndecan-4 reduces the affinity of the tail to the phosphoinositide PIP$_2$. Upon phosphorylation, the cytoplasmic tail loses its capacity to (b) undergo multimerization and (c) activate PKCa in the presence of PIP$_2$. These findings provide the first evidence for a functional role of the recently reported (7) phosphorylation of Ser$^{183}$ in the cytoplasmic tail of syndecan-4.

The capacities of the cytoplasmic tail of syndecan-4 to undergo multimerization and to activate PKCa were manifest only in the presence of PIP$_2$. A recent NMR study reported on PIP$_2$ binding to a lysine-rich 9-amino acid-long variable region (LGKPIYKK) from the middle of the cytoplasmic tail of syn-
decan-4 (8). The interaction of PIP$_2$ with this region could conceivably be mediated in part by electrostatic bonds between the two phosphates attached to the PIP$_2$ inositol ring and the positively charged lysines of the variable region. This electrostatic interaction could be disrupted by the phosphorylation of Ser$^{183}$ located 3 residues upstream of the variable region. An interaction of a similar nature may take place between PIP$_2$ and several basic residues located in the N-terminal actin-binding domain of $\alpha$-actinin (13). The specific binding of PIP$_2$ to the pleckstrin homology domains of several proteins also appears to be mediated by an electrostatic interaction between the PIP$_2$ phosphates and positively charged lysines in the pleckstrin homology domain (14). Moreover, the syndecan-4 variable region resembles the consensus sequences for PIP$_2$-binding motifs (15). A reduction in PIP$_2$ binding following phosphorylation of serine residues, similar to the one we reported here, is thought to occur in a lysine-rich sequence of the myristoylated alanine-rich protein kinase C substrate (16).

The mechanism of PKCa activation by the cytoplasmic tail of syndecan-4 is thought to require formation of cytoplasmic tail multimers (4). Ser$^{183}$ phosphorylation prevents this oligomerization by inhibiting PIP$_2$ binding to the variable region of the syndecan-4 cytoplasmic tail. It follows, therefore, that the loss of PKCa activation by the cytoplasmic tail upon phosphorylation of Ser$^{183}$ is a direct consequence of the concomitant reduction in affinity to PIP$_2$ and impaired multimerization. Because the cytoplasmic tail of syndecan-4 did not activate PKC$\delta$, this activation may be specific to PKCa. On the other hand, Ser$^{183}$ phosphorylation had no effect on the capacity of the syndecan-4 cytoplasmic tail to bind PKCa. The ability of syndecan-4 to activate PKCa signaling in endothelial cells, the regulation of this signaling by syndecan-4 phosphorylation, and the previously demonstrated bFGF-dependent phosphorylation of the state of syndecan-4 cytoplasmic tail phosphorylation suggests the existence of a novel bFGF-dependent signaling pathway. The same signaling pathway may also be employed by other events affecting syndecan-4 phosphorylation.

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