The transmembrane proteoglycan syndecan-4, which is a coreceptor with integrins in cytoskeleton-matrix interactions, appears to be multimerized in vivo. Both purified and recombinant core proteins form sodium dodecyl sulfate-resistant oligomers, and we now report that a synthetic peptide corresponding to the central region of syndecan-4 cytoplasmic domain (4V) also oligomerizes. The degree of oligomerization correlates with the previously reported ability to bind protein kinase C (PKC) and regulate its activity. Only multimeric recombinant syndecan-4 core protein, but not the monomeric protein, potentiated the activity of PKCα, and only oligomeric syndecan-4 cytoplasmic peptides were active. Changes in peptide sequence caused parallel loss of stable oligomeric status and ability to regulate a mixture of PKCβ2 activity. A synthetic peptide encompassing the whole cytoplasmic domain of syndecan-4 (4L) containing a membrane-proximal basic sequence did not form higher order oligomers and could not regulate the activity of PKCβ2 unless induced to aggregate by phosphatidylinositol 4,5-bisphosphate. Oligomerization and PKC regulatory activity of the 4V peptide were both increased by addition of N-terminal cysteine and reduced by phosphorylation of the cysteine thiol group. Concentration of syndecan-4 at sites of focal adhesion formation may enhance multimerization and both localize PKC and potentiate its activity to induce stable complex formation.

Extracellular matrix molecules such as fibronectin regulate many cellular processes through information encoded in the ligand-receptor interaction. Fibronectin has at least two distinct classes of cell-surface receptors: integrins and heparan sulfate proteoglycans. Integrins bind at several sites, but adhesion for many cell types is primarily through the classical RGD sequence in the tenth type III repeat of the molecule (1, 2). Clustering of specific integrins by either immobilized extracellular molecules or anti-integrin antibodies has many biological effects (reviewed in Refs. 3–6). It stimulates tyrosine phosphorylation (7, 8), elevates intracellular calcium (9), activates the Na+/H+ antiporter (10), and activates phosphatidylinositol 4-phosphate 5-kinase (11) with cytoskeletal rearrangement (3, 12). Ligand-induced dimerization or oligomerization is a key event in transmembrane signaling by hormone or growth factor receptors with tyrosine kinase activity. This leads to an increase in tyrosine kinase activity, autophosphorylation of receptors, and the induction of diverse biological responses (13, 14). Although integrins have no intrinsic tyrosine kinase activity, it is clear that their clustering is needed prior to subsequent tyrosine phosphorylation events (reviewed in Refs. 3–6). In addition, integrin-associated kinase(s) has been identified (15, 16).

Interactions between integrins and the cell-binding domain of fibronectin are only sufficient for attachment and spreading in normal primary fibroblasts (17), and the integrin α5β1, which is the primary ligand for adhesion to fibronectin in this system (1, 3, 5), remains in a diffusely punctate distribution in the cell membrane (18, 19). An additional stimulus (17–19) is needed for cytoskeletal and membrane reorganization to form stress fibers and focal adhesions (reviewed in Ref. 3), which appear to be not only structural complexes but also to have signaling functions. This can be provided by further stimulation with the near C-terminal heparin-binding domain of fibronectin (Hep II) (17), a synthetic peptide from this domain (18), or by pharmacological activation of protein kinase C (PKC)3 with phorbol esters (19). This causes a redistribution of α5β1 into large clusters in focal adhesions and a concomitant redistribution of the cytoplasmic components vinculin and talin (18, 19). Circumstantial evidence has indicated that a cell-surface heparan sulfate proteoglycan may transduce the signal from the Hep II domain of fibronectin (reviewed in Ref. 20). Syndecan-4 is one member of the syndecan family of transmembrane heparan sulfate proteoglycans (20, 21) that is selectively concentrated in the focal adhesions of a range of cells (22) in a PKC-dependent manner (23) and may, therefore, function as a coreceptor with integrins.

The four mammalian syndecans all have highly homologous transmembrane and cytoplasmic domains, except for a short variable (V) region in the center of the cytoplasmic domain (20, 21). The extracellular domains, however, bear little homology. All syndecans form homologous dimers or multimers that resist treatment with SDS (20–22, 24, 25). Asundi and Carey (25) have shown that syndecan-3 core protein assembles into stable, noncovalent multimers mediated by the transmembrane domain and ectodomain flanking region and that this is independent of cysteine residues. Multimerization of syndecan-4 has been seen in biochemical studies (22, 24–27), and the high concentration of this molecule in focal adhesions (22) should favor this. Recently, we showed that syndecan-4 could directly activate PKCs in the absence of phospholipid (PL) and potentiate PL-induced activity (26). This ability appears to reside in

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† The abbreviation used are: PKC, protein kinase C; GST, glutathione S-transferase; PL, phospholipid; PIP2, phosphatidylinositol 4,5-bisphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CHAPS, 3-[((3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; PAGE, polyacrylamide gel electrophoresis.
the central portion of the cytoplasmic domain, which is unique to syndecan-4, based on studies with fusion proteins containing or lacking this region, and with synthetic peptides encompassing this amino acid sequence (26). The recombinant proteins capable of activating PKCα were oligomeric, consistent with in vivo data. Preliminary data also suggested that the variable region of syndecan-4 (4V) could also oligomerize. We now report that the degree of oligomerization correlates with the ability to regulate PKCα activity, indicating that clustering of syndecan-4 with ligand may control signaling events.

EXPERIMENTAL PROCEDURES

Materials—Synthetic peptides with sequences corresponding to regions of the cytoplasmic domain of syndecan-4 or syndecan-2 (see Table I) were synthesized and sequenced by the University of Alabama at Birmingham (UAB) Comprehensive Cancer Center Peptide Synthesis and Analysis Shared Facility and analyzed by the UAB Comprehensive Cancer Center Mass Spectrometry Shared Facility. Some peptides (e.g. Cys4V) had an additional cysteine at the N terminus. PKCα and PKCβ1 purified from rabbit brain was purchased from Upstate Biotechnologies (Lake Placid, NY), recombinant PKCα from Molecular Probes (Eugene, OR), γ-[32P]ATP from DuPont NEN, Sepharose CL-4B and glutathione-agarose beads from Pharmacia Biotech Inc., and protein standards for size exclusion chromatography from Bio-Rad. Phosphatidylinositol and diolein were purchased from Avanti Polar Lipids (Alabaster, AL). A peptide encompassing the PKC phosphorylation site in the epidermal growth factor receptor and P-81 phosphoelastase paper were obtained from Biodol Research (Plymouth Meeting, PA) and Whatman, respectively. Antibodies against glutathione S-transferase (GST) were purchased from Molecular Probes, and reduced glutathione was from Janssen Chimica (New Brunswick, NJ). Sephadex G-50 (Fine), Sephadex G-150 (Fine), N-lauroylsarcosine, isopropyl β-D-thiogalactopyranoside, PIP2, insitol hexaphosphate, and other chemicals were all purchased from Sigma.

Expression and Purification of Recombinant GST-Syndecan-4 Core Protein—A cDNA encoding the entire syndecan-4 core protein or lacking the entire cytoplasmic domain was subcloned into GST expression vector pGEX-SK-1 (Pharmacia) and fusion protein in Escherichia coli induced with 0.1 mM isopropyl β-D-thiogalactopyranoside (26). Bacteria were pelleted by centrifugation for 2 min at 10,000 × g. The pellets were resuspended with TBSE (Tris-buffered saline with EDTA; 10 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EDTA) containing 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine-HCl, sonicated on ice for 1 min, and N-lauroylsarcosine was added to a final concentration of 1% in TBSE to solubilize recombinant proteins. After 30 min of incubation on ice, insoluble materials were removed by centrifugation at 10,000 × g for 10 min. Supernatants were diluted 10 times with TBSE containing 4% Triton X-100 and applied to 0.5-ml pre-equilibrated glutathione-agarose columns. After washing with TBSE containing 4% Triton X-100 to remove unbound materials, the column was washed with TBSE containing 1% Triton X-100 and once with TBSE containing 0.1% Triton X-100 to decrease the detergent concentration. Fusion proteins were eluted with 50 mM Tris-HCl, pH 8.0, containing 5 mM reduced glutathione.

Size Exclusion Chromatography—Purified GST-syndecan-4 fusion proteins were loaded onto Sepharose CL-4B or Sephadex G-150 (0.7 × 100 cm) gel filtration columns pre-equilibrated with 50 mM HEPES (pH 7.3), 0.1% CHAPS, and 150 mM NaCl. Proteins were eluted with the same buffer at a flow rate of 6 ml/h at room temperature and detected with a PhosphorImager (Molecular Dynamics) after electrophoresis on 20% SDS-PAGE. In assays using 0.1 μg/ml of the epidermal growth factor receptor agonist peptide of the sequence RKRTLRRL (Biomol Research) as an alternative substrate (see Fig. 6), the reaction mixture contained PIP2 (50 μM) and/or syndecan-4 peptide (4I1, 0.25 mg/ml) in the absence of PL and calcium, and PKCαβγ mixture (3 ng) was used. The reaction was stopped by spotting the whole reaction mixture onto phosphoelastase filters (Whatman, P-81, 2.1 cm) and dropping these into 75 mM phosphoric acid. The phosphoelastase filters were washed once for 10 min each, immersed in 95% ethanol for 5 min, dried, and counted with 4 μl of scintillation mixture in a scintillation counter (Wallac model 1409).

RESULTS

Multimerization of Syndecan-4 Core Protein Correlates with PKC Regulatory Activity—Our previous studies showed that recombinant syndecan-4, in which the core protein formed SDS-resistant oligomers, could increase the ability of PKCα to phosphorylate histone III-S (26). Therefore, we investigated whether this multimeric form is critical for its potentiation activity. Gel chromatography of pure preparations of recombinant syndecan-4 on Sepharose CL-4B under nondenaturing conditions showed that the major species had a Mr ≈ 500,000 (Fig. 1A), indicating that the major form of intact syndecan-4 is oligomeric. We could sometimes also detect minor pools of Mr ≈ 350,000 and ≈ 230,000 (data not shown). These oligomeric core proteins migrated on SDS-PAGE gels as a single population with apparent Mr ≈ 150,000 (Fig. 1B). Since the deduced molecular mass of the fusion protein is 49 kDa, fusion protein resolves on denaturing gels as oligomers, possibly dimers. Reduction had no effect; the core protein of syndecan-4 in any case contained no cysteine. However, a single freeze-thaw cycle dissociated this oligomeric form into monomeric proteins migrating with Mr ≈ 53,000 on Sephadex G-150 gel chromatography (Fig. 1A) and ≈ 50,000 on SDS-PAGE gels (Fig. 1B). Although multimeric forms of intact syndecan-4 core protein could potentiate PKCα activity to phosphorylate histone III-S (Fig. 1C; compare lanes 1 and 2), monomeric forms of syndecan-4 core protein did not have this activity (Fig. 1C, compare lanes 1 and 3). Similar potentiation was seen with both purified PKCαβγ mixture and recombinant PKCα (data not shown).

PKCαβγ Regulation Requires Multimerization of Syndecan-4 Cytoplasmic Domains—As reported previously, mutants of syndecan-4 with partially or totally truncated cytoplasmic domains could not increase PKCα activity, although they did form oligomers (26). A peptide from this domain was also active (26). Since oligomerization of the core protein was needed for PKC regulatory activity, we monitored whether synthetic peptides with sequences from the cytoplasmic domain could oligomerize and if this correlated with the ability to regulate PKC activity. The apparent molecular masses of syndecan-4 peptides encompassing the whole cytoplasmic domain (4L) or just the variable region unique to syndecan-4 (4V) as estimated by Sephadex G-50 gel chromatography are larger than predicted (Table I). Peptide 4L eluted close to putative dimeric size, whereas 4V eluted as a tetramer. Therefore, with respect to syndecan-4, two sites in the core protein are involved in oligomerization: the transmembrane and/or ectodomains as shown for syndecan-3 (25), and the central region of its cytoplasmic domain. However, the highly basic region of syndecan-4 between the two sites was not involved in oligomerization as judged by the ability of the 4V peptide to self-associate at 4L compared with 4V in the absence of phospholipid (see below). In agreement with a need for oligomerization for PKCαβγ regulatory activity, 4V but not 4L could potentiate PL-induced activation of PKCαβγ to phosphorylate histones (Fig. 2A) and directly activate PKCαβγ in the absence of PL (Fig. 2B).
alent syndecan-2 peptides, which lack PKC regulatory activity (26), were also examined, since syndecan-2 is the closest homologue to syndecan-4 but lacks the required cytoplasmic sequence for PKC regulation (20, 21, 26). Both gel chromatography and SDS-PAGE indicated a lack of apparent oligomerization of syndecan-2 peptide (Table I), confirming the unique ability of syndecan-4 cytoplasmic domain to self-associate. GST fusion proteins containing the whole syndecan-2 core protein, however, do migrate with similar properties to syndecan-4 on SDS-PAGE gels, confirming the general ability of syndecan core proteins to self-associate (data not shown).

Taken together, the results show that although transmembrane regions of syndecans promote SDS-resistant oligomer formation (putative dimers), further oligomerization to form tetramers or higher oligomers, as seen with 4V peptides and whole syndecan-4 fusion proteins, is required for PKCoβγ (or PKCoα) activation.

We tested whether the addition of cysteine to the N terminus of 4V and 4L peptides could induce higher order clustering and concomitantly increase PKCoβγ or PKCo regulatory activity. Cys4V eluted at 8.7 kDa (Table I), indicative of an octamer, even though electrospray ionization mass spectrometry showed the molecular mass as 1.1 kDa (data not shown). Thus, the N-terminal cysteine on Cys4V may aid V region multimerization. This had no statistically significant effect on the ability of 4V to potentiate PL-induced PKCoβγ activity (Fig. 2A), but direct activation of PKCoβγ by Cys4V (i.e. in the absence of PL) was greater than that seen with 4V (Fig. 2B). In the presence of PL, both 4V and Cys4V could potentiate the normally maximal activity by 4-fold. In the absence of PL, when PKCoβγ is normally inactive, 4V and Cys4V caused 1.7- and 3.0-fold direct activation. In contrast, Cys4L, having cysteine at the N terminus of 4L, still eluted at 7.4 kDa as a dimer (Table I) and was no more active in regulating PKCoβγ activity than 4L (Fig. 2).

Calcium concentrations can affect protein-protein interactions, and phosphorylation experiments were performed in 750 μM calcium to maximize PKCoβγ activity (28). However, gel filtration at this calcium concentration did not affect the multimeric state of the peptides (Table I).

The mass spectrometer was purchased (National Institutes of Health Grant S10RR06487) and operated by the Comprehensive Cancer Center Mass Spectrometry Shared Facility, University of Alabama at Birmingham (supported in part by National Cancer Institute Grant P30CA13148).
status of the peptides (Table I). These multimeric peptides were resistant to SDS, although the apparent $M_r$ on SDS-PAGE was lower than that seen by gel filtration, being only 1.5- to 3-fold that deduced from the amino acid sequence (Table A). For example, both 4V and Cys4V migrated with an apparent $M_r \sim 3000$. Both methods of estimation depend on the secondary structure of the molecules tested, with gel filtration being less denaturing. Further control experiments with 2V and 2L peptides showed that N-terminal cysteine residues did not induce their oligomerization (Table I).

The results so far indicate that 1) syndecan-4 core protein multimerization correlates with PKC potentiation; 2) the V region of the cytoplasmic domain of syndecan-4 can itself form homopolymers as does recombinant core protein; 3) multimerization is independent of calcium concentration; 4) dimerization is not sufficient for PKC<sub>αβγ</sub> regulatory activity since 4L is inactive; and 5) tetrameric 4V is sufficient for potentiation of PL-induced PKC<sub>αβγ</sub> activity, but direct activation of PKC<sub>αβγ</sub> is more effective with octameric forms seen with Cys4V.

**Reduction of Cys4V Oligomerization Results in Decreased Regulation of PKC Activity**—When incubated with PKC<sub>αβγ</sub> and [32P]ATP in the absence of PL, Cys4V, but not 4V, was itself phosphorylated even though it lacks serine or threonine amino acids (Table A, Fig. 2A). Although the phosphorylation of peptide Cys4V was 10-fold less than that of histone III-S, it was time-dependent (Fig. 3A). Since 4V was not phosphorylated, Cys4V phosphorylation may be on cysteine thiol groups (29). To investigate whether this phosphorylation affected the direct activation of PKC<sub>αβγ</sub> by Cys4V, peptides Cys4V, 4V, or 4L were “cold-phosphorylated” by preincubation with PKC<sub>αβγ</sub> and ATP in the absence of PL (Fig. 3B, lanes 1–3). Their capacities to subsequently activate PKC<sub>αβγ</sub> were compared with those of peptides similarly incubated with PKC<sub>αβγ</sub> but without ATP (Fig. 3B, lanes 4–6). Minimal histone phosphorylation by PKC<sub>αβγ</sub> was seen in the presence of 4L (lane 1), and this was slightly reduced following incubation with cold ATP and PKC<sub>αβγ</sub> (lane 4). Peptide 4V promoted histone phosphorylation to a similar extent with (lane 5) or without (lane 2) pre-treatment. In contrast, the ability of Cys4V to promote histone phosphorylation (lane 3) was markedly reduced (lane 6) by preincubation with ATP and PKC<sub>αβγ</sub>. Thus, preincubation of Cys4V with cold ATP and PKC<sub>αβγ</sub> caused a loss of ability to activate PKC<sub>αβγ</sub>. To determine whether phosphorylation of Cys4V altered its oligomeric structure, we incubated Cys4V with PKC<sub>αβγ</sub> ± 200 μM cold ATP for 10 min at 37°C and analyzed the peptide by Sephadex G-50 gel filtration chromatography (Fig. 4). Cys4V and 4V maintained their original elution profiles (8.7 and 4.6 kDa, respectively) in the absence of enzyme, but elution of phosphorylated Cys4V was markedly altered, with a minor population eluting at 8.7 kDa as before, but most at 3.9 kDa. Thus, phosphorylation appeared to cause a reduction in the multimeric nature of Cys4V, parallelizing a loss in its ability to directly activate PKC<sub>αβγ</sub>. A further control experiment was performed to analyze whether cysteine thiol group phosphorylation itself was inhibitory independent of peptide multimerization. To do this, peptides based on syndecan 4V sequence (CLGKPRFYRK or CLGKPKPYFKK, where two lysine residues were substituted with arginine or the tyrosine residue was substituted with phenylalanine) were used.
were inactive in regulating PKC activity (26) but could be readily phosphorylated. These peptides, when phosphorylated as described above, had no effect on the subsequent ability of "wild type" Cys4V to activate PKCβγ (data not shown). Therefore, reducing the oligomerization status of Cys4V peptide was directly related to decreased PKCβγ regulation.

Aggregation of Syndecan-4 Cytoplasmic Domain by PIP2 Results in PKCβγ Regulatory Activity—As shown before, the sequence KPIYK is critical in the up-regulation of PKC activity (26). This sequence is present in both syndecan 4V and 4L, but 4L activated PKCβγ much less than 4V. Although this appears to parallel multimerization status, it was also possible that the highly basic membrane-proximal region of syndecan-4 cytoplasmic domain (Table I) could directly inhibit the effects of 4V. This was ruled out by addition of five times excess of 4L or 2L, the latter having no ability to regulate PKCβγ (data not shown). Therefore, reducing the oligomerization status of Cys4V peptide was directly related to decreased PKCβγ regulation.

**Protein Kinase C and Syndecan-4**

**FIG. 4.** Preincubation in the presence of PKCβγ and cold ATP reduces the apparent mass of Cys4V. The molecular mass of 4V or Cys4V was analyzed by Sephadex G-50 gel filtration column chromatography before (●, 4V; ○, Cys4V) or after (■, 4V; ●, Cys4V) preincubation in the presence of PKCβγ and cold ATP.

**FIG. 5.** Neither 4L nor 2L affect the ability of 4V to potentiate PL-mediated activation of PKCβγ (A) or to directly activate PKCβγ (B). PKC assays were performed as described under "Experimental Procedures" in the presence (A) or absence (B) of PL and with increasing concentrations of 4L or 2L as shown above each autoradiograph of phosphorylated histone on 20% SDS-PAGE. 50 μg/ml 4V were used for potentiation (A), and 50 μg/ml Cys4V were used for direct activation (B).

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3 J. R. Couchman, A. Woods, E.-S. Oh, A. Theibert, and G. Prestwich, unpublished data.
line replaced by alanine, eluted as a mixture of tetra-, di-, and monomer, with 30% being monomeric. CysKR, in which two lysine residues were replaced by arginine, was insoluble at the high concentration needed for these experiments. CysYF, with tyrosine replaced by phenylalanine, eluted as three peaks, the largest material being of similar size to that of Cys4V (octameric). In parallel with this, CysIS and CysPA showed very limited ability in our previous studies (data not shown, but see Ref. 26) to directly activate PKCβγ to phosphorylate histones or to potentiate PL-induced activation. In contrast, CysYF showed more, although somewhat variable, activity, but it was never as active as Cys4V or 4V. Since 4V is more active than those peptides that also form tetramers (CysIS and CysPA) and even CysYF, which forms some octamers, both multimerization state and specific amino acid sequence seem to regulate PKCβγ activity.

**DISCUSSION**

It is a common characteristic of syndecans that their core proteins form homologous dimers or multimers. Isolated core proteins migrate on SDS-PAGE with apparent molecular masses significantly higher than those deduced from cDNA sequence analysis (21, 27). This occurs even with fusion proteins isolated from bacteria in which glycosylation can have no effect (24–26) and with fusion proteins lacking the cytoplasmic domain (25, 26). We confirm here that GST fusion proteins containing the core protein of syndecan-4 formed stable oligomers when analyzed by both gel chromatography and SDS-PAGE. Gel chromatography indicated that syndecan-4 core protein can form higher order oligomers (tetramers and perhaps octamers) under nondenaturing conditions. These forms of syndecan-4 had potent PKC up-regulatory activity (Ref. 26 and this study). Oligomerization did not involve cystine formation, since syndecan-4 lacks cysteine residues, and oligomers could be dissociated by a single freeze-thaw cycle, demonstrating that the interactions were noncovalent. Dissociation into monomeric forms correlated with the loss of ability of syndecan-4 fusion proteins to regulate PL-induced PKC activity. This supports previous data (25) that the cytoplasmic domain of syndecans is not necessary for self-association. These fusion proteins still contained the full ectodomain of syndecan-4, but the lysine critical for self-association of syndecan-3 is not present in syndecan-4 (21, 25, 31–33), suggesting that other interaction sites must exist in the ectodomain of syndecan-4. It is improbable that the GST portion of the syndecan-4 fusion protein was responsible for SDS-resistant self-association, since this is not a common occurrence in this type of fusion protein and other studies indicate that oligomerization is a common property of syndecans (21–27).

A major finding here was that, in addition to sites in the transmembrane and/or ectodomain promoting oligomerization, the V region of syndecan-4 cytoplasmic domain, but not of syndecan-2, could itself oligomerize. Peptides corresponding to this region migrated on SDS-PAGE with apparent molecular masses significantly higher than those deduced from cDNA sequence analysis (21, 27). This occurs even with fusion proteins isolated from bacteria in which glycosylation can have no effect (24–26) and with fusion proteins lacking the cytoplasmic domain (25, 26). We confirm here that GST fusion proteins containing the core protein of syndecan-4 formed stable oligomers when analyzed by both gel chromatography and SDS-PAGE. Gel chromatography indicated that syndecan-4 core protein can form higher order oligomers (tetramers and perhaps octamers) under nondenaturing conditions. These forms of syndecan-4 had potent PKC up-regulatory activity (Ref. 26 and this study). Oligomerization did not involve cystine formation, since syndecan-4 lacks cysteine residues, and oligomers could be dissociated by a single freeze-thaw cycle, demonstrating that the interactions were noncovalent. Dissociation into monomeric forms correlated with the loss of ability of syndecan-4 fusion proteins to regulate PL-induced PKC activity. This supports previous data (25) that the cytoplasmic domain of syndecans is not necessary for self-association. These fusion proteins still contained the full ectodomain of syndecan-4, but the lysine critical for self-association of syndecan-3 is not present in syndecan-4 (21, 25, 31–33), suggesting that other interaction sites must exist in the ectodomain of syndecan-4. It is improbable that the GST portion of the syndecan-4 fusion protein was responsible for SDS-resistant self-association, since this is not a common occurrence in this type of fusion protein and other studies indicate that oligomerization is a common property of syndecans (21–27).

**FIG. 6.** PIP2 induces a higher oligomeric status of 4L peptide consistent with PKC potentiation activity. A, Sephadex G-50 gel filtration chromatography of 4L peptide in the absence of phospholipid (▲) and in the presence of PIP2 (●) or inositol hexaphosphate (○) are shown. B, oligomeric forms of 4L promoted by PIP2 potentiate PKCβγ (lane 4) phosphorylation of epidermal growth factor receptor peptide. Relative activity is indicated by mean ± S.E. (n = 4) compared with that in the absence of peptide and effector (lane 1).

**FIG. 7.** The amino acid sequence KPIYK is needed for oligomerization of 4V. Sephadex G-50 gel filtration chromatography of the substituted peptides with N-terminal cysteine are shown: Cys4V (CLGKKPIYKK, ●), CysPA (CLGKKAIYKK, ○), CysIS (CLGKKPSYKK, △), and CysYF CLGKKPIFKK, □). The approximate elution positions for peptides of various oligomerization states are shown based on the elution of protein standards.
the whole cytoplasmic domain showed less tendency to oligomerize than those containing just the V region, probably due to the high charge of the C1 region normally proximal to the membrane. However, the reduced tendency of the 4L peptide to oligomerize was partly overcome by PI2P in vitro, since PI2P induced higher oligomeric structures. This also increased the ability of the 4L peptide to potentiate PKC activity. We believe that the interaction of PI2P with syndecan-4 may be of biological significance, and this is under investigation in our laboratory. Of particular relevance may be that PI2P could partially activate PKC, and this was further enhanced by 4V or 4L peptides. Thus, as seen previously in vivo during cell adhesion, accumulated PI2P resulting from PI2P kinase activity induced on integrin-mediated adhesion (reviewed in Refs. 3 and 6) may increase or stabilize the oligomerization status of syndecan-4 cytoplasmic domain and induce the potentiation of PKC activity. Regulation of other focal adhesion components, such as vinculin and a-actinin, by PI2P has also been shown (3, 34–36), and we hypothesize that PI2P may regulate syndecan-4 and PKCa, two additional focal adhesion components.

Biologically active of the syndecan-4 cytoplasmic domain is not due solely to oligomeric status, since peptides of altered sequence, even when tetrameric or partially octameric (e.g. CLGKKPIFKK, where a single tyrosine was replaced by a phenylalanine residue), were much less active than the corresponding oligomeric forms of the native peptides. Preliminary data4 using circular dichroism spectroscopy also indicate that although the 4V peptide has secondary structure, this is par
tially or totally lost when residue substitutions are made or the peptide sequence is scrambled. Further long term studies with nuclear magnetic resonance spectroscopy are underway to examine peptide-peptide and peptide-PI2P interactions.

Dimerization or oligomerization of membrane receptors often activate signaling cascades with phosphorylated tyrosines binding SH2 domain-containing adapter proteins (3–6). Receptors with intrinsic tyrosine kinase activity undergo transphos
tors with intrinsic tyrosine kinase activity undergo transphos
tory. Of particular relevance may be that PI2P could partially regulate syndecan-4 and vinculin and a-actinin, by PI2P has also been shown (3, 34–36), and we hypothesize that PI2P may regulate syndecan-4 and PKCa, two additional focal adhesion components.

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