THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 51, Issue of December 20, pp. 49296–49303, 2002
Printed in U.S.A.

Regulation of Inositol Phospholipid Binding and Signaling through Syndecan-4*

Received for publication, September 20, 2002
Published, JBC Papers in Press, October 10, 2002, DOI 10.1074/jbc.M209679200

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Syndecan-4 is a transmembrane heparan sulfate proteoglycan that can regulate cell-matrix interactions and is enriched in focal adhesions. Its cytoplasmic domain contains a central region unlike that of any other vertebrate or invertebrate syndecan core protein with a cationic motif that binds inositol phospholipids. In turn, lipid binding stabilizes the syndecan in oligomeric form, with subsequent binding and activation of protein kinase C. The specificity of phospholipid binding and its potential regulation are investigated here. Highest affinity of the syndecan-4 cytoplasmic domain was seen with phosphatidylinositol 4,5-bisphosphate (PtdIns-(4,5P)$_2$) and phosphatidylinositol 4-phosphate, and both promoted syndecan-4 oligomerization. Affinity was much reduced for 3-phosphorylated inositides while no binding of diacylglycerol was detected. Syndecan-2 cytoplasmic domain had negligible affinity for any lipid examined. Inositol hexakisphosphate, but not inositol tetrakisphosphate, also had high affinity for the syndecan-4 cytoplasmic domain and could compete effectively with PtdIns(4,5)P$_2$. Since inositol hexaphosphate binding to syndecan-4 does not promote oligomer formation, it is a potential down-regulator of syndecan-4 signaling. Similarly, phosphorylation of serine 183 in syndecan-4 cytoplasmic domain reduced PtdIns(4,5)P$_2$ binding affinity by over 100-fold, although interaction could still be detected by nuclear magnetic resonance spectroscopy. Only protein kinase Cα was up-regulated in activity by the combination of syndecan-4 and PtdIns(4,5)P$_2$, with all other isoforms tested showing minimal response. This is consistent with the codistribution of syndecan-4 with the α isoform of protein kinase C in focal adhesions.

Phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2)[1] has multiple roles in cell signaling and the regulation of cell adhesion, morphology, and trafficking (1–4). It can be cleaved by phospholipases to generate diacylglycerol and inositol trisphosphate (InsP_3). These are second messengers that activate some serine/threonine kinases, including conventional and novel protein kinase C (PKC) isoforms (5, 6) and trigger calcium release from intracellular stores (6), respectively. InsP_3 can also be the target of kinases that sequentially convert it through InsP_4 and InsP_5 to InsP_6 (inositol hexaphosphate) that has been proposed to have various regulatory functions in phosphatase inhibition, trafficking, calcium influx, and cell growth (7–9). PtdIns(4,5)P_2 can also be converted to PtdIns(3,4,5)P_3 by PI 3-kinases that have also been implicated in regulation of protein trafficking, cell growth and survival, and cytoskeletal organization (10, 11). In addition, PtdIns(4,5)P_2 may have roles itself, such as binding and regulation of the actin-associated proteins vinculin, α-actinin, and gelsolin (2). Binding of PtdIns(4,5)P_2 to specific sites on these proteins influences their interactive properties with, for example, actin (12–14). Many proteins interact with this phospholipid through defined motifs including pleckstrin homology and epsin N-terminal homology domains (15–17).

One cell surface heparan sulfate proteoglycan, syndecan-4, also binds PtdIns(4,5)P_2 through a motif in the central portion of its cytoplasmic domain, known as the V (variable) region. The V region of syndecan-4 is unlike that of any other family member and has the sequence LGKKPIYKKA (18, 19). The two pairs of lysine residues appear to be critical for this activity, and the motif KK*XXX*KK is known to bind inositol polyphosphate based on previous studies, for example of synaptotagmin II (20). The functional consequences of inositol phospholipid binding at this site appears to be the stabilization of a dimer of syndecan-4 cytoplasmic domain in an unusual twisted clamp motif, determined by nuclear magnetic resonance (NMR) spectroscopy (21, 22). Dimers, or more probably, higher order oligomers (23), then can bind protein kinase Cα (PKCα), and cause it to be strongly activated (18, 23, 24). This may explain why syndecan-4 and PKCα are both focal adhesion components in many cell types (25, 26). Our current hypothesis is that

* This work was supported by National Institutes of Health Grants GM50194 (to J.R.C. and A. W.) and NS29632 (to G. D. P.), the Sankyo Chemical Company (to J. R. C., and A. W.), Wellcome Trust program Grant 065940 (to J. R. C.), a grant from the Ministry of Science and Technology/The Korea Science and Engineering Foundation through the Protein Network Research Center at Yonsei University (to W. L.), and Korea Research Foundation Grant KRF-99-042-D00096 (to E. S. O.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PtdIns(3,4,5)P_3, phosphatidylinositol 3,4,5-trisphosphate; InsP_3, inositol 1,4,5-trisphosphate; InsP_4, inositol 1,3,4,5-tetrakisphosphate; InsP_6, inositol hexakisphosphate; InsS_6, inositol hexasulfate; PKC, protein kinase C; BZDC, benzoyldihydrocinnamide; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

syndecan-4, when clustered, signals through the kinase at nascent focal adhesions and contributes to focal adhesion assembly, with possible involvement of the G protein RhoA (19, 23–25, 27).

One unresolved issue is the regulation of the syndecan-4, PtdIns(4,5)P_2, and PKCα signaling complex. There are several possibilities. First, all syndecans can interact with PDZ domain proteins through a C-terminal FYA sequence (19, 28). Such interactions could lead to complex disassembly or internalization or to stabilization. This has not been examined. Second, Horowitz *et al.* (24, 29, 30) showed that phosphorylation of the single serine residue at the boundary of the membrane proximal C1 region and V region of syndecan-4 cytoplasmic domain (Ser^{183}) can lead to decreased signaling through PKCα. In part this may be due to decreased affinity of PtdIns(4,5)P_2 for the phosphorylated syndecan-4 cytoplasmic domain (24). A third, alternate, hypothesis is that PtdIns(4,5)P_2 may be displaced by another compound, yielding a form of syndecan-4 cytoplasmic domain unable to bind or activate PKCα. One possibility is $InsP_6$. The KK*XXX*KK motif within syndecan-4 V region provides a potential site for $InsP_6$ binding (31), although we showed previously that this inositol phosphate neither promotes syndecan-4 cytoplasmic domain oligomerization nor activates PKCα (18).

Here we have examined these hypotheses by analyzing the specificity of phospholipid and inositol phosphate binding to syndecan-4 cytoplasmic domain peptides, whether $InsP_6$ can compete with PtdIns(4,5)P_2 for binding to the cytoplasmic domain of syndecan-4 core protein and the role of Ser^{183} phosphorylation. Both $InsP_6$ and phosphorylation may down-regulate syndecan-4-mediated signaling, since both strongly affect PtdIns(4,5)P_2 interactions. In addition, $InsP_6$ diminished microfilament bundle formation in fibroblasts, under experimental conditions where syndecan-4 and PKCα are involved.

EXPERIMENTAL PROCEDURES

Materials—Syndecan-2 and −4 peptides corresponding to the entire cytoplasmic domains of human syndecan-4 (4L) and syndecan-2 (2L) were synthesized by SynPep (Dublin, CA), and their sequences confirmed by mass spectroscopy. These sequences were (C)RMKKKDEGSYDLGKKPIYKKAPTNEFYA and RMRKKDEGSYDLGERKPSSAAYQKAPTKEFYA, respectively. Two modified 4L peptides were also synthesized, one lacking the three C-terminal amino acids (FYA; denoted 4ΔE) and a second incorporating a phosphoserine at position 183 in place of serine (p-4L). Also used were peptides corresponding to the central, variable (V) region of syndecan-4 and −2 with the sequences (C)LGKKPIYKK and (C)LGERKPSSAAYQ, respectively. At least two different batches of each peptide were used. All inositol phospholipids and diacylglycerols were purchased from Biomol (Plymouth Meeting, PA). PtdIns(4,5)P_2 was also purchased from Avanti Polar Lipids (Alabaster, AL), as were phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine. $InsP_6$, $InsP_4$, $InsP_3$, and inositol hexasulfate ($InsS_6$), HEPES, CHAPS, and one batch of PtdIns(4,5)P_2 were from Sigma-Aldrich.

Lipid Binding Assay—Lipids were dissolved in a chloroform/methanol solution at 2 mg/ml as described previously (29). Solubilized lipids were dried under N_2 and sonicated for 2 min in ice-cold H_2O at a final concentration of 1 mg/ml. Peptides were incubated on ice for 30 min with the indicated concentrations of lipid in 10 mM Tris-HCl (pH 7.5) in a reaction volume of 100 μl. The samples were spun in filter units (Ultrafree-MC, 30,000 NMWL, Millipore, Bedford, MA) at 2000 × *g* for 70 s following the method described by Haarer *et al.* (32). The filtrates (40 μl of each in 4% SDS, 10% glycerol, 1.5% dithiothreitol, 0.004% bromphenol blue, 50 mM Tris-HCl, pH 6.8) were resolved by SDS-PAGE on 16.5% Tris-Tricine gels, stained with Coomassie Brilliant Blue G-250 (BioRad). The stained peptides were scanned on a BioRad GS670 imaging densitometer and quantitated. All experiments were performed at least in triplicate. Bound and free phospholipid were then calculated and averaged from replicates. The data were used to generate Scatchard plots, from which affinities were estimated. In addition, two forms of displacement experiments were performed. Peptides were first incubated with differing concentrations of inositol phosphate or sulfate for 30 min, then with 50 μM inositol phospholipid for an additional 30 min, or PtdIns(4,5)P_2 was incubated first with the peptide, then the inositol phosphate or sulfate was added.

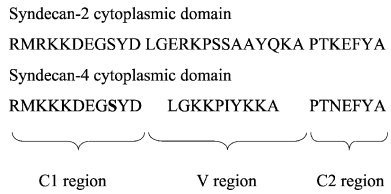

FIG. 1. **Sequences of mammalian syndecan-2 and syndecan-4 cytoplasmic domains.** The two constant regions, homologous with all syndecans (*C1* and *C2*), and the variable domain unique to each syndecan (*V*) are shown. Ser^{183} of syndecan-4 that can be phosphorylated, is shown in *bold*.

TABLE I
Lipid affinities for syndecan-4

Lipid affinities (μM) for cytoplasmic domains of wild-type syndecan-4 (4L), phosphorylated syndecan-4 (p-4L), syndecan-4 with a truncation of the C-terminal last three amino acids (4ΔE), or wild-type syndecan-2 (2L). ND-not detectable.

Lipid	4L	p-4L	4ΔE	2L
PtdIns4,5P_2	5	570	4.5	6100
PtdIns4P	4	ND		1065
PtdIns3,4P_2	480	ND		ND
PtdIns3,4,5P_3	ND	ND		ND
DAG[a]	ND			ND
DAG[b]	ND			ND
Ptd ethanolamine	ND			ND
Ptd serine	365			3700

[a] DAG, 1-stearoyl-2-linoleoyl-*sn*-glycerol.
[b] DAG, 1-stearoyl-2-arachidonyl-*sn*-glycerol.

Photoaffinity Labeling—The preparation and use of [^{3}H]benzoyldihydrocinnamide (BZDC)-$InsP_4$ and -$InsP_6$ probes has been described previously (33). Experiments were performed in 96-well plates. To 21 μl of buffer, consisting of 25 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 1 mM dibasic potassium phosphate, was added 1 μg of peptide in 5 μl of buffer and 3 μl of water, with or without unlabeled $InsP_4$ or $InsP_6$, as appropriate, to a final concentration of 20 μM. After a 10-min incubation on ice, 1 μl of photoactivable probe (0.5 μCi, 20–70 nM) was added. The plate was incubated for 60 min on ice before exposure to UV irradiation. The reaction was stopped by the addition of 4× SDS-PAGE sample buffer. Samples were resolved by 20% SDS-PAGE at ~30 mA, with prestained low molecular mass standards (Invitrogen) in one or two lanes. The gels were impregnated with Entensify (DuPont), dried and fluorographs exposed for 1 week. In other experiments, increasing concentrations of unlabeled $InsP_6$ were included in the assays, in order to ascertain the K_i as an approximate measure of affinity of the inositol phosphate for the V region of syndecan-4.

Size Exclusion Chromatography—Gel filtration procedures were as previously (22). Synthetic peptides or a mixture of synthetic peptide and phosphoinositide were loaded onto a Sephadex G-50 gel filtration column (0.7 × 50 cm) equilibrated with 50 mM HEPES (pH 7.3), 0.1% CHAPS, and 150 mM NaCl. Peptides were eluted with the same buffer at a flow rate of 3 ml/h at room temperature, and 1-ml fractions were assayed on a UV monitor at 280 nm. The column was calibrated with molecular standards containing thyroglobulin (670 kDa), bovine γ-globulin (158 kDa), chicken ovalbumin (44 kDa), equine myoglobin (17 kDa), and vitamin B_{12} (1.3 kDa).

Cell Adhesion Studies—Primary rat embryo fibroblasts were seeded onto fibronectin-coated glass coverslips in 24-well plates as previously (34) for 30 min. At this time $InsP_4$, $InsP_6$, or $InsS_6$ were added to the serum-free medium at various concentrations for up to an additional 2.5 h. In some cases, after 1.5 h, adherent cells were returned to normal growth medium, as a control to ascertain that adhesion inhibition was reversible. For interference reflection microscopy analysis of focal adhesion formation (35), cultures were fixed in 3% glutaraldehyde in phosphate-buffered saline for 15 min and washed and mounted in serum-free medium. Other cultures were fixed in 4% paraformaldehyde in phosphate-buffered saline, containing 0.1% Triton X-100. Texas Red-conjugated phalloidin (Molecular Probes, Eugene, OR) staining of microfilaments was performed as before (35). The percentage of cells with focal adhesions or microfilament bundles was counted in three separate

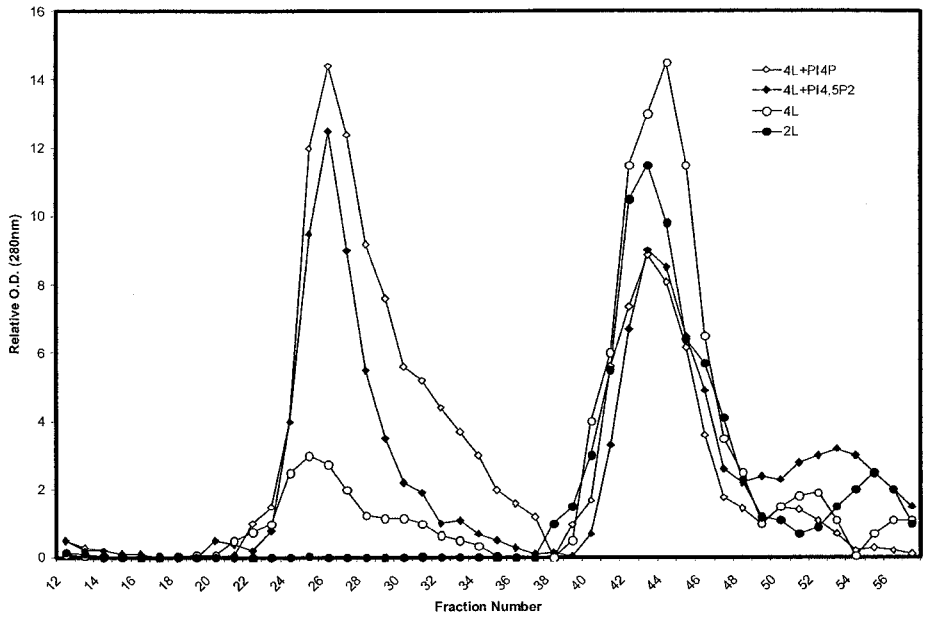

FIG. 2. **Inositide promotion of syndecan-4 cytoplasmic domain oligomerization.** Gel chromatograms of syndecan-4 (4L) and syndecan-2 (2L) cytoplasmic domains in the presence or absence of PtdIns-4P or PtdIns(4,5)P_2. Both inositides promoted oligomerization of 4L. 2L had no tendency to oligomerize and eluted as a monomer.

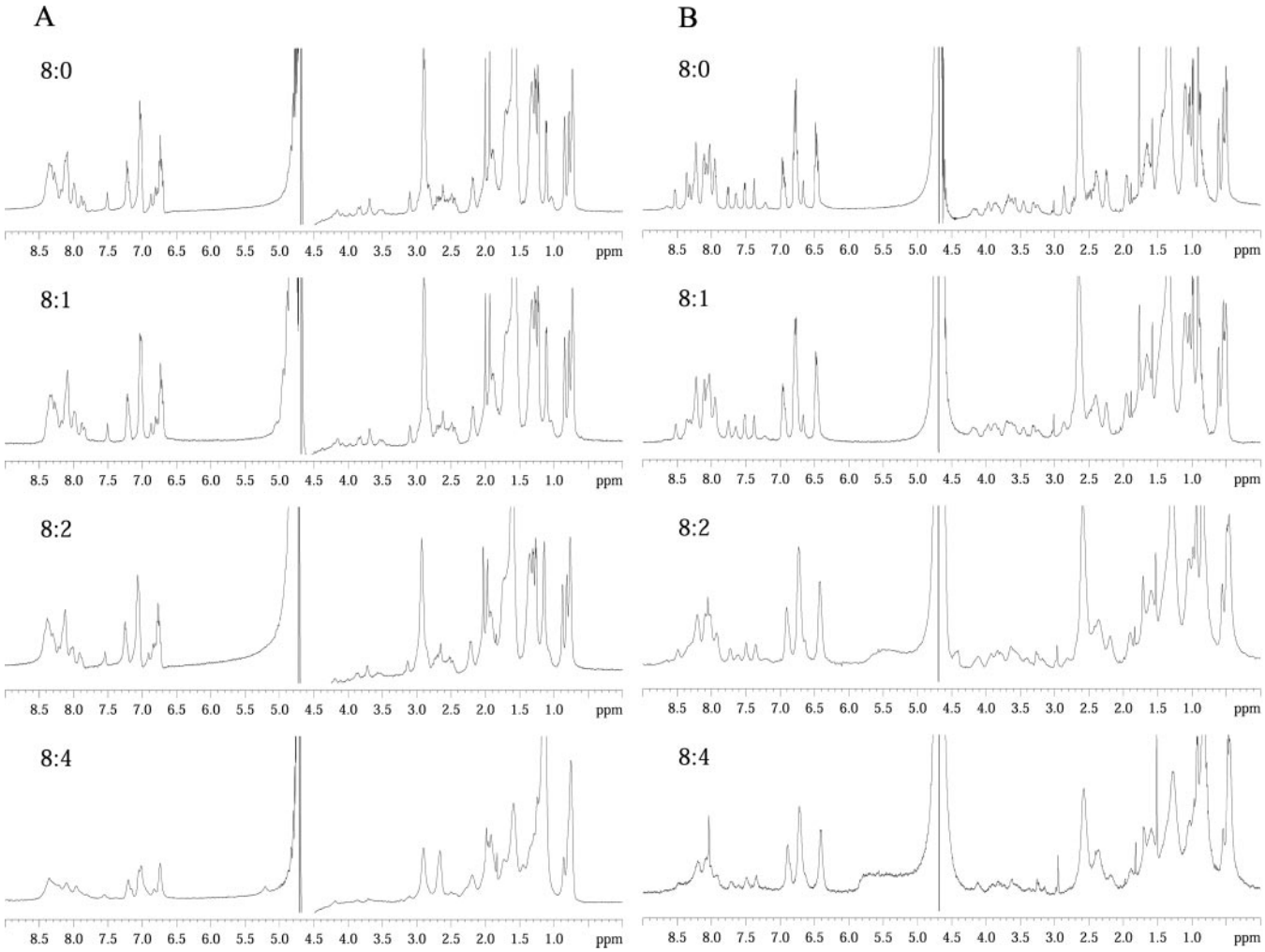

FIG. 3. **Syndecan cytoplasmic domain interactions with PtdIns(4,5)P_2.** Proton one-dimensional NMR spectra of 4L peptide (*A*), p-4L peptide (*B*), and 2L peptide (*C*) with titration of PtdIns(4,5)P_2. Molar ratios of peptide to inositide are shown in the *upper left* of each panel.

replicates, with at least 100 cells per coverslip counted in each case. In further studies, cells were seeded on the integrin-binding 110-kDa fragment of plasma fibronectin (19, 34, 35) for 1.5 h, then stimulated for 30 min at 37 °C with 10 ng/ml recombinant 31-kDa hepII domain of fibronectin (35) or 25 μg/ml purified monoclonal antibody 150.9 against the N-terminal of syndecan-4 (36) to promote focal adhesion formation (19, 27). Some cultures were treated with $InsP_6$ or $InsS_6$ for the final 35 min. Cells were fixed and stained for microfilament bundles with Texas Red-conjugated phalloidin as before.

Protein Kinase C Assays—Recombinant isoforms of PKC were incu-

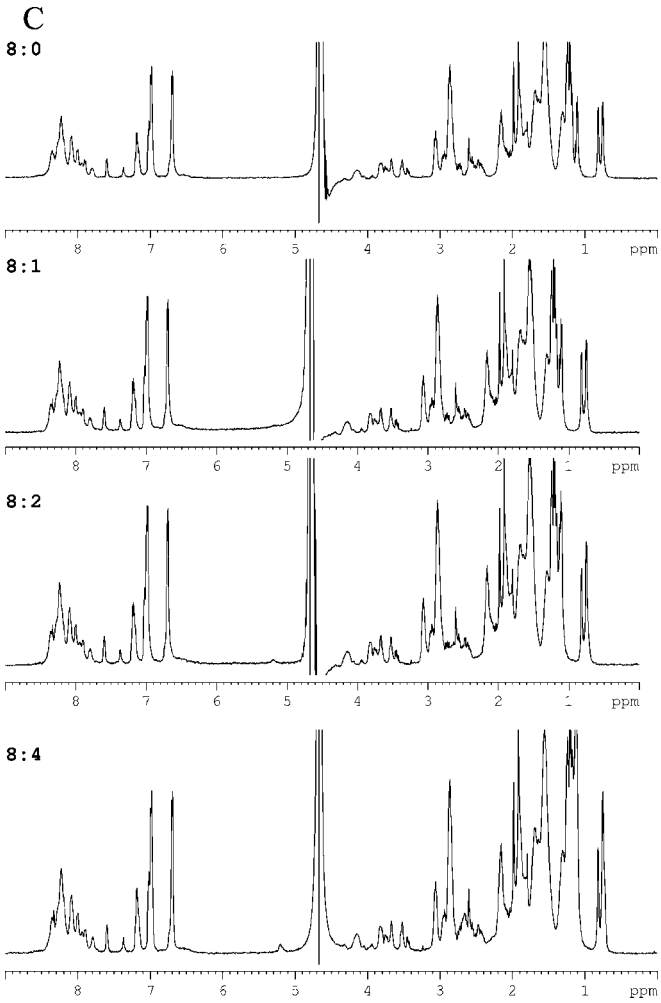

FIG. 3—*continued*

bated with, or without, the addition of PtdIns(4,5)P_2 and syndecan-4L cytoplasmic domain peptide, using identical methods as previously (18, 23). Phosphorylated histone IIIS was resolved by 20% SDS-PAGE and autoradiography. Quantitation was by laser scanning densitometry as before (18).

Nuclear Magnetic Resonance Spectroscopy—Phosphoinositide titration experiments were performed on a Bruker DRX500 spectrometer in quadrature detection mode, equipped with a triple resonance probe head with triple-axis gradient coils. A series of one-dimensional NMR spectra were recorded for 4L, p-4L, and 2L peptides with different concentrations of PtdIns(4,5)P_2. All data were collected at 25 °C, and the strong solvent resonance was suppressed by water-gated pulse sequence combined with pulsed-field gradient (PFG) pulses. All NMR data were processed using Bruker XWIN-NMR (Bruker Instruments) software on an SGI Indigo[2] work station. The proton chemical shifts were referenced to internal sodium 4,4-dimethyl-4-silapentane 1-sulfonate (DSS).

RESULTS

Specificity of Phospholipid Binding to Syndecan-4—Fig. 1 shows the sequences of syndecans-2 and −4 with their constant (C1 and C2) and variable (V) regions denoted. Lipid micelles were allowed to bind to peptides corresponding either to the full-length cytoplasmic domain of syndecan-4 or syndecan-2, and the unbound peptide recovered and quantified after centrifugation through a molecular mass cut-off filter. Affinities of the lipids for the peptides are shown in Table I. There was a significant preference of syndecan-4 cytoplasmic domain for PtdIns(4,5)P_2 and PtdIns(4)P over inositol phospholipids containing phosphate at the 3-position on the inositol ring. Phosphatidylserine had weak affinity for syndecan-4, while no binding to phosphatidylethanolamine or two forms of diacylglycerol was noted. Since diacylglycerol is a cleavage product of PtdIns(4,5)P_2 generated by phospholipase C, the data suggest that interactions of the inositol phosphate moiety of PtdIns(4,5)P_2 with syndecan-4 are key. Very little binding of any lipid to syndecan-2 cytoplasmic domain was seen, confirming that only the central V region of syndecan-4 contains the lipid binding site(s), since the C1 and C2 regions of these two core proteins are highly homologous. This was confirmed with a synthetic peptide corresponding to the V region of syndecan-4 cytoplasmic domains, which had similar affinity to 4L for PtdIns(4,5)P_2 (not shown).

The almost identical affinities of PtdIns(4,5)P_2 and PtdIns(4)P for the cytoplasmic domain of syndecan-4 (4L) raised the question of whether both could stabilize the peptide in dimeric conformation. Fig. 2 shows that both phospholipids markedly promoted 4L dimer formation, which is only limited in their absence. Syndecan-2 cytoplasmic domain (2L) showed no tendency to form dimers (Fig. 2), even in the presence of PtdIns (4)P or PtdIns(4,5)P_2 (not shown).

Modification of Syndecan-4 Cytoplasmic Domain and Its Influence on PtdIns(4,5)P_2 Binding—Phosphorylation of the single Ser^{183} residue in syndecan-4 cytoplasmic domain has been reported to decrease phospholipid binding and subsequent PKCα activity (24). A synthetic peptide corresponding to the entire cytoplasmic domain of syndecan-4, but incorporating phosphorylated Ser^{183} had markedly lower affinity for PtdIns(4,5)P_2 than the unphosphorylated peptide (570 μM compared with 5 μM; Table I). Moreover, this decrease was not accompanied by any change in preference for phospholipid interactions. There was still no detectable binding to D3 inositol phospholipids, and the affinity of the 4L peptides for PtdIns (4)P was also reduced to undetectable levels (Table I).

One-dimensional NMR spectroscopy was used as a further sensitive indicator of syndecan cytoplasmic domain interactions with PtdIns(4,5)P_2 (Fig. 3). The spectra demonstrated that each of the three peptides, 4L, p-4L and 2L, have different characteristics with respect to PtdIns(4,5)P_2 binding. Most resonances for 4L and p-4L were changed and broadened upon inositide titration, indicative of oligomerization in the presence of the phospholipid. Therefore, even though the membrane filter assay showed a much decreased affinity of p-4L for PtdIns(4,5)P_2, an interaction was still clearly detectable by NMR spectroscopy. However, the spectra of 4L and p-4L were distinct, which indicate differences in oligomer organization. Consistent with results of gel chromatography and lipid binding assays, the spectra of 2L peptide were unchanged with increasing inositide, indicative of no detectable interaction (Fig. 3*C*).

Published data suggest that the C2 region of syndecan-4 cytoplasmic domain is flexible and may not participate in interactions with inositides (22). This region, and particularly its terminal FYA motif, does interact with PDZ domain proteins (28). These may stabilize the C2 structure *in situ*. Deletion of the FYA sequence did not alter *in vitro* binding affinity for PtdIns(4,5)P_2 (Table I).

Interactions of Inositol Phosphates with Syndecan Cytoplasmic Domains—Peptides corresponding to the V region and entire cytoplasmic domains of syndecan-2 and −4 were exposed to [^{3}H]BZDC-InsP_4 or [^{3}H]BZDC-InsP_6. Fig. 4 shows the structure of these compounds. Covalent cross-linking was achieved by UV irradiation, with subsequent SDS-PAGE, fluorography, and quantitation. Control experiments contained an ~400-fold excess of unlabeled InsP_4 or InsP_6. The results in Fig. 5*A* show

[2] W. Lee and J. R. Couchman, unpublished data.

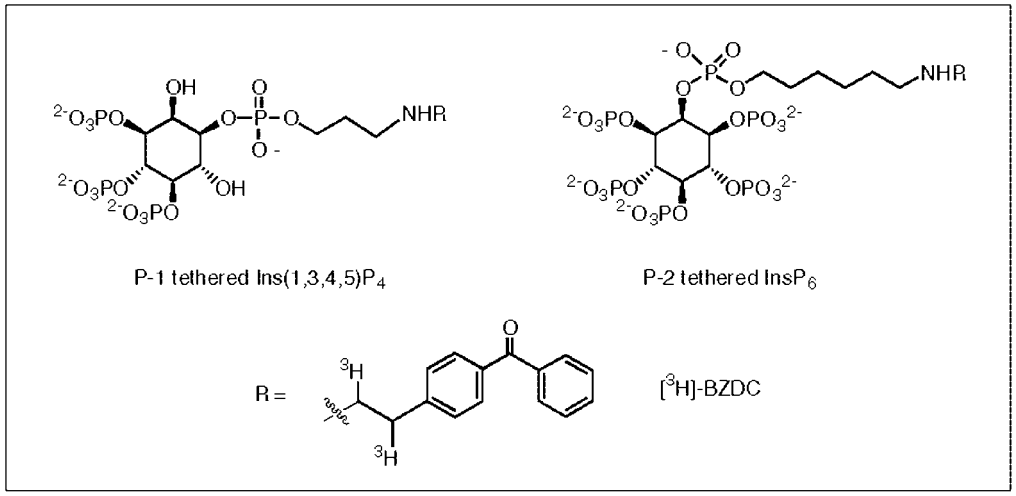

FIG. 4. **Structure of photoaffinity labels for Ins(1,3,4,5)P_4 and $InsP_6$.**

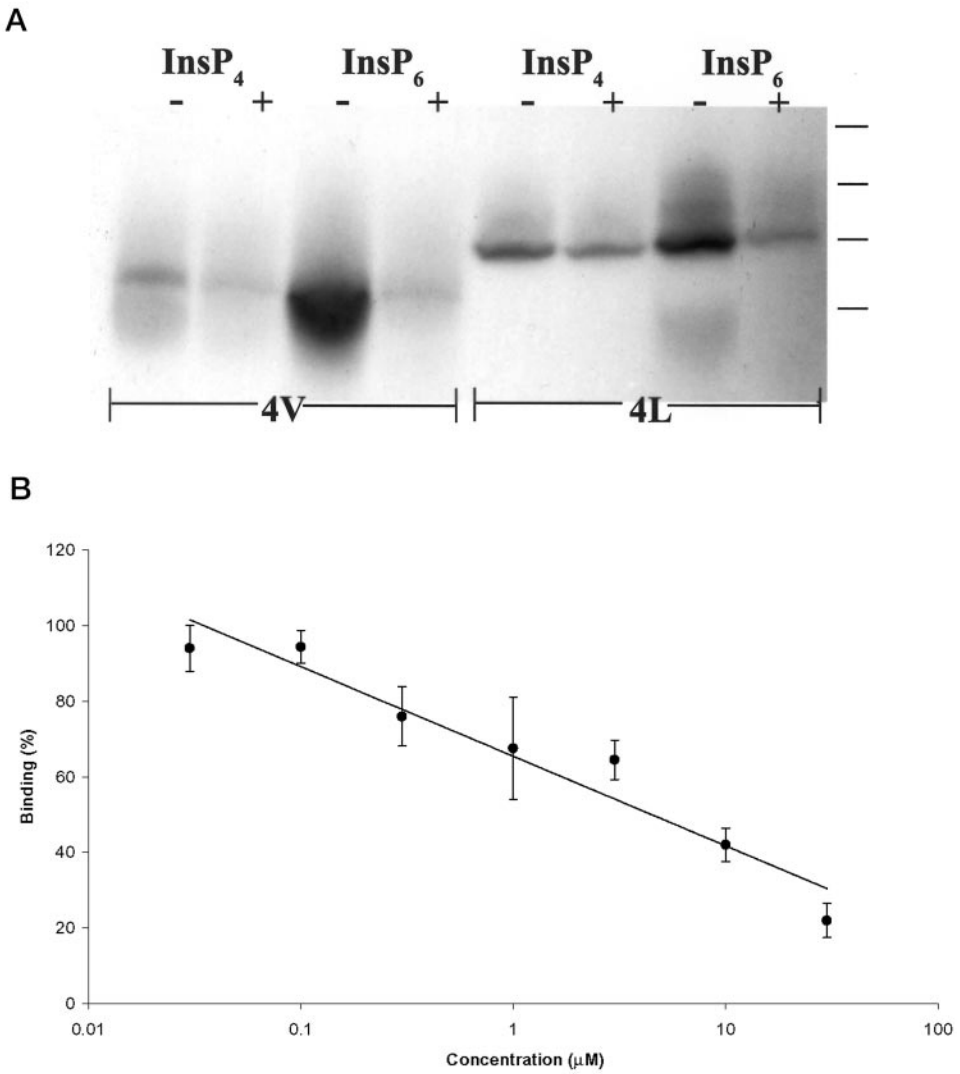

FIG. 5. **Interaction between $InsP_6$ and syndecan-4 cytoplasmic domain.** *A*, photoaffinity-labeled $InsP_4$ and $InsP_6$ were mixed with the syndecan 4L or 4V peptides in the presence (+) or absence (−) of excess unlabeled inositide. Specific binding of the $InsP_6$ probe was observed. *B*, competition experiments performed with increasing levels of unlabeled $InsP_6$, but constant amount of photoaffinity-labeled $InsP_6$ showed an approximate IC_{50} of 4.5 μM.

that both the full-length cytoplasmic domain of syndecan-4 and its central V region specifically bound the [^{3}H]BZDC-$InsP_6$ probe. Quantification of the $InsP_6$ probe bound to 4V and 4L peptides, by scanning densitometry, showed reductions of 92–94% in the presence of excess unlabeled $InsP_6$. In contrast, there was limited binding of the [^{3}H]BZDC-$InsP_4$, and this was

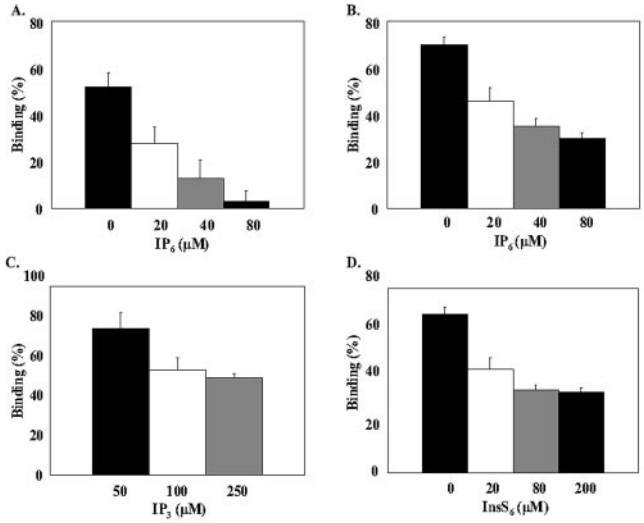

FIG. 6. **$InsP_6$ is a competitor for PtdIns(4,5)P_2 binding to syndecan-4 cytoplasmic domain.** *A*, *C*, and *D*, syndecan 4L peptide was exposed to varying concentrations of $InsP_6$ (*A*), $InsP_3$ (*C*), or $InsS_6$ (*D*), then to 50 μM PtdIns(4,5)P_2. In *B* the peptide was exposed first to PtdIns(4,5)P_2, then to $InsP_6$. The amount of PtdIns(4,5)P_2 bound was calculated from a membrane filter separation of peptide that was either bound or unbound to lipid.

reduced by 58–61% by excess unlabeled compound. Consistent with the fact that the syndecan-4 V region bound the $InsP_6$ probe, no specific binding of syndecan-2 peptides (with their dissimilar V region; Fig. 1) to either probe was detected (not shown).

To gauge the affinity of $InsP_6$ for syndecan-4 cytoplasmic domain, 50 nM [^{3}H]BZDC-$InsP_6$ was incubated with increasing concentrations of unlabeled $InsP_6$ in the presence of 5 μg of 4V peptide. The data show (Fig. 5*B*) the IC_{50} was ~4.5 μM. However, it should be emphasized that the radiolabeled compound is more hydrophobic than the competitor, and so the true affinity of $InsP_6$ itself may be greater.

$InsP_6$ Is an Effective Competitor of PtdIns(4,5)P_2 Binding to Syndecan-4 Cytoplasmic Domain—As a second measure of $InsP_6$ affinity for syndecan-4 cytoplasmic domain, two assays were performed. In the first, the ability of PtdIns(4,5)P_2 to displace bound $InsP_6$ from the 4L peptide, was measured. Inositol polyphosphate was partially displaced (Fig. 6*A*) by Ptdins(4,5)P_2 (just 5% of added PtdIns(4,5)P_2 bound to peptide that was preincubated with 80 μM $InsP_6$, whereas over 50% bound in the absence of $InsP_6$). In the second assay, the ability of $InsP_6$ to displace already bound PtdIns(4,5)P_2 was calculated. The higher affinity of $InsP_6$ for syndecan-4 cytoplasmic domain was again suggested, since the inositol phospholipid was readily displaced in a dose-dependent manner (Fig. 6*B*; PtdIns(4,5)P_2 binding was reduced to 30% by 80 μM $InsP_6$). In a control experiment for that shown in Fig. 6*A*, $InsP_3$ was used in place of $InsP_6$ (Fig. 6*C*). Here, even preincubation of peptide with high quantities of $InsP_3$ did not reduce the amount of PtdIns(4,5)P_2 that subsequently bound. In another control experiment, inositol hexasulfate was used in place of $InsP_6$ (Fig. 6*D*). Preincubation with sulfated inositol was less efficient than with $InsP_6$ in reducing PtdIns(4,5)P_2 binding to the syndecan-4 cytoplasmic peptide, but more efficient than $InsP_3$. This suggests that ionic interactions are largely responsible for interactions between $InsP_6$ and the syndecan-4 cytoplasmic domain and that this interaction is of higher affinity than that between PtdIns(4,5)P_2 and the 4L peptide.

Inositol Hexaphosphate Inhibits Cell Adhesion—Since $InsP_6$ at levels consistent with those found *in vivo* (ranging as high as 15–60 μM;37) could compete with PtdIns(4,5)P_2 for binding to the syndecan-4 cytoplasmic domain, cell adhesion studies were performed. Syndecan-4 and PtdIns(4,5)P_2, together with PKC, have roles in focal adhesion formation in fibroblasts seeded on fibronectin substrates (13, 19, 25, 27). Since $InsP_6$, when bound to the core protein of syndecan-4, does not facilitate PKCα signaling, it was ascertained whether exogenous inositol phosphates influenced fibroblast adhesion. Table II and Fig. 7 show that while cell spreading was not affected, focal adhesion and microfilament bundle assembly were strongly inhibited by $InsP_6$. This effect was not seen with $InsP_4$, while partial inhibition was seen with $InsS_6$. The effects of both $InsP_6$ and $InsS_6$ were reversible, as focal adhesion formation was completed within 1 h of returning cells to normal culture medium. A further test of $InsP_6$ was in a focal adhesion formation assay. Cells spread on the integrin-binding 110-kDa fragment of plasma fibronectin spread but do not form focal adhesions. This last stage can be stimulated by the 31-kDa HepII domain of fibronectin, or by antibodies against the core protein that cluster syndecan-4 (reviewed in Ref. 19). However, treatment of fibroblasts with 0.1 mM $InsP_6$, after 1.5 h of spreading on the 110-kDa integrin-binding domain of fibronectin, prevented a response to either the recombinant fibronectin HepII domain or clustering antibody against the N terminus of syndecan-4 (Fig. 8). Cells remained spread but did not form prominent microfilament bundles that terminate in focal adhesions. Equivalent treatment with $InsS_6$ had much reduced inhibitory effects (not shown).

TABLE II
The effects of inositol phosphates and inositol hexasulfate on focal adhesion and microfilament bundle formation

Treatment	Time after seeding	% Cells with focal adhesions
	h	
$InsP_4$ (0.2 mM)	1.5	95.0 ± 1.7
	3.0	86.7 ± 0.6
$InsP_4$ (0.4 mM)	1.5	69.7 ± 3.1
	3.0	80.0 ± 1.7
$InsP_6$ (0.2 mM)	1.5	37.7 ± 4.9
	3.0	6.0 ± 3.5
$InsP_6$ (0.4 mM)	1.5	40.3 ± 5.7
	3.0	12.3 ± 1.2
None	1.5	89.0 ± 2.0
	3.0	93.3 ± 2.1

Treatment	Time after seeding	% Cells with microfilament bundles
	h	
$InsP_6$ (0.1 mM)	2.5	19.0 ± 5.6
$InsP_6$ (0.1 mM)	2.5 (with 1h recovery)	87.7 ± 4.0
$InsS_6$ (0.1 mM)	2.5	43.3 ± 14.5
$InsS_6$ (0.1 mM)	2.5 (with 1h recovery)	91.0 ± 2.6
None	2.5	90.0 ± 3.0
None	2.5 (with 1h medium change)	90.5 ± 3.5

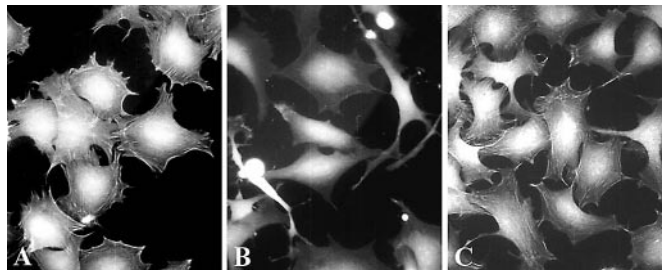

FIG. 7. **$InsP_6$ blocks the final stage of cell adhesion to fibronectin.** Primary rat embryo fibroblast attachment and spreading were unaffected by 0.2 mM InsP6 (*B*) or InsP4 (*C*), but microfilament bundle formation, seen in untreated controls (*A*) and InsP4-treated cells (*C*) was abrogated by $InsP_6$ (*B*). Original magnification, ×50.

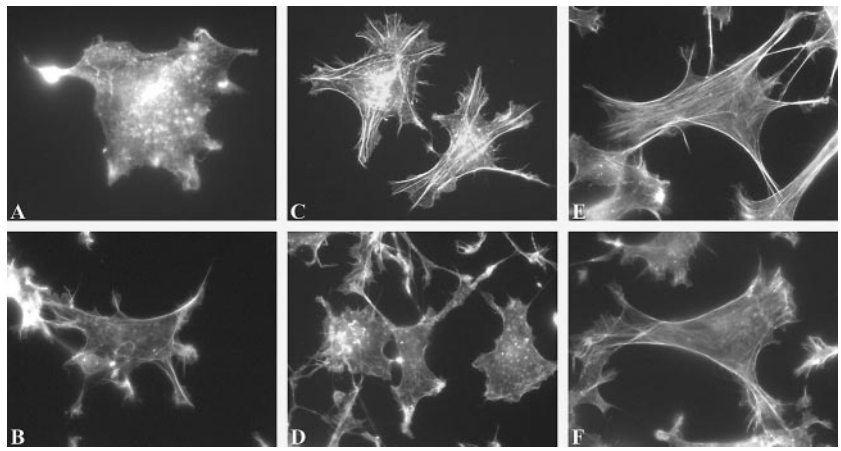

FIG. 8. **Microfilament bundle formation promoted by fibronectin HepII domain and clustering antibodies is inhibited by $InsP_6$.** Fibroblasts spread on the 110-kDa integrin-binding fragment of fibronectin (*A* and *B*) do not form microfilament bundles or focal adhesions, but these are stimulated by fibronectin HepII domain (*C*) or clustering antibodies binding to the N terminus of syndecan-4 core protein (*E*). In both cases, 0.1 mM $InsP_6$ blocks these events (*D* and *F*, respectively). Original magnification, ×60.

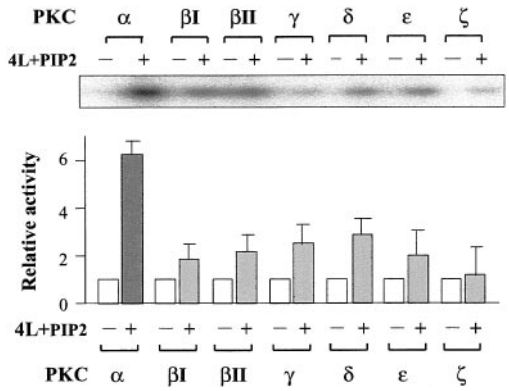

FIG. 9. **Protein kinase Cα is activated by a combination of syndecan-4 cytoplasmic domain and PtdIns(4,5)P_2.** Recombinant isoforms of PKC were assayed for phosphorylation of histone IIIS substrate in the presence or absence of syndecan 4L peptide and PtdIns(4,5)P_2. All isoforms except ζ (which had almost no activity) showed low activity while only α was strongly up-regulated.

Specificity of PtdIns(4,5)P_2/Syndecan-4-mediated PKC Activation—Many isoforms of PKC have a lipid binding capability, but only PKCα has been shown to co-distribute with syndecan-4. A number of recombinant PKC isoforms were tested to determine whether their activity could also be up-regulated by a combination of PtdIns(4,5)P_2 and syndecan-4 cytoplasmic domain. As shown in Fig. 9, only PKCα was markedly activated, but several other conventional (βI, βII, and γ) and novel isoforms (δ and ϵ) could be modestly activated. As expected from a lack of inositide or diacylglycerol binding site, the atypical PKCζ isoform was not affected by the presence of PtdIns(4,5)P2 and syndecan-4 cytoplasmic domain.

DISCUSSION

Syndecan-4 is alone among the family of cell surface heparan sulfate proteoglycans in having a cationic motif in its cytoplasmic V region that can bind inositol phospholipids and PKCα (18, 19, 21–24). We have shown previously by NMR spectroscopy that a dimer of V region is stabilized by one molecule of PtdIns(4,5)P_2, and the cytoplasmic domain forms a unique "twisted clamp" structure that may be relevant to its function (21). Syndecan-4 has a role in focal adhesion formation, and evidence also suggests that PKC activity is required both for focal adhesion formation and syndecan-4 insertion into forming adhesions (25, 26, 38). Here we show specificity in terms of inositol phospholipid binding, with a distinct preference for PtdIns(4,5)P_2 over alternately phosphorylated inositol phospholipids. In particular, D3 forms had low, or no measurable affinity for syndecan-4 cytoplasmic domain peptides. This is unlike the situation with some proteins that bind inositol phospholipids through pleckstrin homology domains, where either D3 or D4 lipids may bind (11, 16). Our preliminary two-dimensional NMR data provide a possible explanation; the 3-hydroxyl group of PtdIns(4,5)P_2 may interact with a residue of syndecan-4 core protein.[2] In turn, this would be disrupted by 3-phosphorylation. This raises the possibility that PI 3-kinase activity could down-regulate syndecan-4 signaling if the PtdIns(4,5)P_2 bound to its cytoplasmic domain is susceptible to 3-phosphorylation. In this context, PI 3-kinase activity has been implicated in focal adhesion disassembly (39), and actin polymerization related to membrane protrusion can be controlled by PtdIns(3,4,5)P_3 (11).

Previously we showed that $InsP_6$ could not stimulate PKCα activity in the presence of syndecan-4 cytoplasmic domain (18) since it did not promote 4L oligomerization (23) that is required for PKC activation. However, as we show here, the 4V peptide does bind to $InsP_6$ with a K_i of ~4.5 μM. This is consistent with the presence of a KK*XXXX*KK motif, shown in synaptotagmin C2B domain to be a binding site for inositol polyphosphate (20, 31). Since the amount of ligand in these assays was very low, we can assume that the K_i may approximate to the affinity of $InsP_6$ for syndecan-4. This is approximately the same as the measured affinity of PtdIns(4,5)P2 for this core protein. Two different displacement assays indicated, however, that the affinity of $InsP_6$ for syndecan-4 cytoplasmic domain is higher than that of the inositol phospholipid. Importantly, the assays for inositol phospholipid and inositol polyphosphate binding were different and cannot be strictly compared. Moreover, measurement of $InsP_6$ affinity utilized unlabeled compound to compete with a BZDC-$InsP_6$ derivative. The latter is more hydrophobic, and this may have led to a requirement for higher concentrations of $InsP_6$ to compete. In turn, the true affinity of $InsP_6$ may be higher than that calculated here, consistent with the fact that $InsP_6$ could compete with PtdIns(4,5)P_2 for the syndecan-4 cytoplasmic domain. The less charged variant, inositol hexasulfate, was accordingly less efficient as a competitor of PtdIns(4,5)P_2.

Proposed roles for $InsP_6$ in mammalian cells include regulation of vesicular traffic, calcium influx, and tumor cell proliferation (7–9). It has been suggested that inositol polyphosphates may compete with phosphoinositides in the regulation of phospholipase Cδ (40), AP-2, Bruton's tyrosine kinase, synaptotagmin II (20), and AMP deaminase (reviewed in Ref. 41). Levels of $InsP_6$ in some cells may reach 15–60 μM (37), and it is the most abundant inositol polyphosphate in most vertebrate cells. At these levels it could compete with PtdInds(4,5)P_2 for syndecan-4 cytoplasmic domain. Experiments here showed that exogenous $InsP_6$ does not block fibroblast attachment or spreading on fibronectin substrata, but focal adhesion and microfilament bundle formation were severely limited. These

data are entirely consistent with the early stages of adhesion being integrin-dependent, but the late stages requiring syndecan-4 (19, 27). Moreover, $InsP_6$ could act as an inhibitor in an assay where antibody clustering of syndecan-4 was used to stimulate microfilament bundle formation in cells prespread on the 110-kDa integrin-binding domain of fibronectin. Similarly, $InsP_6$ also blocked microfilament bundle formation promoted by recombinant HepII domain of fibronectin. $InsP_4$ had no impact, consistent with *in vitro* data showing limited binding of this inositol polyphosphate to the syndecan-4 core protein. It cannot be ruled out that $InsP_6$ competes not only for PtdIns(4,5)P_2 binding to syndecan-4, but other inositide-mediated processes. However, the ability of $InsP_6$ to affect stages of cell adhesion suspected to have a syndecan-4 dependence is a striking result, and these effects on the cytoskeleton have not been noted previously.

One effect of $InsP_6$ binding to syndecan-4 may be to limit oligomerization. Syndecan-4 cytoplasmic domain peptides do not oligomerize in response to $InsP_6$, and correspondingly, do not activate PKCα (23). Evidence points to a critical role of PtdIns(4,5)P_2 in promoting, or stabilizing, oligomers of syndecan-4 that can then interact with PKCα and strongly activate it (Fig. 8 and Refs. 19 and 25). Protein binding specificity for $InsP_6$ over $InsP_4$ has been reported previously, for example with myelin proteolipid protein (42), and, in some cases, $InsP_4$-binding correlates with an ability to interact with PtdIns(3,4,5)P_3, rather than phosphatidylinositol bisphosphates (43). Similarly, rabphilin 3A, a regulator of vesicle traffic and linkage to the actin cytoskeleton, binds PtdIns(4,5$P)_2$ (44), but does not have high affinity for $InsP_4$ (31). Consistent with this, we have found little binding of syndecan-4 cytoplasmic domain to PtdIns(3,4,5)P_3, or $InsP_4$.

Syndecan-4 cytoplasmic domain of 3T3 cells can be serine-phosphorylated in response to serum-free conditions, and reversed by a heparin-binding growth factor (29). The kinase involved was thought not to be PKCα, but perhaps a novel isoform of PKC, such as PKCδ or PKCϵ (30). As previously suggested by Horowitz *et al.* (24), phosphorylation of Ser^{183} markedly decreased the binding of PtdIns(4,5)P_2 in our assays and abrogated binding of PtdIns (4)P. Nor was there increased binding of other phosphoinositides. NMR spectroscopy showed that despite decreased affinity, p-4L was subject to oligomerization in the presence of PtdIns(4,5)P_2. Since the spectra generated by 4L and p-4L were distinct in these experiments, it suggests that the structures of the 4L and p-4L dimers differ from each other.

The ability of the syndecan-4 cytoplasmic domain to bind and activate PKC isoforms seems to be restricted to PKCα alone. The up-regulation of novel PKC isoforms is modest by comparison, and this effect was almost completely due to the inositol phospholipid, rather than an impact of the syndecan-4 peptide (not shown). Consistent with this is the observation that PKCζ, an atypical isoform lacking a diacylglycerol and phorbol ester binding site, was unaffected by the addition of PtdIns(4,5)P_2 and syndecan 4L peptide. If PKCδ or PKCϵ is responsible for phosphorylation of syndecan-4 cytoplasmic domain, it is presumably regulated in a manner distinct from that shown here for PKCα.

Syndecan-4 regulates focal adhesion formation (19, 25–27). Over-expression of full-length syndecan-4 in CHO cells promotes increased focal adhesion formation, with concomitantly enhanced microfilament bundle assembly (36). In contrast, transfection of constructs encoding syndecan-4 truncated to delete the inositol phospholipid and PKC-binding sites act as dominant negatives. These decrease both spreading and microfilament bundle assembly, together with a reduction in the number and size of focal adhesions (36). It has been shown that PtdIns(4,5)P_2 is essential for focal adhesion assembly (13), and our data suggest that one function may revolve around the capacity of syndecan-4 to signal through PKCα. Syndecan-4 may, therefore, be a nidus for cytoskeleton-membrane association and stabilization.

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Regulation of Inositol Phospholipid Binding and Signaling through Syndecan-4

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J. Biol. Chem. 2002, 277:49296-49303.
doi: 10.1074/jbc.M209679200 originally published online October 10, 2002
