Syndecan-4 and integrins are the primary transmembrane receptors of focal adhesions in cells adherent to extracellular matrix molecules. Syndecans is a cytoplasmic protein that interacts specifically with the cytoplasmic domain of syndecan-4, and it co-localizes with syndecan-4 in focal contacts. In the present study we sought possible interactors with syndecans. We find that syndecans interacts with the focal adhesion adaptor protein paxillin. The binding of syndecans to paxillin is direct, and these interactions are triggered by the activation of protein kinase C. Syndecans also binds the paxillin homolog, Hic-5. The connection of syndecan-4 with paxillin through syndecans parallels the connection between paxillin and integrins and may thus reflect the cooperative signaling of these two receptors in the assembly of focal adhesions and actin stress fibers.

Cell adhesion is important for such biological functions as cell growth, proliferation, survival, and migration. Fibroblasts adherent to extracellular matrix (ECM) proteins (i.e. fibronectin, vitronectin, collagen) in vitro, cluster transmembrane adhesion receptors and intracellular-cytoskeletal adaptors and signaling molecules into macromolecular complexes termed focal adhesions (1). The primary transmembrane receptors associated with the focal adhesion are members of the integrin superfamily and the heparan-sulfate proteoglycan (HSPG) syndecan-4 (2–5). Many integrins (such as the αβ2 and αβ3) bind to an Arg-Gly-Asp (RGD) sequence found within their ECM ligands. Previous studies have demonstrated that fibroblasts seeded onto an RGD-containing cell-binding domain fragment (CBD) of fibronectin will attach but not spread or form focal adhesions and actin stress fibers (6, 7). This laboratory demonstrated that syndecan-4, working in cooperation with the αβ2 integrin, can stimulate a Rho-dependent pathway that will elicit focal adhesion and stress fiber formation in CBD-adherent cells (6).

The cytoplasmic face of the focal adhesion serves two purposes, physical organization of the adhesion complex and a convergence point for various signaling pathways (1, 4, 8–10). Structural proteins (such as actin, α-actinin, talin, vinculin, zyxin, actopaxin, paxillin, and Hic-5) (11–13) serve to stabilize the focal adhesion complex, couple cell surface receptors to the actomyosin contractile apparatus (9), and act as a scaffold to which other proteins can bind. Paxillin, in particular, is a multifaceted scaffolding protein containing two main structural domains: an SH2- and SH3-containing N terminus and a C-terminal region composed of four sequential LIM (lin-11, isl-1, mec-3) domains (14). Between these two domains paxillin is capable of binding both structural (α-actinin and actopaxin) as well as signaling (focal adhesion kinase [FAK], Csk, and c-Abl) molecules, allowing it to play an intimate role in the dynamics of the focal adhesion (12–15).

Signaling molecules act as conduits between the extracellular environment and the cell, influencing focal adhesion turnover, nuclear transcription, cell morphology, and cell survival (4, 8). Tyrosine kinases (FAK, Src, Csk, Fyn, c-Abl, and Pyk2) are activated upon integrin engagement and subsequently trigger such cellular events as mitosis, cytoskeletal rearrangement (4, 16, 17) and activation of the Ras-extracellular signal-regulated kinase (ERK) MAPK cascade (4). In addition, enhanced tyrosine phosphorylation is associated with increased focal adhesion and stress fiber formation (10).

The serine/threonine kinase protein kinase C (PKC) is also associated with focal adhesions. Cells adherent to the CBD of fibronectin and treated with phorbol esters to directly stimulate PKC generate focal adhesions and actin stress fibers (18), indicating that this kinase is involved in the focal adhesion assembly process. PKC activation directs the localization of syndecan-4 to focal adhesion sites (2), and in turn, PKC activation is potentiated following oligomerization of syndecan-4 core proteins (19–21) and the binding of phosphatidylinositol 4,5-bisphosphate (PIP2) to syndecan-4 (22). Interestingly, PKC stimulation also enhances the shedding of syndecan-4 from the plasma membrane, thereby modifying its function from a cell surface receptor to an ECM-associated molecule (3, 23). Syndecan-4 is a member of a family of transmembrane HSPG (syndecans 1–4) that are characterized by divergent extracellular domains and cytoplasmic domains that contain two constant regions separated by a variable region that is unique to each family member (3, 24). Recently, several proteins have been identified that bind to the cytoplasmic domain.
of the syndecan family members. These proteins (syntenin, synectin, synbindin, and CASK) contain PDZ domains that ligate the invariant C-terminal 4 amino acids (EFYA) present in all 4 syndecan molecules (25–29). Other molecules ligate the unique variable region and, therefore, bind selectively to one family member. PIP$_2$ binds the variable region of syndecan-4 (22), as does the cytoplasmic protein syndesmos (30).

Syndesmos was isolated from a yeast two-hybrid screen in which the cytoplasmic domain of syndecan-4 was used as bait and was found to co-localize with syndecan-4 in the focal contacts of ventral plasma membranes of fibronectin adherent cells. Syndesmos can be myristylated suggesting that it may interact directly with the plasma membrane in addition to its association with syndecan-4. When overexpressed in NIH3T3 cells, syndesmos accelerates cell spreading and the assembly of actin stress fibers (30). Due to the impact syndesmos has on cell morphology, we deduced that it might interact with other focal adhesion-associated proteins and sought to identify other potential cytoplasmic interactors. Here we report that syndesmos binds the adaptor protein paxillin. The connection of syndecan-4 with paxillin and possible downstream signaling events allows the integrin to form focal adhesions and actin stress fibers (30). Syndesmos can be myristylated suggesting that it may interact directly with the plasma membrane in addition to its association with syndecan-4. When overexpressed in NIH3T3 cells, syndesmos accelerates cell spreading and the assembly of actin stress fibers (30). Due to the impact syndesmos has on cell morphology, we deduced that it might interact with other focal adhesion-associated proteins and sought to identify other potential cytoplasmic interactors. Here we report that syndesmos binds the adaptor protein paxillin. The connection of syndecan-4 with paxillin and possible downstream signaling events allows the integrin to form focal adhesions and actin stress fibers.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Primary chick embryo fibroblasts (CEFs) were obtained and cultured as previously described (2). All experiments were done on cultures that were 85% confluent.

**Immunoprecipitations and GST Pull-down Experiments**—Syndesmos was immunoprecipitated from cell lysates with anti-syndesmos polyclonal antibodies (30). The immunoprecipitates were washed four times in lysis buffer (phosphate-buffered saline/Triton X-100 (0.1%), protease inhibitors (Complete, protease inhibitor mixture, Roche Molecular Biochemicals) and 1 mM sodium orthovanadate), solubilized in SDS-PAGE buffer, electrophoresed, transferred to an immobilon-P membrane, and analyzed for bound syndesmos by autoradiography.

**GST Pull-down Experiments**—GST-paxillin, GST-Hic-5 and GST-syndesmos (Amersham Biosciences, Inc.). The construction of GST-syndesmos has been previously described (30). The GST-paxillin and GST-Hic-5 constructs were a gift from Dr. Sheila Thomas (Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA). Cell lysates from CEFs were incubated overnight at 4 °C with the glutathione-agarose beads, washed six times in the lysis buffer, solubilized in SDS-PAGE buffer, electrophoresed, transferred to an immobilon-P membrane, and analyzed for bound syndesmos using the anti-syndesmos polyclonal antibody (30) as described above for the immunoprecipitations.

**In Vitro Transcription-Translation Assay**—In vitro transcription-translation was carried out using the TNT in vitro transcription-translation kit from Promega (Madison, WI). Reactions were carried out as recommended by the manufacturer. The template used for these experiments was syndesmos cDNA that was cloned in pBluescript (Stratagene, La Jolla, CA). In vitro cell labeling grade L-$^{[35]S}$methionine (SJ, 1015; >1000 Ci/mmole) was purchased from Amersham Biosciences, Inc.

**Protein Cross-linking**—CEFs were treated for 30 min with the membrane-permeable homobifunctional cross-linking reagent DTSP (3,3'-dithiobispropionic acid N-hydroxysuccinimide ester, C14H16O4S2, Sigma), which contains a cleavable disulfide linkage. Cells were then rinsed with phosphate-buffered saline and blocked with 0.2 mM glycine in phosphate-buffered saline for 10 min. After two washes with phosphate-buffered saline, the cells were lysed in radioimmune precipitation buffer, and the lysates were expressed in Escherichia coli (BL21) and purified on glutathione-agarose beads according to the manufacturer’s instructions (Pierce, Rockford, IL).

GST-paxillin, GST-Hic-5, GST-syndesmos, or GST alone were expressed in Escherichia coli (BL21) and purified on glutathione-agarose beads according to the manufacturer’s instructions (Pierce, Rockford, IL).
were incubated with either anti-syndesmos serum or preimmune serum coupled to protein-G beads. The immunoprecipitates were washed four times with radioimmune precipitation buffer, resuspended in electrophoresis buffer containing 20% β-mercaptoethanol, boiled for 5 min to cleave the disulfide bond, and then analyzed by SDS-PAGE under reducing conditions and Western blotting using anti-paxillin antibodies.

**TPA Experiments**—After starvation in Dulbecco’s modified Eagle’s medium without fetal bovine serum, CEFs were treated for the indicated times with TPA (Sigma) at a concentration of 100 ng/ml. Cell lysates and immunoprecipitates were obtained as described above.

**RESULTS**

**Syndesmos Binds Paxillin and Hic-5**—To identify possible syndesmos interactors, CEFs were treated with orthovanadate (100 μM) for 2 h in the presence of 10% fetal bovine serum and lysed. Proteins immunoprecipitated from the cell lysate with antibodies against syndesmos were electrophoresed and analyzed by Western blot using an anti-phosphotyrosine antibody. A major tyrosine-phosphorylated protein that migrates with an apparent molecular mass of 70 kDa was observed (Fig. 1A). Based on its molecular mass we hypothesized that this protein is paxillin, one of the major tyrosine-phosphorylated focal adhesion-associated proteins. This hypothesis was confirmed when proteins from cell lysates were immunoprecipitated with syndesmos antibodies and blotted with an antibody against paxillin. Two immunoreactive bands (70 and 42 kDa) could be detected with this antibody (Fig. 1B) as previously reported (31). Although the paxillin antibody used also recognizes the paxillin homolog Hic-5 (12), the lower band is likely not Hic-5, but a proteolytic fragment of paxillin, since we also observe this band in *in vitro* transcription-translation assays using a paxillin template (data not shown).

Interactions of syndesmos with paxillin could also be demonstrated in GST pull-down experiments. GST-syndesmos, but not GST alone, is able to pull-down two paxillin immunoreactive bands from CEF lysates (Fig. 2A) and GST-paxillin, but not GST alone, could pull-down syndesmos from CEF lysates (Fig. 2B). In addition, GST-Hic-5 could also pull-down syndesmos from CEF lysates (Fig. 2B) suggesting that the syndesmos binding site is present in both paxillin and its homolog Hic-5.

**Binding of Syndesmos to Paxillin and Hic-5 Is Direct**—To test if syndesmos-paxillin complexes exist intracellularly, CEFs were treated with the membrane permeable cross-linker DTSP, and immunoprecipitates were obtained from cell lysates with either anti-syndesmos serum or preimmune serum. The analysis of these immunoprecipitates by SDS-PAGE and Western blot with antibodies against paxillin clearly indicates that paxillin was cross-linked to syndesmos (Fig. 3A). It is thus likely that a direct intracellular interaction exists between the two proteins.

To further confirm that the interactions between syndesmos and paxillin or Hic-5 observed in the immunoprecipitations and GST pull-downs in the above experiments are direct, we performed GST pull-down experiments with radiolabeled *in vitro* synthesized syndesmos to eliminate the potential involvement of other associated proteins. GST-paxillin and GST-Hic-5, but not GST alone, bind *in vitro* synthesized syndesmos (Fig. 3B). Together, these data indicate a direct interaction between paxillin (or Hic-5) and syndesmos.

**Activation of PKC Triggers Interactions between Syndesmos and Paxillin**—PKC activation, like syndesmos, is associated with focal adhesion and actin stress fiber formation and also with increased tyrosine phosphorylation in cells. To determine whether PKC activation is involved in promoting the interaction of syndesmos and paxillin, serum-starved CEFs were incubated with TPA, which directly activates PKC, for various periods of time. Paxillin was co-immunoprecipitated with syndesmos upon 1 min of TPA treatment (Fig. 4). Additional treatment with the tyrosine phosphatase inhibitor orthovanadate extends the syndesmos-paxillin interaction for at least 3 min (Fig. 4) indicating that these associations are also dependent on tyrosine phosphorylation events. The cross-linking experiment

---

**Fig. 4. Activation of PKC leads to binding between syndesmos and paxillin in CEFs.** Paxillin is co-immunoprecipitated with syndesmos from serum-starved cells 1 min after treatment with TPA in the absence of orthovanadate. The interaction is extended to 3 min in the presence of orthovanadate.

---

| TPA induction (100ng/ml) | 0' | 1' | 3' | 5' | 8' |
|-------------------------|----|----|----|----|----|
| P - Syndesmos            |    |    |    |    |    |
| IB - Paxillin            |    |    |    |    |    |

**Fig. 5. Binding of syndesmos with paxillin is through a region conserved in both chicken and mouse syndesmos.** Alignment of chicken syndesmos and mouse syndesmos. The nucleotide sequence of mouse syndesmos has been submitted to GenBank™ (accession number: AF435792).
done in the absence of vanadate (Fig. 3) and also the in vitro experiments (Figs. 2, 3, and 6) show that tyrosine phosphorylation is not necessary for the association between syndesmos and paxillin. However, the experiment described in Fig. 4 shows that, if not absolutely necessary for interaction, the tyrosine phosphorylation has a strong enhancing effect on the syndesmos/paxillin interaction possibly by stabilizing the complex.

TPA treatment has been shown to trigger focal adhesion formation and to enhance the recruitment of syndecan-4 in these focal adhesions (18, 2). Now we are showing that TPA treatment also increases the association of syndesmos with paxillin. It is interesting to note that this increase is very transient and again that the presence of vanadate stabilizes the complex. Since our laboratory has shown that the recruitment of syndecan-4 in focal adhesions could be triggered by TPA treatment we speculate that the increase in syndesmos/paxillin association is involved in the focal adhesion assembly process as well. The short duration would suggest a rapid turnover of that complex.

The Binding Site for Paxillin on Chicken Syndesmos Is in a Conserved Region Shared with the Mouse Protein—Screening of a mouse cDNA library with a full-length chicken syndesmos cDNA generated a number of clones that encode a protein of 211 amino acids. This sequence is considerably shorter that the 320 amino acids of chicken syndesmos (30). Most of the mouse sequence has an extremely high homology to a central region of the chicken sequence (Fig. 5). The mouse sequence does not possess either the four potential initiation methionine residues or an 80-amino acid C-terminal portion found in chicken syndesmos. To define the binding site in chicken syndesmos that interacts with paxillin we performed GST pull-down experiments with a number of GST-syndesmos deletion constructs of chicken or mouse origin. The results of these tests indicate that syndesmos binds to paxillin through the amino acid sequence between amino acids 118 and 205 of the chicken sequence (Fig. 6). This sequence is in the region of high homology with the mouse sequence that also binds paxillin.

**DISCUSSION**

Focal adhesions are macromolecular complexes that are formed when cells are plated on extracellular matrix components. They are made up of the transmembrane molecules, integrins and syndecan-4, and a large number of cytoplasmic signaling and adaptor proteins. Paxillin is an important cytoplasmic adaptor protein that binds several other focal adhesion-associated proteins such as vinculin, actopaxin, FAK, Src, Crk, and Csk (15, 31, 32). A molecular connection between paxillin and integrins can be established via a vinculin-talin bridge. Vinculin binds both paxillin and talin, and the latter binds the cytoplasmic domain of the β chain of integrins.

In the present communication we report on a molecular connection between paxillin and syndecan-4, the second transmembrane receptor of focal adhesions. We show that paxillin binds directly to syndesmos, a cytoplasmic protein that binds specifically to the cytoplasmic domain of syndecan-4 (30). Thus, paxillin, as an important focal adhesion complex adaptor protein, can establish molecular connections between the intracellular focal adhesion complex and integrins and syndecan-4, the two transmembrane proteins of that complex. The connection of paxillin with integrins is through a vinculin-talin bridge and that with syndecan-4 is through syndesmos. It is interesting that the interactions between paxillin and syndesmos can be triggered by the activation of PKC. The variable region of the cytoplasmic domain of syndecan-4 binds PIP2 and thus recruits and potentiates PKC activation (27). PKC activation also leads to the assembly of focal adhesions and actin stress fibers in cells plated on the cell binding domain of fibronectin, a condition in which syndecan-4 is not occupied (18). Furthermore, cells plated on the cell binding domain of fibronectin can assemble the focal adhesion complex when treated with antibodies directed against syndecan-4 (6). Thus syndecan-4 and integrins signal cooperatively in the assembly of the focal adhesion complex. We have previously shown that overexpression of

---

**FIG. 6. Analysis of the paxillin binding region of syndesmos.** The region on the full-length chicken syndesmos (GST-Sdos FL) is located in a region conserved in the mouse syndesmos sequence.

**FIG. 7. Model indicating the connection between syndecan-4 and paxillin via syndesmos and downstream signaling events.** A parallel connection between integrins and paxillin is indicated. We suggest that these two pathways may reflect the cooperativity between syndecan-4 and integrins in the assembly of focal adhesions and actin stress fibers.
Syndesmos enhances cell spreading (30). The interaction of syndesmos with paxillin is enhanced following PKC activation. As syndesmos is primarily localized in the cytoplasm it may exist complexed with paxillin. PKC activation may then promote the localization of syndesmos to focal adhesions.

The connection of paxillin with the cytoplasmic domains of both syndecan-4 and integrins could represent one aspect of the cooperativity between these receptors in which they would be linked to intracellular signaling pathways through the binding of paxillin to FAK. These signaling pathways could involve the binding of FAK to Graf, an SH3-containing GTPase-activating protein for Rho or the phosphorylation of tyrosine residue 925 on FAK, which allows an interaction with Grb2 and in turn brings SOS, the guanine nucleotide exchange factor for Ras, to the complex. Similarly, the interactions of FAK-dependent phosphorylation of paxillin generates a binding site for the adapter protein Crk, which in turn binds the guanine nucleotide exchange factor C3G providing another link to the Ras signaling pathway (8). These possible interactions for syndecan-4 are shown diagramatically in Fig. 7.

Acknowledgments—We thank Dr. Sheila Thomas for providing the GST-paxillin and GST-Hic5 constructs. We also thank our colleagues Enzo Calautti and Tokuro Iwabuchi for critical discussions.

REFERENCES
1. Burridge, K., and Chrzanowska-Wodnicka, M. (1996) Annu. Rev. Cell Dev. Biol. 12, 463–518
2. Baciu, P. C., and Goetinck, P. F. (1995) Mol. Biol. Cell 6, 1503–1513
3. Bernfield, M., Gottle, M., Park, P. W., Reizes, O., Fitzgerald, M. L., Lincecum, J., and Zako, M. (1999) Annu. Rev. Biochem. 68, 729–777
4. Giancotti, F. G., and Ruoslahti, E. (1999) Science 285, 1024–1032
5. Woods, A., and Couchman, J. R. (1994) Mol. Biol. Cell 5, 183–192
6. Saoncella, S., Echtermeyer, F., Denhez, F., Nowlen, J. K., Mosher, D. F., Robinson, S. D., Hynes, R. O., and Goetinck, P. F. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 2805–2810
7. Woods, A., Couchman, J. R., Johansson, S., and Hook, M. (1986) EMBO J. 5, 665–670
8. Clark, E. A., and Brugge, J. S. (1995) Science 268, 233–239
9. Critchley, D. R. (2000) Curr. Opin. Cell Biol. 12, 133–139
10. Schoenwaelder, S. M., and Burridge, K. (1999) Curr. Opin. Cell Biol. 11, 274–286
11. Couchman, J. R., and Woods, A. (1999) J. Cell Sci. 112, 3415–3420
12. Nikolopoulos, S. N., and Turner, C. E. (2000) J. Cell Biol. 151, 1435–1448
13. Turner, C. E. (2000) Nat. Cell Biol. 2, E231–E286
14. Turner, C. E. (2000) J. Cell Sci. 113, 4139–4140
15. Schaller, M. D., and Parsons, J. T. (1995) Mol. Cell. Biol. 15, 2635–2645
16. Avraham, H., Park, S. Y., Schinkmann, K., and Avraham, S. (2000) Cell Signal. 12, 123–133
17. Salgia, R., Brunkhorst, B., Piesch, E., Li, J. L., Lo, S. H., Chen, L. B., and Griffin, J. D. (1995) Oncogene 11, 1149–1155
18. Woods, A., and Couchman, J. R. (1995) J. Cell Biol. 101, 277–290
19. Oh, E. S., Woods, A., and Couchman, J. R. (1997) J. Biol. Chem. 272, 8133–8136
20. Oh, E. S., Woods, A., and Couchman, J. R. (1997) J. Biol. Chem. 272, 11805–11811
21. Oh, E. S., Woods, A., Lim, S. T., Theibert, A. W., and Couchman, J. R. (1998) J. Biol. Chem. 273, 10624–10629
22. Horowitz, A., Murakami, M., Gao, Y., and Simons, M. (1999) Biochemistry 38, 15871–15877
23. Subramanian, S. V., Fitzgerald, M. L., and Bernfield, M. (1997) J. Biol. Chem. 272, 14713–14720
24. Zimmermann, P., and David, G. (1999) FASEB J. 13, S91–S100
25. Cohen, A. R., Woods, D. F., Marfatia, S. M., Walther, Z., Chiashi, A. H., Anderson, J. M., and Wood, D. F. (1998) J. Cell Biol. 142, 129–138
26. Ethell, I. M., Haghara, K., Miura, Y., Irie, F., and Yamaguchi, Y. (2000) J. Cell Biol. 151, 53–68
27. Gao, Y., Li, M., Chen, W., and Simons, M. (2000) J. Cell. Physiol. 184, 373–379
28. Grootjans, J. J., Zimmermann, P., Reekmans, G., Smets, A., Degeest, G., Durr, J., and Davis, H. (1999) Proc. Natl. Acad. Sci. U. S. A. 94, 13683–13688
29. Hsueh, Y. P., Yang, F. C., Kharazia, V., Naisbitt, S., Cohen, A. R., Weinberg, R. A., and Davis, H. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 13683–13688
30. Oh, E. S., Woods, A., Lim, S. T., Theibert, A. W., and Couchman, J. R. (1998) J. Biol. Chem. 273, 10624–10629
31. Baciu, P. C., Saoncella, S., Lee, S. H., Denhez, F., Leuthardt, D., and Goetinck, P. F. (2000) J. Cell Sci. 113, 315–324
32. Salgia, R., Li, J. L., Lo, S. H., Brunkhorst, B., Kansas, G. S., Sobhany, E. S., Sun, Y., Piesch, E., Hallek, M., Ernst, T., Tantrauahi, R., Chen, L. B., and Griffin, J. D. (1995) J. Biol. Chem. 274, 15039–15047
33. Weng, Z., Taylor, J. A., Turner, C. E., Brugge, J. S., and Seidel-Dagan, C. (1993) J. Biol. Chem. 268, 14956–14963