Phosphatidylinositol-4,5-bisphosphate Mediates the Interaction of Syndecan-4 with Protein Kinase C†

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ABSTRACT: Recent studies have demonstrated that the cytoplasmic tail of syndecan-4, a widely expressed transmembrane proteoglycan, can activate protein kinase Cα in vitro, in combination with phosphatidylinositol-4,5-bisphosphate (PI-4,5-P_2). Syndecan-4 is involved in growth factor binding as well as in adhesion to extracellular matrix proteins, while PI-4,5-P_2 synthesis is modulated by growth factor and adhesion-generated signaling. The cooperative activation of PKCα by the proteoglycan and the phosphatidylinositol may constitute, therefore, an essential part of the cell's response to these extracellular signals. To characterize the activation mechanism of PKCα, we addressed here the nature of the interplay between syndecan-4, PI-4,5-P_2, and PKCα by measuring their mutual binding affinities and the specificity of their interactions. We found that the cytoplasmic tail of syndecan-4 is unlikely to bind directly to PKCα, and that this interaction critically depends on PI-4,5-P_2. The PI-4,5-P_2 specificity of the activation of PKCα is conferred by the cytoplasmic tail of syndecan-4, which has higher binding affinity for this phosphatidylinositol over phosphatidylinositol-3,4-bisphosphate and the -3,4,5-trisphospate. The activation is specific to PKCα and does not encompass the novel protein kinase C δ isoenzyme.

A growing body of evidence implicates the syndecan family of transmembrane proteoglycans in signal transduction as part of the cellular response to growth factors and extracellular matrix proteins (reviewed in ref *1*). In particular, a series of recent studies reported that the cytoplasmic tail of the syndecan-4 transmembrane proteoglycan activates the catalytic activity of the conventional protein kinase C (cPKC)[1] α isoenzyme (*2*−*5*). The significance of this mode of activation lies in its bringing together two ubiquitous membrane-associated elements, the cytoplasmic tail itself, and phosphatidylinositol-4,5-bisphosphate (PI-4,5-P_2), which appears to be an essential participant in the activation. Syndecan-4 is a co-receptor for heparan sulfate-binding growth factors as well as for extracellular matrix proteins (*6*, *7*). PI-4,5-P_2 has also been implicated in the cellular response to extracellular matrix proteins and growth factors, as adhesion to fibronectin causes a sharp increase in PI-4,5-P_2 synthesis, accompanied by sensitization of the cell to platelet derived growth factor (*8*). The existence of a reciprocal relation between the functions of syndecan-4 and PI-4,5-P_2 is also suggested by the involvement of the small G-protein Rho in regulating syndecan-4-mediated assembly of focal adhesions and stress fibers (*9*), since Rho also regulates PI-4,5-P_2 synthesis in response to integrin-mediated adhesion (*10*). Thus, the activation of PKC cooperatively by syndecan-4 and PI-4,5-P_2 may constitute an integral step in the transduction pathway downstream of several commonly occurring extracellular signals.

Several aspects of the manner in which syndecan-4 activates PKCα have since emerged (*2*, *4*, *5*): (a) The activation involves a cooperative interaction between the cytoplasmic tail of syndecan-4 and PI-4,5-P_2, with both components required for effective activation to occur; (b) A combination of the syndecan-4 cytoplasmic domain and PI-4,5-P_2 as cofactors sustains in vitro PKCα activity comparable to the one obtained with the standard cofactors calcium, phosphatidylserine (PS), and diacylglycerol (DAG); (c) When compared to phosphatidylinositol-3,4,5-trisphosphate (PI-3,4,5-P_3) and to inositol tetra- and hexaphosphate, PI-4,5-P_2 sustains a severalfold higher PKCα activity in the presence of the cytoplasmic tail of syndecan-4 (*4*); (d) Phosphorylation of the cytoplasmic tail at Ser^{183} abolishes its capacity to activate PKCα, probably as a result of a concomitant sharp decrease in the affinity of the phosphorylated tail to PI-4,5-P_2; (e) PI-4,5-P_2 can directly bind to the syndecan-4 cytoplasmic tail; (f) Syndecan-4 oligomerization is required for PKCα activation. Therefore, PI-4,5-P_2-dependent oligomerization of syndecan-4 with concomitant binding of PKCα to the variable region of the syndecan-4 cytoplasmic domain was proposed to explain these observations (*3*, *4*).

To further elucidate the activation mechanism of PKC by the cytoplasmic tail of syndecan-4, we tested the possibility

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[1] Abbreviations: DAG, diacylglycerol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LQQ, Y^{192}KK→LQQ mutated syndecan-4 cytoplasmic tail peptide; PI-3,4-P_2, PI-4,5-P_2, PI-3,4,5-P_3, phosphatidylinositol-4,5-bisphosphate, 3,4-bisphosphate, and 3,4,5-trisphosphate, respectively; PKC, protein kinase C; PS, phosphatidylserine; SPR, surface plasmon resonance; RU, resonance unit; S4c, syndecan-4 cytoplasmic tail peptide; S4cP, Ser^{183}-phosphorylated syndecan-4 cytoplasmic tail peptide; SE, standard error.

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of direct binding between the cytoplasmic tail and PKCα, and compared the binding affinities of the cytoplasmic tail and the kinase to several phosphatidylinositols. Additionally, we mapped the PI-4,5-P_2 binding site in the cytoplasmic tail of syndecan-4 and examined the effect of its mutation on the capacity of the cytoplasmic tail to activate PKCα. We find that direct syndecan-4 cytoplasmic tail-PI-4,5-P_2 binding and not syndecan-4-PKCα binding is responsible for PKCα activation. The specificity of the syndecan-4-dependent activation for PKCα is determined by the higher affinity of the syndecan-4 cytoplasmic tail for PI-4,5-P_2 in preference to other phosphatidylinositols, and by the higher affinity of PI-4,5-P_2 binding to conventional rather than novel (n)PKCs.

EXPERIMENTAL PROCEDURES

Materials. PI-4,5-P_2, PS, and diolein were purchased from Sigma. Phosphatidylinositol-3,4-bisphosphate (PI-3,4-P_2) and PI-3,4,5-P_3 were purchased from Matreya (Pleasant Gap, PA). Recombinant PKCα and PKCδ (80 and 50% pure, respectively, as determined by silver staining) were synthesized and prepared as described (*11*). The PKCβI optimal substrate peptide (FKLKRKGSFKKFA), a set of six 9-amino acid-long peptides corresponding to the variable domain in the cytoplasmic tail of syndecan-4, a 28 amino acid-long peptide (S4c) (RMKKKDEGSYDLGKKPIYKKAPTNEFYA) corresponding to the wild-type sequence of the syndecan-4 cytoplasmic tail peptide, and a 28 amino acid-long peptide corresponding to the syndecan-4 cytoplasmic tail containing a Y^{192}KK→LQQ mutation (LQQ) were synthesized by Genemed Synthesis (South San Francisco, CA). A 28 amino acid-long syndecan-4 cytoplasmic tail peptide with a phosphorylated Ser (S4cP) was synthesized by the Biopolymers Laboratory, Harvard Medical School (Boston, MA).

Surface Plasmon Resonance (*SPR*) *Measurements*. Ligands were immobilized on CM5 sensor chips (Biacore, Uppsala, Sweden) using the manufacturers' amine-coupling kit, resulting in binding equivalent to 1000−2000 resonance unit (RU) increase in the response level when immobilizing PKC isozymes, or 150−300 RU when immobilizing syndecan-4 cytoplasmic tail peptides. PKC isozymes were immobilized in 150 mM NaCl, 10 mM acetic acid, pH 4.0, and cytoplasmic tail peptides were immobilized in 100 mM NaCl, 50 mM acetic acid, pH 6.0. Analytes were perfused at the indicated concentrations into flow cells at a flow rate of 5 μL/min in 100 mM NaCl, 10 mM HEPES pH 7.4, and the surface was regenerated with a 2 min pulse of 1 M NaCl, 50 mM NaOH at 10 μg/mL. In some experiments, the analyte buffer was supplemented with 0.2 mM $CaCl_2$ and 5 mM $MgCl_2$. PI-4,5-P_2 (dissolved at 2 mg/mL in 20 parts $CHCl_3$, 9 parts MeOH, 1 part H_2O, 0.1 part 1N HCl), PI-3,4-P_2, or PI-3,4,5-P_3 (both dissolved at 1 mg/mL in 1 part $CHCl_3$, 1 part MeOH, 0.3 part H_2O) were dried under N_2 and sonicated for 5 min in ice-cold H_2O. This technique produces a suspension of micelles, which typically contain 80 molecules each (*12*). Syndecan-4 cytoplasmic tail peptides were incubated with PI-4,5-P_2 for 10 min at 25 °C. When a phospholipid was present in the analyte, the regeneration buffer used to remove the bound analyte from the last perfusion was supplemented with 0.5% Triton X-100. SPR responses were measured on a BIACORE 2000 system. As a control, the SPR response to each analyte was measured with a control flow cell subjected to amine coupling without the ligand.

Binding sensorgrams were analyzed using the built-in models and curve fitting algorithms of the Biaevaluation 3.0 software package (Biacore). The SPR response obtained with the control ligand-free flow cells was subtracted from the response curves of each analyte. Binding constants generated in this manner were considered as acceptable based on the visual comparison of the fitted curves to the experimental ones. In most cases, the average squared residual (χ^2) was below 10. The equilibrium constant (K_D) was calculated as the ratio of the kinetic parameters K_d/K_a, where K_a is the association and K_d the dissociation constant, respectively. Similarly, in the case of bivalent analyte model fitting, the first-order equilibrium constant (K_{D1}) was calculated as the ratio of the first-order kinetic parameters K_{d1}/K_{a1}. K_{D1}, which represents the binding between the free ligand and analyte prior to ligand-analyte complex formation, is used below for purposes of data presentation and comparison to fits produced by 1:1 (Langmuir) binding model.

Yeast Two-Hybrid Assay. Rat syndecan-4 cytoplasmic domain cDNA was subcloned into the DNA binding domain of the pAS2−1 vector of a System 2 (Clontech, Palo Alto, CA) yeast two-hybrid assay kit. Human PKCα cDNA or synectin cDNA were subcloned into the activation domain of the pACT2 vector of the kit. Both vectors were cotransformed into yeast host Y190 according to the manufacturer's manual and were allowed to grow at 30 °C in Trp-Leu-His-deficient plates supplemented with 35 mM 3-amino-1,2,4-triazole (Sigma).

PKC In Vitro Assays. Assays were performed and quantified as described (*5*), using PKCα (120 ng/mL) or PKCδ (430 ng/mL), either with or without syndecan-4 cytoplasmic tail peptides S4c, S4cP, and LQQ (all at 50 μM). The PKC βI optimal peptide (100 μM) was used as substrate in all assays.

PIP_2 Binding Assay. Each of a set of six 9-amino acid-long peptides corresponding to the wild-type or mutated sequences of the variable domain of the cytoplasmic tail of syndecan-4 were incubated (200 μM) with 0, 100, 200, and 400 μM PI-4,5-P_2 on ice for 30 min. The rest of the assay as well as the quantitation of the peptide-PI-4,5-P_2 binding were performed as described (*5*).

RESULTS

Binding of the Syndecan-4 Cytoplasmic Tail to PKCα. Association between PKCα and the cytoplasmic tail of syndecan-4 has been suggested by several lines of evidence, including immunoprecipitation and affinity chromatography of cell lysates, immunofluorescence on rat fibroblasts, and solid-phase assays with recombinant PKCα (*2*, *5*). These experiments incorporated, however, several potential cofactors, such as phospholipids or other cell lysate ingredients, in addition to the two tested reactants. To establish whether PKCα and the cytoplasmic tail of syndecan-4 are able to bind directly in the absence of other facilitators, the interaction between these two proteins was observed by SPR, using PKCα as an immobilized ligand and S4c as a soluble analyte. Analysis of SPR sensorgrams using a 1:1 (Langmuir) binding model produced an equilibrium constant (K_D) of 28 ± 1 μM. (Table 1 and Figure 1A). A similar K_D of 15 ± 1 μM was

Table 1: First-Order Equilibrium Constants ($K_{D1} \pm$ SE) Between the Indicated Ligands and Analytes Calculated From their SPR Responses

	analyte						
ligand	S4c	S4c + PI-4,5-P_2	S4cP	S4cP + PI-4,5-P_2	PI-4,5-P_2	PI-3,4-P_2	PI-3,4,5-P_3
PKCα	28 ± 0.1 μM	74 ± 1 nM	15 ± 1 μM	64 ± 0.1 nM	87 ± 2 nM	140 ± 2 nM	60 ± 2 nM
S4c					10 ± 2 nM	10 ± 2.4 μM	63 ± 0.5 nM
S4cP					4.3 ± 0.8 μM		

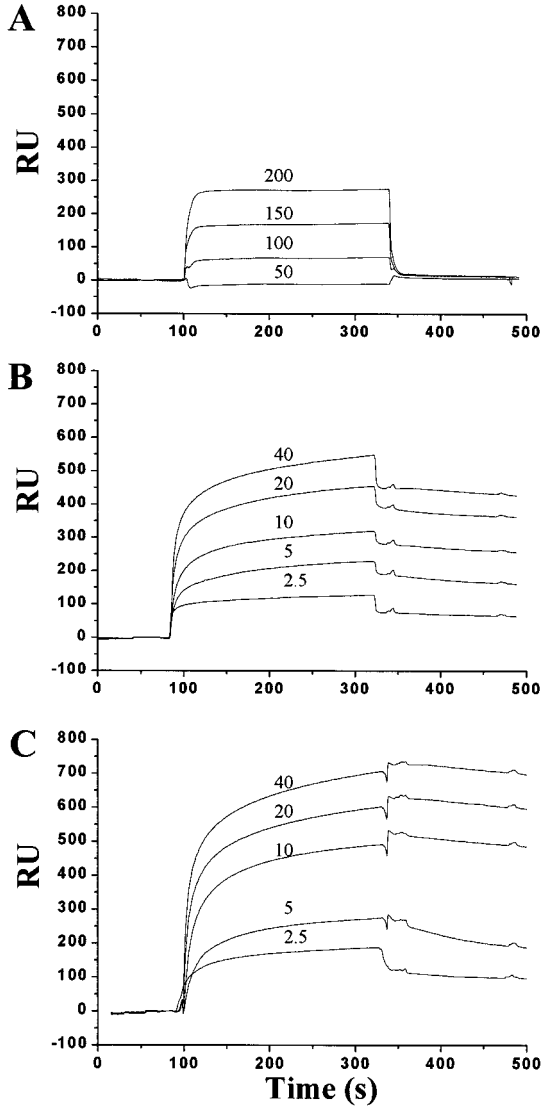

FIGURE 1: SPR-measured binding of the syndecan-4 cytoplasmic tail peptide to PKCα. SPR sensorgrams obtained with PKCα immobilized to CM5 sensor chips and perfused with the indicated concentrations (in μM) of S4c peptide (A), S4c incubated with PI-4,5-P_2 (B), and PI-4,5-P_2 alone (C).

obtained when using S4cP, the phosphorylated cytoplasmic tail peptide, as analyte under the same conditions. These values indicate a relatively low binding affinity when compared to the K_Ds of other peptide−protein interactions, which are typically in the sub-micromolar range (e.g., ref *13*).

As an alternative approach, the interaction between PKCα and the cytoplasmic tail of syndecan-4 was tested by a yeast two-hybrid assay. Yeast cotransformed by rat syndecan-4 cytoplasmic tail and human PKCα cDNAs did not form colonies on nutrient-deficient plates, even after a 14-day incubation at 30 °C. Cotransfection with synectin, a newly identified syndecan-4-binding protein[2] and syndecan-4 cytoplasmic tail cDNAs performed as a positive control did produce, however, LacZ-positive colonies (data not shown). In agreement with the SPR experiments, these results do not support a direct interaction between PKCα and the cytoplasmic tail of syndecan-4. Since both the SPR and the yeast two-hybrid assay are sensitive and highly specific techniques, we consider the latter conclusion as prevailing over immunoprecipitation-based observations concerning binding of the cytoplasmic tail to PKCα (*2*, *5*).

Several recent reports provide evidence for direct binding between the cytoplasmic tail of syndecan-4 and PI-4,5-P_2 (*14*) as well as for the role of PI-4,5-P_2 in facilitating activation of PKCα by the cytoplasmic tail (*4*). In view of these reports, we modified the SPR assays by perfusing an equimolar mixture of S4c peptide and PI-4,5-P_2, present in the buffer as unillamelar vesicles (or micelles), over immobilized PKCα. Under these conditions, the S4c peptide forms dimers and larger oligomers and binds with PI-4,5-P_2 at an approximate molar stoichiometry of 1:2 (*5*). The resulting sensorgrams (Figure 1B) were qualitatively different from those recorded with S4c alone as analyte, and could not be fitted well with a Langmuir model. A significantly better fit ($\chi^2 = 4.0$) was obtained, however, by using a bivalent analyte model instead. The first-order equilibrium constant (K_{D1}) calculated by this model was 74 ± 1 nM, indicating a much higher affinity between PKCα and the S4c-/PI-4,5-P_2 mixture compared to PKCα and S4c alone (Table 1). This result implies that the interaction of the cytoplasmic tail of syndecan-4 with PKCα is mediated by PI-4,5-P_2, which either binds PKCα in tandem with the cytoplasmic tail, or serves as a linker between the cytoplasmic tail and the kinase. In the latter case, the interaction between the cytoplasmic tail of syndecan-4 and PKCα would be indirect, unlike the hitherto held view (*2*, *5*). The combined effects of syndecan-4 and PI-4,5-P_2 on PKCα activity were previously measured in the presence of 5 mM $MgCl_2$ (*5*) and compared to the activity obtained under standard cPKC conditions, which also included 0.2 mM $CaCl_2$. We remeasured, therefore, the affinity between PKCα and an equimolar mixture of S4c peptide and PI-4,5-P_2 in an analyte buffer supplemented with 5 mM $MgCl_2$ and 0.2 mM $CaCl_2$. The first-order equilibrium constant calculated from these experiments was closely similar (75.5 ± 3 nM) to the one obtained in the absence of $MgCl_2$ and 0.2 mM $CaCl_2$.

To further clarify the nature of the interaction between the cytoplasmic tail of syndecan-4 and PKCα, we measured the SPR response of immobilized PKCα reacted with PI-4,5-P_2 alone or with an equimolar mixture of PI-4,5-P_2 and S4cP, the phosphorylated syndecan-4 cytoplasmic tail peptide, instead of S4c. As in the case of the PKCα binding to the S4c/PI-4,5-P_2 mixture, the binding sensorgrams of

[2] Gao, Y., and Simons, M., unpublished observations (see also Gene Bank entry AF104358).

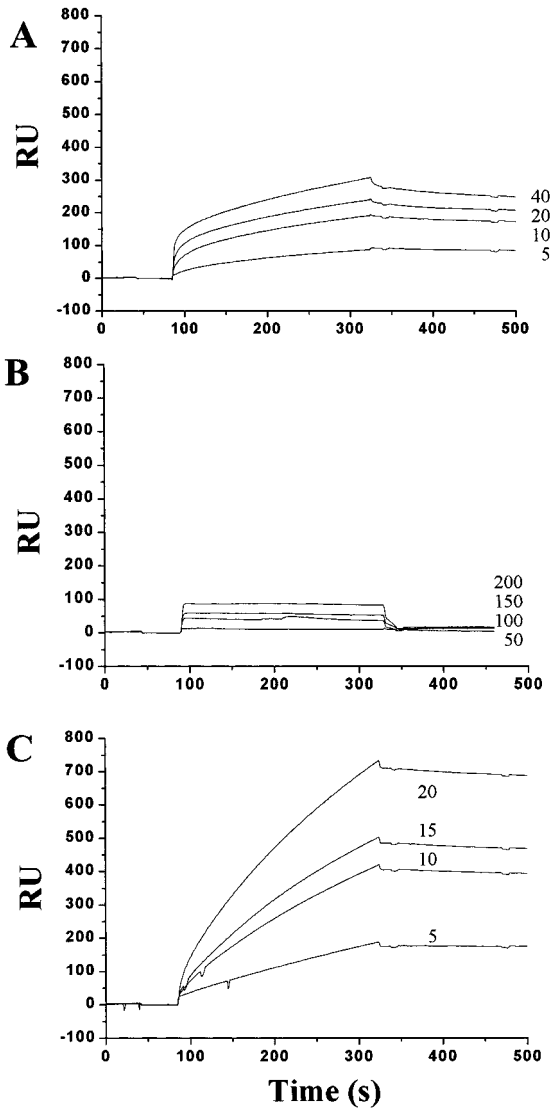

FIGURE 2: SPR-measured binding of phosphatidylinositols to syndecan-4 cytoplasmic tail peptides. SPR sensorgrams obtained with S4c (A, C) or S4cP (B) peptides immobilized to CM5 sensor chips and perfused with the indicated concentrations (in μM) of PI-4,5-P_2 (A, B) or PI-3,4-P_2 (C).

PKCα to PI-4,5-P_2 alone were best fitted by a bivalent analyte model, with a similar affinity (Figure 1C). The presence of S4cP in the analyte mixture produced a comparable SPR response and was characterized by a closely similar affinity ($K_{D1} = 64 \pm 0.1$ nM; Table 1) to that between PKCα and a S4c/PI-4,5-P_2 mixture.

Phosphatidylinositol Binding Specificity of the Syndecan-4 Cytoplasmic Tail. To test the premise that PI-4,5-P_2 serves as a linker between PKCα and S4c, we addressed the binding affinity between the peptide and PI-4,5-P_2 separately from the combined interaction of the two with PKCα. A previous estimate of this affinity was based on the less accurate molecular mass-cutoff filter assay (*5*), which cannot be compared directly with SPR measurements. Thus, the S4c-PI-4,5-P_2 binding affinity was remeasured by SPR, using S4c as an immobilized ligand perfused with PI-4,5-P_2. Similar to the PKCα-PI-4,5-P_2 interaction, the resulting SPR response curves were best fitted by a bivalent binding model, but the calculated first-order equilibrium constant was lower by close to an order of magnitude (Table 1 and Figure 2A), indicating the affinity of PI-4,5-P_2 for S4c is higher than its affinity for PKCα. In accordance with the previous molecular mass-cutoff filter assays (*5*), the SPR-measured affinity of S4cP for PI-4,5-P_2 was lower than that of S4c by more than 2 orders of magnitude (Table 1 and Figure 2B).

The ability of PI-4,5-P_2 to facilitate multimerization of the cytoplasmic tail of syndecan-4 and to activate PKCα have previously been demonstrated by in vitro experiments (*4*, *5*). Though PI-4,5-P_2 is a ubiquitous phospholipid present in unstimulated cells (*15*, *16*), these processes may conceivably be mediated by other phosphatidylinositols in vivo. Of particular interest is the potential role of the phosphoinositide 3-kinase (PI3K) products PI-3,4-P_2 and PI-3,4,5-P_3, which are generated in response to cell stimulation by growth factors.

The SPR response curves of PI-3,4,5-P_3 perfused over immobilized S4c (data not shown) yielded a K_{D1} of 63 $\pm$ 0.5 nM, which, though lower, is still comparable to that of PI-4,5-P_2. The corresponding SPR response curves of PI-3,4-P_2, however, indicated that the affinity of S4c for this phospholipid was lower by 3 orders of magnitude than its affinity for PI-4,5-P_2 (Table 1 and Figure 2C). Thus, the cytoplasmic tail of syndecan-4 appears to specifically bind PI-4,5-P_2, favoring it over PI-3,4-P_2 and PI-3,4,5-P_3. By comparison, PKCα did not exhibit a similar preference for PI-4,5-P_2, as its SPR-measured (data not shown) affinities for PI-3,4-P_2 and PI-3,4,5-P_3 were similar (K_Ds of 140 $\pm$ 2 and 60 $\pm$ 2 nM, respectively).

Mapping of the PI-4,5-P_2 Binding Site in the Cytoplasmic Tail of Syndecan-4. Since binding between the cytoplasmic tail of syndecan-4 and PI-4,5-P_2 seems to be an essential component of the complex formation with PKCα, as well as of the activation of the kinase by the cytoplasmic tail, we set out to determine which tail residues are involved in this interaction. Previous results (*4*, *14*) as well as comparison to known PI-4,5-P_2 binding regions in other proteins (*17*) suggested a 7-residue long sequence located in the central region of the syndecan-4 cytoplasmic tail. This sequence is spanned by two pairs of lysines on each end and contains a single tyrosine (K^{188}KPIYKK). The positively charged lysines are thought to contribute to PI-4,5-P_2 binding by forming electrostatic bonds with the phosphate groups of the latter, as in the case of PI-4,5-P_2 binding to α-actinin (*18*). The tyrosine residue could also participate in the interaction of the syndecan-4 cytoplasmic tail with PI-4,5-P_2 by alignment of its aromatic ring with the PI-4,5-P_2 inositol ring and formation of hydrogen bonds between the two.

To investigate these possibilities, we synthesized a set of five peptides corresponding to the 9-residue variable domain of the syndecan-4 cytoplasmic tail (LGKKPIYKK). Each of these peptides contained a point mutation of one of the four lysines and the single tyrosine. The lysines were replaced by glutamines, which lack the lysine's positively charged amine, and the tyrosine was replaced by a leucine, which lacks the former's aromatic ring. As a preliminary screen for the effect of these point mutations, the binding between each peptide to PI-4,5-P_2 micelles was compared to that of the wild-type variable domain peptide by molecular mass-cutoff filter assays. While single lysine replacements had little or no effect on the affinity of the variable domain to PI-4,5-P_2, the Tyr→Leu mutation caused a 3-fold increase in the variable domain's K_D (Figure 3A). Since the replace-

A

Peptide	*K_D (nM)*
WT (L^{186}GKKPIYKK)	10
$K^{188} \rightarrow Q$	11
$K^{189} \rightarrow Q$	11
$Y^{192} \rightarrow L$	29
$K^{193} \rightarrow Q$	13
$K^{194} \rightarrow Q$	15
$Y^{192}KK \rightarrow LQQ$	>> 10 μM*

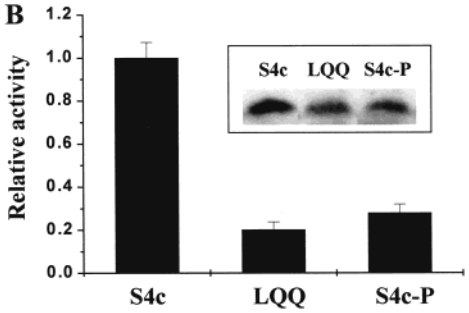

FIGURE 3: Effect of syndecan-4 cytoplasmic tail mutations on its PI-4,5-P_2 binding and PKCα activation. (A) Equilibrium constants for PI-4,5-P_2 binding of 9-amino-acid-long peptides corresponding to the wild-type or point-mutated sequence of the variable domain in the cytoplasmic tail of syndecan-4 were measured by molecular mass-cutoff assays. The bottom K_D value for the LQQ 28-amino-acid-long mutated cytoplasmic tail peptide (*) is an approximation based on its markedly low SPR response when used as an immobilized ligand and perfused with PI-4,5-P_2. (B) In vitro PKCα activity measured in the presence of the indicated S4c peptides together with PI-4,5-P_2 ($n = 3$, bars denote standard deviations). Inset: autoradiographic images of PKC βI optimal substrate peptide phosphorylated under the assay conditions (see Experimental Procedures and ref *5*).

ments of the two lysines located carboxy-terminally to the tyrosine also caused a small increase in the K_D, we generated a 28 amino acid-long peptide corresponding to the full length cytoplasmic tail of syndecan-4 containing a $Y^{192}KK \rightarrow LQQ$ replacement (residue numbering corresponds to the rat syndecan-4 sequence (*19*)) and measured its interaction to PI-4,5-P_2 by SPR. The SPR response of the immobilized peptide containing the LQQ mutation to perfused PI-4,5-P_2 was, however, too low to be quantitated reliably (data not shown), indicating this combined 3-residue mutation drastically reduced their mutual affinity.

We have previously reported that Ser^{183} phosphorylation of the cytoplasmic tail of syndecan-4 reduced its capacity to activate PKCα and attributed this reduction to the decreased affinity of the phosphorylated cytoplasmic tail to PI-4,5-P_2 (*5*). To further test the correlation between the affinity of the cytoplasmic tail to PI-4,5-P_2 and its activation of PKCα, we compared the in vitro kinase activities of PKCα in the presence of S4c to those obtained in the presence of the LQQ-mutated peptide and S4cP. The LQQ mutation caused a 5-fold decrease in the capacity of the cytoplasmic tail to activate PKCα, similar to the effect of phosphorylation (Figure 3B), or to the activity level in the presence of PI-4,5-P_2 alone (*5*), supporting the notion that the binding of PI-4,5-P_2 to the cytoplasmic tail of syndecan-4 is essential for its capacity to activate PKCα.

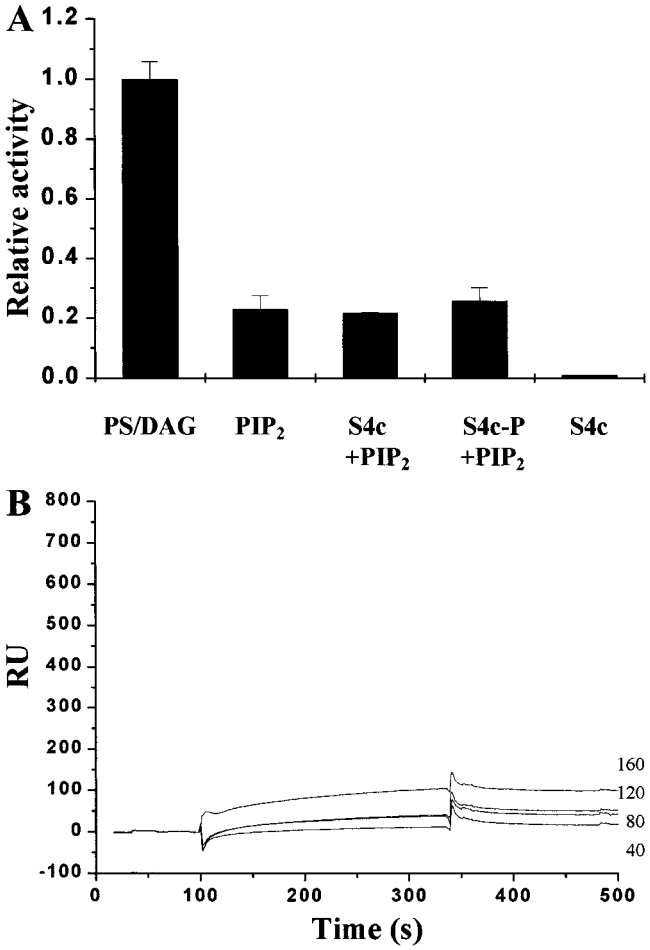

FIGURE 4: Activation of PKCδ by the syndecan-4 cytoplasmic tail and its binding to PI-4,5-P_2. (A) In vitro PKCδ activity measured in the presence of the indicated cofactors ($n = 3$). The activity levels are normalized relative to the in vitro PKC activity in the presence of PS (20 μG/mL), diolein (DAG) (6.2 μg/mL), and $CaCl_2$ (0.2 mM), and in the absence of S4c (left-hand column). (B) SPR sensorgrams obtained with PKCδ immobilized to CM5 sensor chips and perfused with the indicated concentrations (in μM) of PI-4,5-P_2.

Isozyme-Specificity of PKC Activation by Syndecan-4. The capacity of the cytoplasmic tail of syndecan-4 to distinctively activate PKCα was singled out because of the physical association between the two proteins, suggested by immunoprecipitation and cellular co-localization (*2*, *5*). This evidence does not rule out, however, that the cytoplasmic tail of syndecan-4 could equally activate other PKC isozymes, whose association with the cytoplasmic tail may be more transient and less detectable than that of PKCα. This possibility was addressed by testing the effect of the cytoplasmic tail of syndecan-4 on the in vitro activity of recombinant PKCδ. This PKC isozyme is also abundant (*20*) in the endothelial cell line used in our previous studies (*5*) and may be responsible for the phosphorylation of the cytoplasmic tail of syndecan-4 (*21*). Unlike PKCα, which was activated 10-fold in the presence of S4c (*5*), the in vitro activity of PKCδ was not affected by the presence of S4c or S4cP peptides (Figure 4A). Moreover, the activity of PKCδ with PI-4,5-P_2 as a cofactor, either in the presence or absence of S4c and S4cP reached only 20% of its maximal PS/DAG-sustained activity.

Since the interaction of PI-4,5-P_2 with PKCα seems to play an essential part in the activation of the kinase by S4c,

we measured the affinity of PKCδ to PI-4,5-P_2 by SPR. The corresponding equilibrium constant (6.5 $\pm$ 0.6 μM) was larger by close to 2 orders of magnitude than the K_{D1} of PKCα and PI-4,5-P_2 (Figure 4B).

DISCUSSION

The results described above provide several insights into the nature of the interaction between PKCα and the cytoplasmic tail of syndecan-4. As previously reported (*4*), the presence of PI-4,5-P_2 is required for syndecan-4-dependent activation of PKCα. The SPR experiments demonstrate the affinity of S4c to PKCα is very low. The absence of direct PKCα-S4c interaction has been further confirmed by the yeast two-hybrid assay. The S4c peptide, on the other hand, displays a much higher affinity for PI-4,5-P_2, which was significantly reduced by phosphorylation of S4c (i.e., the S4cP peptide). In contrast, the affinity of PKCα for PI-4,5-P_2 was not affected by the presence of either S4c or S4cP, thus indicating that the syndecan-4 cytoplasmic domain neither participates, nor interferes with the PKCα−PI-4,5-P_2 interaction. Put together, these results suggest that the interaction between PKCα and S4c is likely to be indirect, being mediated by PI-4,5-P_2, which brings PKCα and S4c together as a shared ligand. PI-4,5-P_2 may serve as a "linker" between the syndecan-4 cytoplasmic domain and PKCα, or may alter the conformation of either PKCα or of the cytoplasmic tail of syndecan-4 in such a manner so as to allow direct interaction between the two.

The ability of the syndecan-4−PI-4,5-P_2 complex to stimulate PKC is limited to PKCα, while being ineffective for PKCδ. Because of the high homology between the C2, regions of the cPKC isoenzymes, where the binding site of anionic phospholipids is located (*22*), PI-4,5-P_2 and syndecan-4 could conceivably bind and activate also PKCβ and γ. This study focuses on PKCα, however, because of the numerous reports specifically linking this isoenzyme with syndecan-4 (reviewed in (*1*)). The underlying basis for this specificity is most likely the higher PI-4,5-P_2 affinity of the cPKC C2 motif compared to the PI-4,5-P_2 affinity of the nPKC regulatory domain. The phosphatidylinositol specificity of the activation is conferred by the cytoplasmic tail of syndecan-4 and not by PKCα, as the latter has similar affinities for PI-4,5-P_2, PI-3,4-P_2, and PI-3,4,5-P_2. In view of the binding mechanism proposed above, the binding site of the syndecan-4−PI-4,5-P_2 complex to PKC is likely to be located in the C2 part of the regulatory domain and not in the catalytic domain, as previously suggested (*2*).

The activation mechanism described here differs from earlier reports on PI-4,5-P_2-mediated activation of PKC. Previous studies found that PKC activation was not sustained by PI-4,5-P_2 alone (*23*) but required the presence of the additional cofactors calcium and PS (*24*−*28*). While confirming that PI-4,5-P_2 is not sufficient by itself for activating PKC, an earlier study from this laboratory found that in the presence of PI-4,5-P_2, the cytoplasmic tail of syndecan-4 can activate PKCα to virtually the same level as in the presence of calcium, PS, and DAG (*5*). Thus, the cytoplasmic tail of syndecan-4 seems to be able to substitute these otherwise obligatory cofactors. Like PI-4,5-P_2, however, the cytoplasmic tail alone is insufficient for activating PKCα, clearly implying a cooperative process between the tail and the phospholipid.

The reduction in PI-4,5-P_2 affinity caused by the Y^{192}KK→LQQ mutation in the cytoplasmic tail of syndecan-4 correlated with the loss of the mutant peptide's capacity to activate PKCα. The extent of this loss of ability to activate PKCα is similar to that seen following phosphorylation of Ser^{183} in the syndecan-4 cytoplasmic tail (*5*), and confirms the premise that the cooperative interaction between the cytoplasmic tail and PI-4,5-P_2 lies at the basis of the PKCα activation mechanism. One known aspect of this cooperativity is multimerization of the cytoplasmic tail (*3*), which is disrupted once the tail becomes phosphorylated at Ser^{183} (*5*). Since the effect of the Y^{192}KK→LQQ mutation is much larger than the additive effects of mutating each residue separately, it is likely these residues constitute a PI-4,5-P_2 docking site, in which the two lysines interact with the D4 and D5 phosphates (*29*), and the tyrosine with the inositol ring. This site would then be abolished once all the 3 residues are replaced. Alternatively, the sharp reduction in the syndecan-4 cytoplasmic tail affinity to PI-4,5-P_2 produced by the combined 3-residue mutation could results from its impact on the tail's tertiary structure.

The role of PI-4,5-P_2 in the regulation of syndecan-4 dependent activation of PKCα activity is in keeping with the function of a number of phosphatidylinositols as second messengers in cellular signal transduction in general (*30*), and in activating several PKC isozymes in particular (*28*). Together with other recently published studies, the implications of the results presented here on the nature of syndecan-4's cellular function are that the proteoglycan may undergo clustering in response to elevated PI-4,5-P_2 levels, forming membrane subdomains of locally high PI-4,5-P_2 concentration. These subdomains could form, in turn, discrete sites of PKC activation (*31*), as well as of initiation of other associated signal transduction events. In the SPR experiments we conducted here, PI-4,5-P_2 was already present as large multimolecular micelles, therefore, its possible sequestration by the cytoplasmic tail of syndecan-4 and the resulting increase in PKCα affinity to PI-4,5-P_2 could not be observed. The process of syndecan-4 clustering itself appears to be regulated by the phosphorylation state of Ser^{183} in its cytoplasmic tail, which is responsive to growth factors (*5*, *21*).

Finally, the binding specificity of the cytoplasmic tail of syndecan-4 for phosphatidylinositols requires the presence of the D4 and D5 phosphates on the PI-4,5-P_2 inositol ring, while the presence of an additional phosphate in the D3 position reduces the binding affinity. Thus, D4 and D5-phosphatidylinositol kinases, a group consisting of several adhesion and growth factor-responsive members (*32*, *33*), may modulate the signaling function of syndecan-4.

In summary, activation of PKCα by syndecan-4 is dependent on PI-4,5-P_2, that serves either as a bridge between the two proteins or alters the conformations of one or the other, thereby facilitating their interaction. The specificity of this event is determined by the higher affinity of PI-4,5-P_2 for PKCα over PKCδ, and by the higher affinity of S4c for D4/D5- over D3-phosphoinositides.

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