**Syndecan-4 Modulates Focal Adhesion Kinase Phosphorylation***

Sarah A. Wilcox-Adelman‡, Fabienne Denhez, and Paul F. Goetinck§

From the Cutaneous Biology Research Center, Massachusetts General Hospital, Harvard Medical School, Charlestown, Massachusetts 02129

The cell-surface heparan sulfate proteoglycan syndecan-4 acts in conjunction with the α5β1 integrin to promote the formation of actin stress fibers and focal adhesions in fibronectin (FN)-adherent cells. Fibroblasts seeded onto the cell-binding domain (CBD) fragment of FN attach but do not fully spread or form focal adhesions. Activation of Rho, with lysophosphatidic acid (LPA), or protein kinase Cδ, using the phorbol ester phorbol 12-myristate 13-acetate, or clustering of syndecan-4 with antibodies directed against its extracellular domain will stimulate formation of focal adhesions and stress fibers in CBD-adherent fibroblasts. The distinct morphological differences between the cells adherent to the CBD and to full-length FN suggest that syndecan-4 may influence the organization of the focal adhesion or the activation state of the proteins that comprise it. FN-null fibroblasts (which express syndecan-4) exhibit reduced phosphorylation of focal adhesion kinase (FAK) tyrosine 397 (Tyr397) when adherent to CBD compared with FN-adherent cells. Treating the CBD-adherent fibroblasts with LPA, to activate Rho, or the tyrosine phosphatase inhibitor sodium vanadate increased the level of phosphorylation of Tyr397 to match that of cells plated on FN. Treatment of the fibroblasts with PMA did not elicit such an effect. To confirm that this regulatory pathway includes syndecan-4 specifically, we examined fibroblasts derived from syndecan-4-null mice. The phosphorylation levels of FAK Tyr397 were lower in FN-adherent syndecan-4-null fibroblasts compared with syndecan-4-wild type and these levels were rescued by the addition of LPA or re-expression of syndecan-4. These data indicate that syndecan-4 ligation regulates the phosphorylation of FAK Tyr397 and that this mechanism is dependent on Rho but not protein kinase C activation. In addition, the data suggest that this pathway includes the negative regulation of a protein-tyrosine phosphatase. Our results implicate syndecan-4 activation in a direct role in focal adhesion regulation.

Syndecan-4 is a member of a family of transmembrane heparan sulfate proteoglycans (syndecans 1–4) that are characterized by divergent extracellular domains and short cytoplasmic domains that contain two constant regions separated by a variable region that is unique to each family member (reviewed in Refs. 1–4). Although all members of the syndecan family arose from a single ancestral gene, their expression patterns in tissues and during development are highly regulated (3–5). The terminal four amino acids (EFYA) of the cytoplasmic domain of all syndecan family members compose a binding site for the PDZ-containing proteins: synbindin, syntenin, CASK/LIN-2, and syntenin (6–10). Unlike other family members, syndecan-4 binds protein kinase C-α (PKC-α)1 through the intermediary phosphatidylinositol biphosphate (11, 12) at the variable region and the cytoplasmic protein syndesmos through both the variable and the membrane-proximal constant regions (13). Syndecans-1, -2, and -4 have been shown to bind extracellular matrix proteins (14–16). However, syndecan-4 is the only family member to localize to sites of cell-cell adhesion complexes (17). Comparison of the localization of syndecan-4 with the focal adhesion marker protein vinculin suggests that syndecan-4 does not localize to newly formed contacts but with more established adhesion sites (18).

Focal adhesions are macromolecular complexes that localize to sites of closest contact (10–15 nm) between cells and the underlying extracellular matrix substrate (reviewed in Refs. 19–21). Focal adhesions are composed of transmembrane receptors (primarily syndecan-4 and members of the integrin superfamily), structural molecules (such as actin, talin, tensin, vinculin, and α-actinin), and signaling molecules (i.e. focal adhesion kinase (FAK), PKC, and Src). Focal adhesions, therefore, serve not only as structural supports but also as signaling conduits between the actin cytoskeleton and the surrounding environment of the cell. The generation of focal adhesions in fibronectin (FN)-adherent cells is dependent on the ligation of two different transmembrane receptors: integrins and syndecan-4. Fibroblasts seeded on the cell-binding domain (CBD) of FN (which contains only the integrin-binding RGD sequence) will attach but do not form focal adhesions or actin stress fibers (22–24). The addition of an antibody against the extracellular domain of syndecan-4 stimulates focal adhesion and stress fiber formation in cells plated on the CBD (24). The syndecan-4 signal can be bypassed in CBD-adherent fibroblasts by directly stimulating the small GTPase Rho with lysophosphatidic acid (LPA) (24). These data indicate that syndecan-4 acts in cooperation with the α5β1 integrin to direct focal adhesion formation and that the action of syndecan-4 is through a Rho-dependent mechanism (24).

The generation of syndecan-4-null mice demonstrated no initial obvious phenotype and showed, surprisingly, that cells

---

* This work was supported in part by National Institute of Health, NICHD Grant HD-37490 and grants from the Cutaneous Biology Research Center through the MGH/Shiseido Company (to P. F. G.) and the NICHD Grant HD-37490 and grants from the Cutaneous Biology Research Center. 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.

‡ Supported by National Institutes of Health Postdoctoral Fellowship F32 HD41235.

§ To whom correspondence should be addressed: Cutaneous Biology Research Center, MGH-East, Bldg. 149, 13th St., Charlestown, MA 02129. Tel.: 617-726-4183; Fax: 617-726-4189; E-mail: paul.goetinck@cbrc2.mgh.harvard.edu.

1 The abbreviations used are: PKC, protein kinase C; FAK, focal adhesion kinase; FN, fibronectin; CBD, cell-binding domain; LPA, lysophosphatidic acid; WT, wild type; PBS, phosphate-buffered saline; PMA, phorbol 12-myristate 13-acetate.
Syndecan-4 Modulates FAK Phosphorylation

recently, Rho
closer examination of early adhesion events documented in
small GTPase Rho, show increases in FAK phosphorylation
site for the Grb2 adaptor protein leading to activation of the
Phosphotyrosines 576 and 577 are required for maximal FAK
interactions may occur between FAK and Rho to facilitate cell
transient inhibition of Rho during early cell spreading
FAK to the deficient cells and showed that Rho activity was
constitutive activation of Rho and this activity level is inversely
shown to be intimately involved in focal adhesion turnover (30,
loss of FAK is associated with decreased cell migration and
increased focal adhesion size (30, 32–34), whereas overexpression of FAK increases cell migration (34–36).
FAK serves as both a scaffolding and signaling protein in the focal adhesion. A nonreceptor tyrosine kinase, FAK, is composed of a central catalytic domain flanked by two noncatalytic regions (37). FAK contains multiple tyrosine residues that, upon their phosphorylation, are capable of binding proteins containing SH2 domains. Tyrosine 397 (Tyr397) is a critical phosphorylation site that results from autophosphorylation upon antibody-induced clustering of cell-surface integrins or cell adhesion to extracellular matrix proteins (such as collagen, fibronectin, and vitronectin) (Refs. 38 and 39 and reviewed in Refs. 40 and 41). FAK-mediated cell migration is dependent on phosphorylation of FAK Tyr397 (35). The phosphorylated form of Tyr397 binds Src (38), the p85 subunit of phosphatidylinositol 3-kinase (42), phospholipase Cγ1 (43), the adaptor proteins Grb7 (44) and Shc (45), and the phosphatase PTEN (46). The unlikely possibility that all of these proteins bind FAK simultaneously suggests that distinct FAK-containing signaling complexes probably exist within the focal adhesion (47). The phosphorylation of the remaining tyrosine residues (407, 576, 577, 861, and 925) occurs in a Src-dependent manner (48–51). Phosphotyrosines 576 and 577 are required for maximal FAK kinase activity (48) and phosphosynoise 925 acts as a binding site for the Grb2 adaptor protein leading to activation of the Ras pathway (50).

fibroblasts treated with LPA, which directly stimulates the
small GTPase Rho, show increases in FAK phosphorylation and its subsequent localization to focal adhesions (52–57). Closer examination of early adhesion events documented initial FAK phosphorylation occurring in a Rho-independent manner followed by Rho-mediated FAK phosphorylation (58). Recently, Ren et al. (31) demonstrated that FAK-null cells exhibit constitutive activation of Rho and this activity level is inversely correlated with focal adhesion turnover. They reintroduced FAK to the deficient cells and showed that Rho activity was restored to normal levels. This suggests that FAK is responsible for the transient inhibition of Rho during early cell spreading (31). All of these studies indicate that reciprocal interactions may occur between FAK and Rho to facilitate cell spreading and focal adhesion and stress fiber formation.

General FAK phosphorylation levels increase during early cell spreading (59) but maximal phosphorylation requires both the integrin-binding and heparin-binding domains of FN (60). As syndecan-4 binds the heparin-binding domain of FN (61) and has been shown to act in a Rho-dependent manner to influence focal adhesions and actin stress fibers (24), we were interested in determining what effect syndecan-4 signaling might have on the autophosphorylation site of FAK. We now demonstrate that increased phosphorylation of FAK Tyr397 is dependent on syndecan-4 ligation, and that the syndecan-4 signal may be superseded by direct activation of Rho.

MATERIALS AND METHODS

Cell Culture—Three types of cells were used in this study. Fibronectin-null cells which express syndecan-4 (24) and fibroblasts derived from newborn littermates that were either wild type (+/+) or null (−/−) for the syndecan-4 core protein gene. These cells are referred to as FN-null and syndecan-4-WT or syndecan-4-null, respectively. All cell types were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen). For experimental assays, 1.5 × 10⁴ cells/100-mm tissue culture dish were seeded in serum overnight and subsequently serum starved for 18 h. The cells were washed three times with phosphate-buffered saline minus calcium chloride and magnesium chloride (PBS, Invitrogen) containing 0.5 mM EDTA and lifted from the dishes with a 1:1 dilution of PBS/EDTA and 0.25% trypsin/EDTA (Invitrogen). The tissue culture plates and glass coverslips used for the experiments were coated with either 30 μg/ml FN (BD Biosciences, San Jose, CA) or 10 μg/ml CBD (Invitrogen) diluted in PBS overnight at 4 °C. The plates and coverslips were then washed with PBS, blocked with 1% bovine serum albumin for 60 min at room temperature, and given a final wash before the cells were seeded. The fibroblasts were allowed to attach and spread for 3 h in serum-free Dulbecco’s modified Eagle’s medium. Following this incubation the cell medium was replaced with serum-free medium containing either 500 ng/ml LPA (Sigma), 250 nM phorbol myristate acetate (PMA) (Sigma), 0.5 mM sodium vanadate (Sigma), or medium alone for 30 (for the FN-null cells) or 60 min (for the syndecan-4-WT and syndecan-4-null cells). All experiments were done on cultures that were 50% confluent.

Transient Transfection—Syndecan-4-null fibroblasts were transiently transfected with the rat syndecan-4 cDNA cloned in pcDNA3.1 Hygro (Invitrogen) or pcDNA3.1 Hygro alone using LipofectAMINE 2000 (Invitrogen) according to the manufacturer’s protocol. Detection of protein expression was ascertained by Western blotting. Proteins containing either 500 ng/ml LPA (Sigma), 250 nM phorbol myristate acetate (PMA) (Sigma), 0.5 mM sodium vanadate (Sigma), or medium alone for 30 (for the FN-null cells) or 60 min (for the syndecan-4-WT and syndecan-4-null cells). All experiments were done on cultures that were 50% confluent.

Cell Harvesting and Western Blotting—Fibroblasts were placed on ice and washed twice with cold PBS containing calcium chloride and magnesium chloride and 1 mM sodium vanadate. The cells were lysed with an extraction buffer containing 25 mM β-glycerolphosphate (pH 7.3), 10 mM EDTA, 2 mM EGTA, 0.1 mM NaCl, 1% Triton X-100, 10 mM β-mercaptoethanol, 0.2 mM sodium vanadate, 1 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 1 μg/ml pepstatin A, and 1 μg/ml aprotinin (63). The lysates were centrifuged at 14,000 rpm for 20 min (4 °C) to remove insoluble material and protein concentration was determined. Equal protein concentrations were resolved on 12% SDS-PAGE and transferred electrophoretically to polyvinylidene difluoride membranes. The membranes were blocked with 5% bovine serum albumin for 2 h at room temperature and incubated with primary antibodies diluted in 1% bovine serum albumin overnight at 4 °C. The monoclonal phosphotyrosine antibody directly conjugated to horseradish peroxidase (clone PY20) was purchased from BD Transduction Laboratories (Lexington, KY). The polyclonal FAK and phosphospecific FAK Tyr397 antibodies were obtained from Upstate Biotechnology Inc. (Lake Placid, NY) and the α-actinin antibody was purchased from Sigma. After washing the blots, secondary antibodies conjugated to horseradish peroxidase were added for 60 min at room temperature. The membranes were subsequently washed again and detection of signal was obtained using the West Pico enhanced chemiluminescent kit according to the manufacturer’s instructions (Pierce). Figures are representative results of experiments that were performed at least three times.

Immunofluorescence Assay—Syndecan-4 WT and syndecan-4 null cells were seeded on glass coverslips at a concentration of 9,000 cells/well of a 24-well plate for 3 h in serum-free medium and then incubated for an additional 60 min in the presence or absence of 500 ng/ml LPA or 250 nM PMA or an additional 2 h in the presence or absence of 15 μg/ml C3 exotoxerase (List Biological Laboratories, Campbell, CA). The
Syndecan-4 Modulates FAK Phosphorylation

**RESULTS**

Cell Adhesion to Fibronectin Increases Phosphorylation of FAK Tyr<sup>397</sup>—Localization of syndecan-4 to focal adhesion sites is a late event in cell spreading. Significant levels of focal adhesion-associated syndecan-4 are not apparent until 2 h post-seeding but levels increase steadily thereafter (18). To address the contribution of syndecan-4 signaling to FAK Tyr<sup>397</sup> phosphorylation, FN-null fibroblasts were seeded onto FN or CBD for 3 h prior to treatment with either activators or inhibitors. FN-null cells were used so as to eliminate any contamination by cell-associated FN and because they have been previously shown to generate focal adhesions and actin stress fibers in response to syndecan-4 signaling (24). Initially whole cell lysates were analyzed for FAK protein expression and general tyrosine phosphorylation. As shown in Fig. 1A, although the relative levels of FAK protein remained constant, FN-null cells exhibited a lower tyrosine phosphorylation level when adherent to the CBD than when adherent to full-length FN as has been shown previously (60). To determine whether the difference in phosphorytrosine levels under the two adhesion conditions included the FAK autophosphorylation site, a phosphospecific antibody against FAK phosphorylated at Tyr<sup>397</sup> was used. Analysis of the FAK autophosphorylation site domain revealed that the phosphorylation of Tyr<sup>397</sup> was significantly lower in cells attached to the CBD compared with the full-length molecule. Reduced phosphorylation under CBD-adherent conditions could be because of either decreased FAK autophosphorylation or increased activity of an endogenous tyrosine phosphatase. To discern between these two possibilities, FN-null cells seeded on the CBD were treated with the tyrosine phosphatase inhibitor, sodium vanadate. Phosphorylation of Tyr<sup>397</sup> in CBD-adherent fibroblasts increased under these conditions to a level comparable with vanadate-treated FN-adherent fibroblasts (Fig. 1A). General tyrosine phosphorylation of FAK also increased in FN and CBD-adherent cells in the presence of vanadate (Fig. 1A). These data indicate that cell adhesion to full-length FN, compared with CBD, leads to an accumulation of FAK phosphorylated on Tyr<sup>397</sup>. The results suggest that a signal arising from a non-CBD part of the FN molecule is involved in this process. The data also suggest that a tyrosine phosphatase may be involved in maintaining a low phosphorylation state of FAK Tyr<sup>397</sup> in cells seeded on the CBD.

**Protein Kinase C Activity Is Not Involved in Regulating Phosphorylation of FAK Tyr<sup>397</sup>**—Protein kinase C activation precedes cell spreading on FN and its stimulation has been shown to increase FN-mediated cell adhesion (66, 67). Direct activation of PKC by the phorbol ester PMA has been shown to stimulate focal adhesion formation (68) and FAK tyrosine phosphorylation (69). Recruitment of syndecan-4 to focal adhesions is dependent on PKC (18), and syndecan-4, in turn, modulates PKC localization and activity through the binding intermediate phosphatidylinositol bisphosphate (11, 12). As syndecan-4 has no intrinsic catalytic activity, interactions with PKC may serve to initiate syndecan-4-directed signaling events. FN-null cells were seeded on FN or the CBD and subsequently treated...
with the phorbol ester PMA to induce PKC activation. Tyrosine phosphorylation increased after incubation of both FN- and CBD-adherent cells with PMA but this amplification did not translate to increased FAK Tyr397 phosphorylation under either condition (Fig. 1B). The Me2SO control had no effect. These results demonstrate that FAK Tyr397 is not phosphorylated in response to PKC activation and confirm that PKC activation increases overall tyrosine phosphorylation.

**Rho Activation Stimulates Phosphorylation of FAK Tyr397**

Focal adhesion formation is a Rho-dependent process (70) and syndecan-4-mediated focal adhesion formation is inhibited by treatment with C3 exotransferase, which ADP-ribosylates and inactivates Rho (24). Addition of LPA, which activates Rho, or constitutively active Rho into serum-starved Swiss 3T3 cells stimulates FAK localization to focal adhesions and also increases FAK tyrosine phosphorylation levels (55, 56). To determine whether Rho activation was involved in FAK Tyr397 phosphorylation, FN-null fibroblasts were incubated with LPA following seeding onto FN or CBD substrates. In the absence of Rho stimulation general tyrosine phosphorylation and FAK Tyr397 phosphorylation were reduced in CBD-adherent cells compared with the syndecan-4 WT controls. The level of phosphorylation of Tyr397 is lower in syndecan-4-null cells compared with the syndecan-4-WT controls. The level of Tyr397 phosphorylation is not as low as that of FN-null cells seeded on a FN substrate. They also, however, confirmed that syndecan-4 can stimulate focal adhesion formation by showing that only wild type cells containing syndecan-4 form focal adhesions when seeded solely on CBD and subsequently incubated with either the heparin-binding domain of FN or a syndecan-4 antibody (25). Fibroblasts isolated from syndecan-4-null mice show reduced migration compared with wild type controls (26) and overexpression of full-length syndecan-4 core protein as well as core proteins that contain deletions in the cytoplasmic region also decrease the rate of migration of CHO-K1 cells (71).

Like the heparan sulfate chains of syndecan-4, those from the glycosylphosphatidylinositol-linked proteoglycan glypican-1 have been shown to ligate the Hep-II domain of fibronectin (61). To determine whether the FAK Tyr397 phosphorylation response seen in the FN-adherent cells is dependent on the presence of syndecan-4 and not another cell-surface heparan sulfate proteoglycan, syndecan-4-null fibroblasts (26) were seeded on full-length FN and analyzed for phosphorylation of FAK Tyr397. The results depicted in Fig. 2A show that the level of phosphorylation of Tyr397 is lower in syndecan-4-null cells compared with the syndecan-4-WT controls. The level of Tyr397 phosphorylation is not as low as that of FN-null cells seeded on CBD and these results are consistent with the observation that fibroblasts that lack syndecan-4 still spread, form focal adhesions and stress fibers, and are motile (25, 26). LPA treatment, to activate the Rho GTPase, increased Tyr397 phosphorylation to that of control syndecan-4-WT cells (Fig. 2A).

Cell-surface expression of syndecan-4 is considered ubiqui-
Syndecan-4 Modulates FAK Phosphorylation

**Fig. 4.** Syndecan-4-null and C3 exotransferase-treated syndecan-4-WT cells do not localize FAK phosphorylated on Tyr^397 to focal adhesions. Syndecan-4-WT (A–F) and syndecan-4-null (G–J) fibroblasts were seeded on FN, then fixed and stained with antibodies to vinculin (B, D, F, H, and J), a marker of focal adhesions, and phosphospecific FAK Tyr^397 (A, C, E, G, and I) antibodies. Arrows indicate co-localization of FAK phosphorylated on Tyr^397 in vinculin-containing adhesion sites of WT cells in the absence or presence of LPA (A–B and C–D) and in syndecan-4-null cells treated with LPA (I–J), whereas arrowheads indicate the lack of phosphorylated FAK Tyr^397 in the focal adhesions in syndecan-4-null cells (Fig. 1A–I). Double arrowheads indicate the absence of FAK phosphorylated at Tyr^397 in the focal contacts of syndecan-4-WT cells treated with C3 exotransferase (E–F) to inactivate Rho.

**Fig. 5.** Rho activation is diminished in the absence of syndecan-4. Syndecan-4-WT and syndecan-4-null fibroblast lysates were treated with LPA and incubated with beads conjugated to the Rho-binding domain of Rhotekin. Beads were washed and then electrophoresed onto SDS-PAGE and blotted for Rho (Rho:GTP). Equal amounts of lysates were also electrophoresed and blotted for total Rho and actin as loading controls.

The localization of FAK phosphorylated on Tyr^397 to focal adhesion sites or helps to maintain the autophosphorylated form of FAK in focal adhesions in a Rho-dependent manner.

Rho Activity Is Lower in Syndecan-4-null Cells—The small GTPase Rho cycles between an active GTP-bound state and an inactive GDP-bound state (72–74). Rho activity is regulated through its ability to bind GTP. Guanine-nucleotide exchange factors catalyze the exchange of GDP for GTP, whereas GTPase-activating proteins enhance the endogenous Rho GTPase activity (72, 73). The diminished FAK Tyr^397 phosphorylation in syndecan-4-null cells might be the result of reduced Rho activity. To ascertain whether the level of active Rho is affected by the lack of syndecan-4 expression, syndecan-4-null and syndecan-4-WT fibroblasts were plated onto FN, treated with LPA, and Rho activity was determined using a pull-down assay with the Rho-binding domain of Rhotekin (31, 75) that selectively binds GTP-bound Rho (65, 75).

The Rho activity level of syndecan-4-null fibroblasts, as depicted in Fig. 5, is significantly lower than the activity level of...
Syndecan-4 Modulates FAK Phosphorylation

DISCUSSION

The heparan sulfate proteoglycan syndecan-4 has two main cellular functions. It acts as a co-receptor for heparin-binding growth factors (such as the family of fibroblast growth factors and heparin-binding vascular endothelial growth factor isofoms) regulating the ligand-dependent activation of the primary receptor (3). Syndecan-4 also functions, in a Rho-dependent manner, with the α5β1 integrin to promote the adhesion-dependent formation of actin stress fibers and focal adhesions (24). Syndecan-4-null fibroblasts will form focal adhesions and actin stress fibers when plated on a FN substrate (25, 26). However, the cells are unable to generate focal adhesions when seeded on the CBD of FN and incubated with either syndecan-4 antibodies or the heparin-binding domain of FN (25). Therefore, although a compensatory cellular mechanism exists to bypass syndecan-4 deficiency, it is possible to solely evaluate the syndecan-4 signaling pathway.

Transfection of full-length syndecan-4 core protein enhances cell spreading and focal adhesion formation and decreases cell migration (71, 76). Interestingly, transfection of cells with syndecan-4 constructs that lack cytoplasmic domains also exhibit decreased cell migration although they do not form stress fibers or extensive focal adhesions when seeded on a FN or vitronectin substrate (71). Fibroblasts generated from mice lacking syndecan-4 also show migration delays compared with wild type controls (26). The impaired migration in syndecan-4-null cells may result from the inability of the adhesions to generate enough tension required for migration or from the inability to disengage established contacts so that new adhesions may form (29). Alternatively, although not mutually exclusive from the former, syndecan-4 may regulate components of a signaling pathway involved in cell migration and the absence of syndecan-4 disrupts the efficiency with which the pathway acts.

It has previously been shown that integrin ligation to the CBD of FN does not induce tyrosine phosphorylation of FAK to the same level as cells that are ligated to full-length FN (60). We analyzed the autophosphorylated form of FAK and now demonstrate that FAK Tyr 397 phosphorylation is lower in the absence of the heparin-binding domain of FN. Incubation of CBD-adherent FN-null cells with the tyrosine phosphatase inhibitor sodium vanadate augmented the phosphorylation levels of FAK Tyr 397 to that of vanadate-treated cells seeded on full-length FN suggesting that syndecan-4 may influence FAK Tyr 397 phosphorylation by regulating the activity of a cellular tyrosine phosphatase. Candidates for such tyrosine phosphatases are: PTEN (77), PTP-PEST (78), and Shp-2 (79), which have been shown to dephosphorylate FAK in vitro. Incubation of our cells with the Shp-2 inhibitor calpeptin did not enhance phosphorylation of FAK Tyr 397 (data not shown) suggesting that under our conditions Shp-2 is not involved in modulating the phosphorylation state of FAK Tyr 397.

Syndecan-4-regulated focal adhesion formation is primarily associated with two signaling molecules: the serine/threonine kinase PKC and the small GTPase Rho. Fibroblasts seeded on CBD can be stimulated to generate actin stress fibers and focal adhesions by incubating the cells with either PMA (to directly activate PKC) (68) or LPA (to directly stimulate Rho) (24). It is unclear whether these signaling molecules collaborate in one signaling pathway or if they work in parallel but separate pathways. Our study shows that increased phosphorylation of FAK Tyr 397 is associated with the activation of the Rho pathway and not the PKC pathway, as only LPA and not PMA increased Tyr 397 phosphorylation in our conditions. Similarly, LPA, but not PMA, treatment also induced the localization of FAK phosphorylated on Tyr 397 to vinculin-containing focal adhesions in syndecan-4-null cells plated on FN. Therefore, our data indicate that syndecan-4-mediated FAK phosphorylation occurs only through a Rho-mediated process and not through a collaboration of both Rho and PKC stimulation.

Fig. 6. Model of syndecan-4 signaling influencing FAK phosphorylation of Tyr 397. A, ligation of both integrin and syndecan-4 (syndecan-4-WT cells on FN and FN-null cells on FN) lead to increased levels of active Rho and diminished activity of a tyrosine phosphatase. This shifts the equilibrium of FAK Tyr 397 to the phosphorylated state. B, in the absence of syndecan-4 ligation (syndecan-4-null cells on FN) Rho is not activated. FAK is phosphorylated on Tyr 397 but is subsequently dephosphorylated through the action of a tyrosine phosphatase. Under these conditions FAK potentially would be cycling between the phosphorylated and dephosphorylated state more rapidly than in the situation described in A. This scenario is also valid for FN-null cells seeded on CBD.
PKC activation augmented general tyrosine phosphorylation in CBD-adherent cells but the stimulation did not translate into increased phosphorylation of FAK Tyr^{397}. The lack of effect with PMA treatment does not imply that a syndecan-4-PKC pathway does not exist. Indeed, syndecan-4 and PKC have a close functional association. PKC recruits syndecan-4 to focal adhesion sites (18) and, conversely, syndecan-4 binds PKC through the intermediary phosphatidylinositol bisphosphate (11, 80) and potentiates its activity (12, 81).

It is possible that PKC promotes focal adhesion formation through an alternative cell-surface heparan sulfate proteoglycan. Syndecan-4-null cells will generate actin stress fibers and focal adhesions when seeded on a combination substrate of CBD and heparin-binding domain FN fragments, and this effect can be inhibited with the addition of heparin to the cell medium (25). These data indicate that another cell-surface heparan sulfate proteoglycan can compensate for the lack of syndecan-4 to promote focal adhesion and stress fiber formation.

Alternatively, the PKC pathway may be activated following the association of syndecan-4 with a heparin-binding growth factor receptor. Growth factor ligation (such as fibroblast growth factor-2) may promote the association of syndecan-4 with PKC, leading to its activation and the subsequent formation of focal adhesions and actin stress fibers. Syndecan-4 acts as a co-receptor for several heparin-binding growth factors, regulating the ligand-dependent activation of the primary receptors (3). The cytoplasmic domain of syndecan-4 has been implicated in promoting fibroblast growth factor 2-dependent cell proliferation and migration (82) and fibroblast growth factor-2 receptor. Growth factor ligation (such as fibroblast growth factor-2) may promote the association of syndecan-4 with PKC, leading to its activation and the subsequent formation of focal adhesions and actin stress fibers. Syndecan-4 acts as a co-receptor for several heparin-binding growth factors, regulating the ligand-dependent activation of the primary receptors (3). The cytoplasmic domain of syndecan-4 has been implicated in promoting fibroblast growth factor 2-dependent cell proliferation and migration (82) and fibroblast growth factor-2 regulates the syndecan-4 dependent activation of PKC (11, 83).

To demonstrate conclusively that the signaling mechanism affecting the phosphorylation of FAK Tyr^{397} acts specifically through syndecan-4, syndecan-4-null fibroblasts were used. The cells were seeded on a FN substrate as they have been shown to develop focal adhesions under these conditions. The lack of syndecan-4 expression in these cells dictates that adhesion to a FN substrate will not stimulate a syndecan-4 signaling pathway but will activate the unknown complementary pathway, if it is involved (25). Our experiments reveal that there is less FAK phosphorylated on Tyr^{397} in syndecan-4-null fibroblasts than wild type fibroblasts under basal conditions and this can be rescued through re-expression of syndecan-4, demonstrating that syndecan-4 is directly involved in influencing FAK Tyr^{397} phosphorylation. The lack of FAK phosphorylated on Tyr^{397} in the syndecan-4-null cells was not because of an inability of Tyr^{397} to be phosphorylated, as treatment with LPA or sodium vanadate (data not shown) increased Tyr^{397} phosphorylation to syndecan-4-WT control levels, but was attributable to a decrease in the levels of active Rho in the cells. Correspondingly, direct inactivation of Rho using C3 exotransferase resulted in the loss of FAK phosphorylated at Tyr^{397} in the focal adhesions of syndecan-4-WT cells. The syndecan-4-null fibroblasts still generate vinculin-containing focal complexes so the limited level of GDP-bound Rho present in the null cells is sufficient to generate stress fibers and focal adhesions. Incubation of syndecan-4-null fibroblasts with LPA augmented the level of active Rho, indicating that the molecule is capable of functioning appropriately but is either not activated to the same degree as in wild type cells or is unable to maintain the active state for the “normal” length of time. Rho activation has been shown to increase FAK phosphorylation (58) and cell motility (84), whereas inhibition of Rho attenuates both (85, 86). Although studies have demonstrated that FAK can inhibit Rho activity, this attenuation occurs during early cell spreading (within 40 min of cell plating) (31). The cells in our experiments were adherent for 4 h during which Rho activity is higher (75). This is the first description, to our knowledge, that the heparan sulfate proteoglycan syndecan-4 influences the level of active Rho in cells.

Rho cycles between an active GTP-bound state and an inactive GDP-bound state. While active it can bind to its effector molecules: Dia, ROCKs (Rho kinases), and phosphatidylinositol-4-phosphate 5-kinase to induce actin polymerization, cell body contraction, and stress fiber and focal adhesion formation (73, 87). Rho also stimulates cell motility (88–90) and this may be a function separate to that of forming focal adhesions and stress fibers (91, 92). Phosphorylation of FAK at Tyr^{397} is necessary for optimal cell migration (34, 36, 93). Phosphorylation of FAK on Tyr^{397} generates a binding site for SH2-containing proteins. Some of these binding partners (phosphatidylinositol-3-kinase, Grb7, and Src) increase cell migration (35, 42, 44, 94, 95) and it may be that FAK serves to enhance the association of these proteins with their downstream effectors by binding them within the focal adhesion (95). Indeed, recent studies have linked Rho activity with the targeting of Src to focal adhesion sites (96).

As syndecan-4-null cells are deficient in active Rho and phosphorylated FAK Tyr^{397} and show impaired cell migration (26) it seems likely that syndecan-4 signaling is associated with cell migration. Decreased cell migration is associated with either lack of syndecan-4 expression or by overexpressing full-length syndecan-4 or syndecan-4 containing cytoplasmic deletion mutants (26, 71). That both ends of the spectrum (overexpression of syndecan-4 and lack of syndecan-4) inhibit cell migration suggests that a homeostasis is generated through syndecan-4 signaling and a balance may be required for optimal cell migration. We hypothesize that under conditions in which syndecan-4 is not ligated (FN-null cells seeded on the CBD or syndecan-4-null cells seeded on full-length FN) integrin ligation induces the phosphorylation of FAK Tyr^{397}. Tyrosine phosphatase activity leads to the subsequent dephosphorylation of this tyrosine residue. The levels of GTP-bound Rho are also low. This situation is most likely not a static event but probably encourages the cycling of FAK between a phosphorylated and nonphosphorylated state on Tyr^{397}. Ligation of syndecan-4 (whether in FN-null or syndecan-4-WT cells plated on full-length FN) results in the increased activation of Rho. This attenuates the tyrosine phosphatase activity causing FAK Tyr^{397} to remain phosphorylated longer. The tyrosine phosphatase activity is not completely inhibited, therefore, cycling between phosphorylation and dephosphorylation of Tyr^{397} probably occurs but under a different kinetic. This is pictured diagrammatically in Fig. 6.

Acknowledgments—We thank Hui Su for confocal assistance and Stefania Saoncella, Tokuro Iwabuchi, and Enzo Cala"ultti for helpful discussions.

REFERENCES

1. Woods, A. (2001) J. Clin. Invest. 107, 935–941
2. Woods, A., Oh, E. S., and Couchman, J. R. (1998) Matrix Biol. 17, 477–483
3. Carey, D. J. (1997) Biochem. J. 327, 1–16
4. Bernfield, M., Grote, M., Park, P. W., Reizes, O., Fitzgerald, M. L., Lincecum, J., and Zako, M. (1999) Annu. Rev. Biochem. 68, 729–777
5. Elnius, K., and Jalkanen, M. (1984) J. Cell Sci. 77, 2975–2982
6. Ethell, J. M., Hagiwara, K., Miura, I., Irie, F., and Yamaguchi, Y. (2000) J. Cell Biol. 151, 53–68
7. Hsueh, Y.-P., and Sheng, M. (1999) J. Neurosci. 19, 7415–7425
8. Gao, Y., Li, M., Chen, W., and Simons, M. (2000) J. Cell. Physiol. 184, 373–379
9. Grootjans, J. J., Zimmermann, P., Reekmans, G., Smets, A., Degeest, G., Darr, J., and David, G. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 13683–13688
10. Cohen, D. J., Woods, D. F., Marfatia, S. M., Walther, Z., Chishiti, A. H., Anderson, J. M., and Wood, D. F. (1998) J. Cell Biol. 142, 129–138
Syndecan-4 Modulates FAK Phosphorylation

50. Schlaepfer, D. D., Hanks, S. K., Hunter, T., and van der Geer, P. (1994) Nature 372, 786–791
51. Schlaepfer, D. D., and Hunter, T. (1998) Mol. Cell. Biol. 18, 5623–5633
52. Kimani, M., Morii, N., Fujisawa, K., Yotsumara, T., Nakao, K., and Narumiya, S. (1993) FEBS Lett. 329, 273–276
53. Chhrzanowska-Wodnicka, M., and Burridge, K. (1994) J. Cell Sci. 107, 3643–3654
54. Ridley, A. J., and Hall, A. (1994) EMBO J. 13, 3600–3610
55. Barry, S. T., and Critchley, D. R. (1994) J. Cell Sci. 107, 2033–2045
56. Flinn, H. M., and Ridley, A. J. (1996) J. Cell Sci. 109, 1133–1141
57. Rodriguez-Fernandez, J. L., and Rozengurt, E. (1998) J. Biol. Chem. 273, 19123–19126
58. Clark, E. A., King, W. G., Brugge, J. S., Symons, M., and Hynes, R. O. (1998) J. Cell Biol. 142, 573–586
59. Burridge, K., Turner, C. E., and Rimmer, L. H. (1992) J. Cell Biol. 119, 893–903
60. Jeong, J., Jun, I., Lim, Y., Kim, J., Park, I., Woods, A., Couchman, J. R., and Oh, E. S. (2001) Biochem. J. 356, 531–537
61. Tsumura, S., Woods, A., and Couchman, J. R. (2000) J. Biol. Chem. 275, 9410–9417
62. Schlaepfer, A. C., and Ott, V. L. (1998) J. Biol. Chem. 273, 35291–35298
63. Xu, F., and Zhao, Z. J. (2001) Exp. Cell Res. 262, 49–58
64. Small, J. V., and Celis, J. E. (1978) J. Cell Sci. 31, 393–409
65. Reid, T., Furuysuchi, T., Ishizaki, T., Watanabe, G., Watanabe, N., Fujisawa, K., Morii, N., Madura, P., and Narumiya, S. (1996) J. Biol. Chem. 271, 13556–13560
66. Vuori, K., and Ruoslahti, E. (1993) J. Biol. Chem. 268, 21459–21462
67. Brown, P. J. (1988) Biochem. Biophys. Res. Commun. 155, 603–607
68. Woods, A., and Courchman, J. R. (1992) J. Cell Sci. 101, 277–290
69. Bruce-Staskal, P. J., and Boush, A. H. (2001) Exp. Cell Res. 264, 296–306
70. Ridley, A. J., and Hall, A. (1992) Cell 76, 389–399
71. Longley, R. L., woods, A., Fleetwood, A. J., Gallagher, J. T., and Couchman, J. R. (1999) J. Cell Sci. 112, 3421–3431
72. Zohn, I. M., Campbell, S. L., Kishore-Far, F., Rossman, K. L., and Der, C. J. (1998) Oncogene 17, 1415–1438
73. Ridley, A. J. (2001) J. Cell Sci. 114, 2713–2722
74. Mackay, D. J., and Hall, A. (1998) J. Biol. Chem. 273, 20685–20688
75. Ren, X.-D., Kosses, W. B., and Schwartz, M. A. (1999) EMBO J. 18, 578–585
76. Echtermeyer, P., Bucic, P. C., Saoncella, S., Ge, Y., and Goetinck, P. F. (1995) J. Biol. Chem. 270, 29812–29819
77. Angers-Loustau, A., Cote, J.-F., Charest, A., Dobken, D., Spencer, S., Lasky, L. A., and Tremblay, M. L. (1999) J. Cell Biol. 144, 1019–1031
78. Mantes, S., Mira, E., Gomez-Mouton, C., Zhao, Z. J., Laluce, R. A., and Martinez, A. C. (1999) Mol. Biol. Cell. 19, 3125–3135
79. Oh, E. S., Woods, A., Lim, S. T., Theibert, A. W., and Couchman, J. R. (1998) J. Biol. Chem. 273, 10624–10629
80. Oh, E. S., Woods, A., and Couchman, J. R. (1997) J. Biol. Chem. 272, 11085–11101
81. Volk, R., Schwartz, J. J., Li, J., Rosenberg, R. D., and Simons, M. (1999) J. Biol. Chem. 274, 24417–24424
82. Horwitz, A., and Simons, M. (1998) J. Biol. Chem. 273, 10914–10918
83. Matsumoto, Y., Tanaka, K., Harimaya, K., Nakatani, F., Matsuda, S., and Iwamoto, Y. (2001) Jpn. J. Cancer Res. 92, 429–438
84. Bobak, D., Moorman, J., Guanzon, A., Gilmer, L., and Hahn, C. (1997) Oncogene 15, 2179–2189
85. Imamura, F., Mukai, M., Ayaki, M., and Akedo, H. (2000) Jpn. J. Cancer Res. 91, 811–816
86. Geiger, B., and Bershadsky, A. (2001) Curr. Opin. Cell Biol. 13, 584–592
87. Yoshikawa, K., Matsumura, F., Akedo, H., and Itoh, K. (1998) J. Biol. Chem. 273, 5146–5154
88. Takashi, K., Kikuchi, A., Kuroda, S., Kotasai, K., Sasaki, T., and Takai, Y. (1993) Mol. Cell. Biol. 13, 72–79
89. Staas, J. M., Joura, A., Bournemeyer, N., Bocquet, P., and Vignais, P. V. (1991) Biochem. Biophys. Res. Commun. 186, 615–622
90. Nabi, I. R. (1999) J. Cell Sci. 112, 1803–1811
91. Machesky, L. M., and Hall, A. (1997) J. Cell Biol. 138, 913–926
92. Wang, H. B., Dembo, M., Hanks, S. K., and Wang, Y. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 11295–11300
93. Reiske, H. R., Kao, S. C., Cary, L. A., Guan, J. L., Lai, J. F., and Chen, H. C. (1999) J. Biol. Chem. 274, 12361–12366
94. Shen, T. L., and Guan, J. L. (2001) FEBS Lett. 499, 176–181
95. Timpone, P., Jones, G. E., Frame, M. C., and Brunton, V. G. (2001) Curr. Biol. 11, 1836–1846