ADAM12/Syndecan-4 Signaling Promotes β1 Integrin-dependent Cell Spreading through Protein Kinase Ca and RhoA*

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The ADAMs (a disintegrin and metalloprotease) comprise a large family of multidomain proteins with cell-binding and metalloprotease activities. The ADAM12 cysteine-rich domain (rADAM12-cys) supports cell attachment using syndecan-4 as a primary cell surface receptor that subsequently triggers β1 integrin-dependent cell spreading, stress fiber assembly, and focal adhesion formation. This process contrasts with cell adhesion on fibronectin, which is integrin-initiated but syndecan-4-dependent. In the present study, we investigated ADAM12/syndecan-4 signaling leading to cell spreading and stress fiber formation. We demonstrate that syndecan-4, when present in significant amounts, promotes β1 integrin-dependent cell spreading and stress fiber formation in response to rADAM12-cys. A mutant form of syndecan-4 deficient in protein kinase C (PKC)α activation or a different member of the syndecan family, syndecan-2, was unable to promote cell spreading. GF109203X and G60976, inhibitors of PKC, completely inhibited ADAM12/syndecan-4-induced cell spreading. Expression of syndecan-4, but not synd4A1, resulted in the accumulation of activated β1 integrins at the cell periphery in Chinese hamster ovary (CHO) cells as revealed by 12G10 staining. Further, expression of myristoylated, constitutively active PKCα resulted in β1 integrin-dependent cell spreading, but additional activation of RhoA was required to induce stress fiber formation. In summary, these data provide novel insights into syndecan-4 signaling. Syndecan-4 can promote cell spreading in a β1 integrin-dependent fashion through PKCα and RhoA, and PKCα and RhoA likely function in separate pathways.

The extracellular environment profoundly influences cell shape. Following an initial cell attachment event, cells may or may not spread depending on cell type and the nature of the molecular signal they receive. Cell spreading is a fundamental cellular process required for cell migration, survival, proliferation, and differentiation (1). Cell spreading on the extracellular matrix requires reorganization of the actin cytoskeleton and activation of integrins (2), resulting in stable adhesion through formation of stress fibers and focal adhesions. Several signaling proteins, including PKC, phosphatidylinositol 3-kinase and R-Ras, have been shown to regulate cell spreading in different cell types (3–6).

ADAMs (a disintegrin and metalloprotease) constitute a recently characterized family of metalloproteases that also mediates cell adhesion. The prototype ADAM is a multidomain protein composed of pro-, metalloprotease, disintegrin-like, cysteine-rich, epidermal growth-factor-like repeat, transmembrane, and cytoplasmic tail domains (7–9). The disintegrin domain of several different ADAMs including ADAM 2, 9, 12, 15, and 23 has been shown to support cell attachment (10–14). We have previously demonstrated that cell attachment of several different cell lines and primary muscle cell cultures to rADAM12-cys (recombinant ADAM12 cysteine-rich domain) is mediated primarily through α5β1 integrin in an RGD-independent manner but cell spreading does not occur (11). The cysteine-rich domain of ADAM12 also possesses important cell binding activities (16, 17). We have previously demonstrated that cell attachment of several different cell lines and primary muscle cell cultures to rADAM12-cys (recombinant ADAM12 cysteine-rich domain) is mediated through syndecans, specifically syndecan-4. Notably, mesenchymal cells attach, spread, and form stress fibers and focal adhesions upon attaching to rADAM12-cys (17). Carcinoma cells also attach through syndecans, but only form cellular actin-containing projections rather than being spread fully. Cell spreading is only obtained upon further integrin activation by the addition of either Mn2+ or activating monoclonal antibodies (16, 17). The importance of the cysteine-rich domain in cellular interactions in vivo was recently underscored by the studies of Gaultier et al. (18), which demonstrated the importance of the interaction of ADAM13 disintegrin-cysteine rich domains with extracellular...
matrix proteins like fibronectin. The molecular mechanisms of the downstream events following binding of ADAMs and syndecan-4, however, are completely unknown.

Syndecan-4 is a member of a family of four (syndecans 1–4) transmembrane heparan sulfate proteoglycans (19–22). The core protein of syndecans is characterized by divergent extracellular domains and highly conserved cytoplasmic tails that contain two constant regions (C1 and C2) separated by a variable region (V) unique to each family member (23). Specifically, the V-region of syndecan-4 contains a unique seven-residue binding site for phosphatidylinositol 4,5-bisphosphate (PIP2) involved in PKC activation (23–25, 66) and multimerization (23, 26), which is critical for cell spreading and cytoskeletal reorganization (27). Syndecan-4 is a widespread component of focal adhesions and appears to be a co-receptor in cell adhesion to many extracellular matrix ligands, modifying the integrin-mediated responses (23, 28, 29). Syndecan-4 has been implicated in the pathogenesis of numerous diseases. Syndecan-4 levels were shown to be up-regulated in fibroblasts and endothelial cells during wound repair (30). Delayed wound repair and impaired angiogenesis were demonstrated in syndecan-4-deficient mice (31). Syndecan-4 was up-regulated in proliferative renal disease (32) and mice deficient in syndecan-4 were more susceptible to α-carageenan induced renal damage (33) indicating that syndecan-4 plays an important role in renal diseases. Most of these studies suggest that syndecan-4 plays a role in cell adhesion to extracellular matrix substrates and in the regulation of cell migration, but the mechanism of syndecan-4 signaling during cell adhesion, spreading, and migration is not clearly understood.

In the present study, we determined how cell spreading is regulated by ADAM12/syndecan-4 signaling. Our data indicate a critical role for syndecan-4 as a primary receptor for rADAM12-cys and demonstrate that ADAM12/syndecan-4 regulates cell spreading in a β1 integrin-dependent manner through PKCα and RhoA, which function in separate pathways.

MATERIALS AND METHODS

Cell Lines—The cell lines used in this study were: CHO-K1 (CCL-61 ATCC), CHO-pgsD677 (CRL-2244 ATCC), and CHO cells stably transfected with full-length rat syndecan-4 (S4) (27), a mutant form of syndecan-4 terminated at isoleucine 191 in the center of the V (variable) region of the cytoplasmic tail (S4ΔI) (27) or with human β1 integrin (CHOβ1) (34), RKO colon carcinoma (35), and MG-63 osteosarcoma (CRL-1427 ATCC). CHO cells were grown in Dulbecco’s modified Eagle’s medium (DMEM/F12 medium and the other cell lines were grown in DMEM, supplemented with glutamax I and 4,500 mg/liter glucose and 10% fetal bovine serum. Stably transfected cell lines were cultured in serum-free medium, according to the manufacturer s modified Ea-

FIG. 1. CHO-K1 cell attachment and spreading on ADAM12 and fibronectin. CHO-K1 (A, B, D, E, and F) or CHOpgsD-677 (C) were plated on rADAM12-cys (B–D) or fibronectin (A), and cell attachment was examined. E, effect of heparin (10 μg/ml) and suramin (10 μM) on CHO cell attachment (control) to rADAM12-cys (white bar) and fibronectin (black bar). F, cell area for CHO cells plated on fibronectin (FN) or on rADAM12-cys alone (A12) and treated with 1 mM Mn2+ (A12 + Mn2+).

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cDNAs and Transfections—A bicistronic vector (pIRES2-EGFP) containing rat full-length syndecan-4 (20) was kindly provided by Dr. A. Horowitz and E. Tkachenko (Harvard Medical School, Boston, MA). cDNAs encoding full-length rat syndecan-2 and a mutant form of syndecan-4 terminated at isoleucine 191 in the center of the V (variable) region of the cytoplasmic tail (syn4ΔI) cloned into the pcDNA3 were described previously (27). Another mutant form of syndecan-4, syn4ΔEE, truncated at glutamate 199, removing the C-terminal FYA motif that binds PDZ (postsynaptic density 95, disk large, zona occludens-1) proteins, was constructed essentially as described previously (27). The eukaryotic expression vector for Myr-PKCαEGFP was described previously (38). The cDNA for PKCα MyrI/II/SII fragment from full-length PKCα in the pEGFP-N1 vector (39) was subcloned into the same Myr-EGFP vector. The cDNAs for RhoGTPases, L63RhoA, and N19RhoA were kindly provided by Dr. Alan Hall (University College, London, UK). Except for the pEGFP-bicistronic constructs, the pEGFP-N1 vector was co-transfected with the signaling constructs in order to visualize transfected cells. Transient transfections were performed using LipofectAMINE plus reagent (Invitrogen) and 3–10 μg of plasmid DNA/ml in serum-free medium, according to the manufacturer’s protocol. For the transfection of MG-63 cells, FuGENE 6 transfection reagent (Roche Molecular Biochemicals, Hvidovre, Denmark) was used.
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**Cell Attachment Assays**—The cell attachment assay was performed as described previously (16, 17). Briefly, BNL-ImmunoMAX® 96-well plates with MaxiSorp™ surface (Nunc AS) or bacterial culture dishes (35 × 10 mm; Corning Incorporate Ltd., Corning, NY) were coated with 20 μg/ml of rADAM12-cys or fibronectin (10 μg/ml) in 0.1 M NaHCO₃ buffer, pH 9.5, overnight at 4 °C. In some experiments, Mn²⁺ (1 mM), heparin (10 μg/ml), or suramin (10 μM) was added, or cells were pre-treated for 15 min with PKC inhibitors: 10 μM GF109203X or 2 μM Go6976 (Calbiochem-Novabiochem GmbH, Bad SodenTS, Germany), with 300 ng/ml of CNF1 toxin for 1 h or a mixture of 7 μg/ml of C3 exoenzyme (Cytoskeleton Inc., Denver, CO) and 5 μg/ml of LipofectAMINE for 12 h at 37 °C in culture medium. Each assay point was derived from 3–6 separate wells and repeated at least three times.

**Morphological and Immunocytochemical Analysis**—Adherent cells were rinsed in phosphate-buffered saline (PBS), fixed with 3.5% paraformaldehyde, and permeabilized with 0.25% Triton X-100 in PBS for 5 min at room temperature. Cells were then washed and incubated with TRITC-phalloidin for 30 min for F-actin staining, and washed and mounted with fluorescent mounting medium (DAKO). For detection of focal adhesions, cells were incubated with mAbs for vinculin or paxillin for 1 h, followed by rinsing and incubation with secondary antibodies. Activated β₁ integrin was detected by using the monoclonal antibodies 12G10. To detect activated and total β₁ integrins, respectively, MG-63 cells were incubated with β₁ integrin antibodies (12G10 and M-106, respectively) and double-stained by incubating with FITC-conjugated goat anti-mouse and rhodamine-conjugated swine anti-rabbit secondary antibodies. Cells were examined using an inverted microscope equipped (Zeiss Axiovert) equipped with phase contrast optics and connected to a PentaMAX chilled charge-coupled device camera (Princeton Instruments). Images were processed using Metamorph Software Program. Spread cell areas were measured electronically using the Metamorph program. The images shown were representative from at least three separate experiments.

**RESULTS**

**Overexpression of Syndecan-4 in Carcinoma and CHO-K1 Cells Promotes Cell Spreading in Response to ADAM12**—We have shown that RKO colon carcinoma cells attach to rADAM12-cys via syndecan(s) as the primary cell surface receptor, whereas additional activation of β₁ integrin with Mn²⁺ or the activating mAb 12G10 is required for cell spreading to occur (16, 17). In contrast, mesenchymal cells, such as MG-63 osteosarcoma cells, attach, spread, and form stress fibers and focal adhesions spontaneously on rADAM12-cys. These results suggest that depending on cell type, rADAM12-cys can activate cross-talk between syndecan(s) and β₁ integrin, resulting in cell spreading. In the present study, we have characterized the downstream signaling pathway activated in rADAM12-cys mediated cell attachment and spreading. In addition to human MG-63 osteosarcoma cells and human RKO colon carcinoma cells (17), we also analyzed hamster CHO-K1 ovarian epithelial cells. CHO-K1 cells express endogenous syndecans 1, 2, and 4 as well as β₁ integrins (27, 40, 41) and have been extensively used to define the molecular mechanisms of cell attachment and spreading on fibronectin (27).

When CHO-K1 cells are plated on fibronectin they attach and spread (Fig. 1, A, E, and F). However, when plated on rADAM12-cys they attach but remain round (Fig. 1, B and F). Overall, less than 2% of cells spread following attachment to rADAM12-cys, while close to 100% spread when plated on fibronectin. Heparin and suramin, which interfere with the function of cell surface heparan sulfate glycosaminoglycan (GAG) chains completely inhibited cell attachment to rADAM12-cys, but did not inhibit cell attachment or spreading on fibronectin (Fig. 1E). CHO-K1 pgsD-677 cells, which are deficient in the synthesis of GAG chains, did not attach to overexpression by co-expression of EGFP. Data are presented as percentage of spread cells and are mean ± S.D. from three separate experiments. **Inset in J** shows syndecan-4 immunostaining of the CHO cells stably transfected with full-length syndecan-4 (S4).
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Fig. 3. Syndecan-4-induced cell spreading requires activation of β1 integrins. RKO colon carcinoma cells were transfected with the syndecan-4-EGFP expression construct (A–D) and plated on rADAM12-cys for 4 h. In C and D, the cells were pretreated with function-blocking β1 integrin antibodies, AIIIB2 (10 μg/ml), for 15 min.

Fig. 4. PKC activity is essential for cell spreading. MG-63 osteosarcoma cells untreated (A, D, G, and H) or pretreated with the PKC inhibitors GF109203X (B, E, I, and J) or Go6976 (C, F, K, and L) as described under “Materials and Methods” and plated on either rADAM12-cys (A–C, G, I, and K) or fibronectin (D–F, H, J, and L) for 1 h. A–F, phase contrast micrographs; G–L, stained for F-actin with TRITC-phalloidin.

Expression of a different member of the syndecan family, syndecan-2, failed to induce cell spreading on rADAM12-cys (Fig. 2, H and I). Both syn4ΔΔ- and syndecan-2-expressing cells were round in morphology and exhibited only peripheral F-actin staining (Fig. 2, G and I). An estimate of cell spreading revealed that around 15% of syn4- and 10% of syn4ΔE-expressing cells were spread, whereas only 2% of the EGFP-3% of the syn4ΔΔ-, and 3% of the syn2-expressing cells were spread (Fig. 2J).

Next, we used CHO cells that are stably expressing full-length wild type syndecan-4 (S4) and a mutant form lacking the PKCα binding site, syn4ΔI (S4ΔI). These cells have been FACS-selected and shown to express high levels of syndecan-4 at the cell surface (27). Labeling for syndecan-4 using an ectodomain-specific monoclonal antibody (150.9) revealed distinct dot-like staining at the cell periphery and at the peripheral ruffles in S4 cells (Fig. 2J, inset). An estimate of spreading assays using these cells revealed that spreading was increased from 15 to 26% in S4 stably transfected cells compared with synd transiently transfected cells. In contrast, the spreading of cells stably transfected with S4ΔI was still only around 2% (Fig. 2F). Transfection of RKO colon carcinoma cells with full-length syndecan-4 similarly induced spreading (Fig. 3A), but cells did not form typical stress fibers (not shown).

We next asked whether overexpression of syndecan-4 promoted activation of β1 integrin following binding to rADAM12-cys. For this experiment, we used a function-blocking antibody to β1 integrin (AIIIB2). Only RKO colon carcinoma cells were used because this antibody is specific for human β1 integrin. As
expected, pretreatment of RKO cells with AIIB2, but not with isotype-matched control antibodies, completely abolished cell attachment to fibronectin (data not shown). Pretreatment of syndecan-4-expressing RKO colon carcinoma cells with AIIB2 completely inhibited cell spreading on rADAM12-cys (Fig. 3, C and D) without affecting cell attachment.

These results demonstrate that transfection of full-length syndecan-4 into CHO-K1 and RKO colon carcinoma cells is sufficient to promote cell spreading on rADAM12-cys in a β1 integrin-dependent manner. Furthermore, the fact that deletion of the PKCα-activating domain from syndecan-4 abrogates this effect indicates a role for PKCα in this process.

PKCα Is Downstream of Syndecan-4 in Response to ADAM12—To characterize the potential role of PKCα downstream of syndecan-4, we first examined MG-63 osteosarcoma cells, mesenchymal cells that are known to attach and spontaneously spread on rADAM12-cys (Ref. 17 and Fig. 4A). These cells were treated with a general PKC inhibitor, GP109203X, and a compound that only inhibits classical PKC isoforms, Go6976, and the response of cells to rADAM12-cys was analyzed. As shown in Fig. 4, B–F, both compounds significantly inhibited cell spreading on rADAM12-cys but not on fibronectin. Moreover, treatment with PKC inhibitors completely inhibited formation of stress fibers in MG-63 cells plated on rADAM12-cys (Fig. 3, G, I, and K) but not on fibronectin (Fig. 4, H, J, and L). These results suggest that activation of a conventional PKC isoform occurs downstream of the syndecan-4, leading to mesenchymal cell attachment and spreading on rADAM12-cys. This observation supports the finding that syndecan-4 binds to and activates PKCα (45).

To determine the consequences of PKCα activation, RKO and CHO-K1 cells were subsequently transfected with a constitutively active, myristoylated PKCα (Myr-PKCα) construct. Transient expression of Myr-PKCα resulted in cell spreading (Fig. 5, A and B), while transient transfection with a different myristoylated isoform of PKCα, Myr-PKCδ, known to restore cell spreading in cells deficient in integrin β1 cytoplasmic signaling (1), did not result in spreading (Fig. 5, C and D). Myr-PKCα-transfected cells were clearly spread but less so than syndecan-4-transfected cells. F-actin staining was observed only in the periphery of both Myr-PKCα-expressing and control cells, and no stress fibers were formed (Fig. 5B).

To determine whether the effect of Myr-PKCα on cell spreading requires its catalytic activity, we treated Myr-PKCα-transfected CHO-K1 cells with PKC inhibitors GF109203X (not shown) and Go6976 (Fig. 5, E and F). As expected, both inhibitors completely abolished cell spreading on rADAM12-cys. In addition, treatment of syndecan-4-transfected CHO-K1 cells with Go6976 completely abolished cell spreading (Fig. 5, G and H). Together, these findings indicate that activation of PKCα is critical for cell spreading. Furthermore a signal other than or in addition to PKCα is required for cells to form stress fibers on rADAM12-cys.

β1 Integrin Activation Is Downstream of PKCα in Syndecan-4 Signaling—We next investigated the molecular mechanism through which PKCα mediates cell spreading in response to rADAM12-cys. Syndecan-4-mediated cell spreading is dependent on β1 integrin activation (Fig. 3), and PKC has been shown to regulate β1 integrin trafficking (37, 46). We therefore studied the activation of β1 integrin itself during cell spreading, using CHO cells stably transfected with human β1 integrin (CHOβ1)
and a monoclonal β1 integrin antibody, 12G10, which specifically recognizes the activated form of β1 integrin (36, 37, 47–49). These cells were transiently transfected with syn4 or synΔI expression vectors and were thereafter plated on rADAM12-cys and stained for activated β1 integrin using mAb 12G10. As shown in Fig. 6A, syndecan-4 transfection resulted in cell spreading and accumulation of activated β1 integrins (12G10). Activated β1 integrin could be detected in peripheral ruffles and cell edges (Fig. 6A, arrows). 12G10 labeling showed a typical dot-like staining and closely resembled that of syndecan-4 staining in S4 cells (Fig. 2J, inset). In contrast, no typical 12G10 staining was observed at cell edges and peripheral ruffles in cells expressing synΔI that lack PKCα binding site (Fig. 6B). These findings indicate that syndecan-4 activates β1 integrin through PKCα. CHO-β1 cells plated on fibronectin served as positive controls for β1 integrin staining (Fig. 6C). To further confirm the role of PKCα in the activation of β1 integrins, we stained Myr-PKCα-expressing CHO-β1 cells (Fig. 6D) with 12G10 antibodies. As shown in Fig. 6E, Myr-PKCα expression resulted in prominent accumulation of 12G10 immunostaining along the cell membranes (arrows).

We next plated the mesenchymal human MG-63 cells on rADAM12-cys, on which they attach and spread, and on fibronectin as a control, and then stained for both activated β1 integrins (12G10 mAb) and total β1 integrins with a polyclonal β1 integrin antibody (M-106). Staining with the 12G10 antibody demonstrated the presence of activated β1 integrins at the cell membranes in MG-63 cells plated on both rADAM12-cys and fibronectin (Fig. 7, A and C), whereas the polyclonal antibodies to β1 integrins stained throughout the cytoplasm (Fig. 7, B, D, and F). Pretreatment with the PKC inhibitor Go6976, completely inhibited the activated integrin staining at the membrane as well as cell spreading (Fig. 7E). These findings indicate that syndecan-4 indeed activates β1 integrins through PKCa. To determine a direct role for syndecan-4 in the activation of β1 integrins, we transfected MG-63 cells with the synΔI construct and determined its ability to inhibit cell spreading and β1 integrin activation. As shown in Fig. 8 expression of synΔI (C and D) but not EGFP alone (A and B) significantly inhibited 12G10 staining and cell spreading in MG-63 cells plated on rADAM12-cys. The 12G10 staining demonstrated the presence of activated β1 integrins at the cell membrane in EGFP-expressing cells (Fig. 8, A and B) but no staining of 12G10 was observed at the cell membrane in synΔI-transfected cells (Fig. 8, C and D). SynΔI transfection resulted in ~60% inhibition of MG-63 cell spreading on rADAM12-cys (Fig. 8E).

These findings (Figs. 6–8) together with experiments using function-blocking β1 integrin antibodies, such as AIIB2 (Fig. 3) strongly suggest that syndecan-4 activates β1 integrin through PKCα. Activation of Both PKCa and RhoA Is Required for Stress Fiber Formation in Response to ADAM12—Since RhoA is the principal regulator of stress fiber assembly (50), we asked whether modulation of RhoA activity might alter rADAM12-cys-induced cell spreading and stress fiber formation. CHO-K1 cells were transfected with vectors encoding constitutively active (L63RhoA) or dominant negative (N19RhoA) forms of RhoA (Fig. 9). Forty hours after transfection cells were plated on rADAM12-cys and stained for F-actin. Neither active RhoA (Fig. 9B, arrow) nor dominant negative RhoA (not shown) restored cell spreading or stress fiber formation.
Since activated RhoA alone did not induce cell spreading or stress fiber formation in RKO colon carcinoma or CHO-K1 cells, we asked whether activating both RhoA and PKCα would do so. We used CNF1 toxin to activate RhoA (51–53). Similar to the findings with the L63RhoA construct, treating CHO-K1 cells with CNF1 alone did not induce cell spreading in response to rADAM12-cys (data not shown). However, upon treatment of Myr-PKCa-expressing CHO-K1 cells (Fig. 9C) with CNF1, the cells spread, and phalloidin staining revealed formation of prominent actin stress fibers (Fig. 9D), but focal adhesions were not formed (not shown). Morphologically these flattened and well-spread cells resembled those of syndecan-4 overexpressing CHO-K1 cells (compare Fig. 9, D and E). In agreement with these results, CHO-K1 cells transfected with both syndecan-4 and the dominant-negative N19RhoA constructs did not exhibit impaired cell spreading, but stress fiber formation was significantly inhibited (Fig. 9F). Likewise, inhibition of RhoA with C3 exoenzyme in mesenchymal cells (MG-63) completely abolished syndecan-4-mediated stress fiber formation without inhibiting cell spreading in response to rADAM12-cys (data not shown). These findings suggest that PKCα and RhoA function in separate pathways and that RhoA requires prior cell spreading mediated by PKCα-β1 integrin to induce stress fiber formation in response to rADAM12-cys.

**DISCUSSION**

ADAM12 supports cell attachment and cell spreading of a variety of cells in a process that is syndecan-4-initiated but β1 integrin-dependent (17). In contrast, cell attachment and spreading on fibronectin are integrin-initiated events, but stress fiber and focal adhesion formation are syndecan-4-dependent (29, 54). Therefore, by using rADAM12-cys as a substrate, it is possible to obtain direct and novel information about syndecan-4 signaling. We show here that ADAM12/syndecan-4 signaling through PKCα and RhoA activates β integrin-dependent cell spreading and stress fiber formation. PKCα and RhoA appear to be activated in an ordered way and in two distinct pathways.

ADAM12 has been demonstrated to be a potent modulator of cell function in vivo and in vitro (55–58). It is up-regulated in carcinoma cells (16) and may therefore dramatically influence cell-cell interactions as well as the local microenvironment at the interface between the tumor cells and the surrounding stroma. To determine the function of ADAM12, we and others have performed cell attachment assays (11, 12, 16, 17, 59). We found that rADAM12-cys is a substrate for cell attachment using syndecan(s) as the primary receptor (16, 17). Subsequent cell spreading depends on cross-talk between syndecan and β1 integrin. In carcinoma and CHO-K1 epithelial cells the pathway leading to activation of β integrin is not functional but can be mimicked by activating β integrin activity with exogenous Mn(II) or activating monoclonal antibodies (Ref. 17 and this study). We postulate that in carcinoma cells, cross-talk between syndecan and β1 integrin is uncoupled, potentially influencing tumor cell behavior and/or survival. In support of this hypothesis, syndecan-4 levels have been shown to be down-regulated in certain carcinoma cells (42, 43). In the present study, we investigated downstream signaling pathways following ADAM12-syndecan engagement and found that overexpression of syndecan-4 in carcinoma and CHO cells promoted...
spreading on rADAM12-cys in a β1 integrin-dependent manner. Overexpression of syndecan-4 could conceivably induce this effect either by promoting dimerization with endogenous syndecan-4 or through accumulation of high levels of syndecan-4 in the cell, or both.

ADAM12/syndecan-4 signaling observed in the present study was dependent on specific sequence information residing in the V-region of syndecan-4, as evidenced by the failure of syn4ΔI and of syndecan-2 to induce cell spreading. The V-region of syndecan-4 contains the motif for binding to PIP2, an essential cofactor for activation of PKCα by syndecan-4 (25). Further evidence that the ADAM12/syndecan effect is mediated via PKCα is that (a) cell spreading induced by syndecan-4 transfection could be completely inhibited by both a general PKC inhibitor (GF109023X) and a specific inhibitor of classical PKC isoforms (Go6976) and (b) transient expression of a constitutively active form of PKC, Myr-PKCα, induced spreading of carcinoma cells on rADAM12-cys.

Although expression of Myr-PKCα could restore cell spreading, it did not lead to stress fiber assembly. However, activation of RhoA with CNF1 toxin in cells expressing Myr-PKCα induced reorganization of actin into stress fibers. On the other hand, isolated activation of RhoA by CNF-1 toxin or by constitutively active RhoA failed to induce spreading and stress fiber assembly, indicating that RhoA activation alone is not sufficient to mimic the syndecan-4 pathway. Finally, a dominant negative mutant of RhoA, N19RhoA, inhibited syndecan-4-induced stress fiber formation but not cell spreading. These results indicate that ADAM12/syndecan signaling activates PKCα and RhoA through separate pathways (Fig. 10, scheme).

The observation that ADAM12/syndecan signaling activates PKCα and RhoA in apparently separate pathways in response to rADAM12-cys is novel. When fibroblasts are plated on the cell-binding domain of fibronectin, full spreading and stress fiber formation can be restored by activating either PKC or Rho, suggesting that both PKC and Rho are situated in the same pathway downstream of syndecan-4 (29, 60, 61). In fact in the latter study (29), addition of syndecan-4 antibodies induced full spreading, stress fibers and focal adhesions in fibroblasts attached to the integrin-binding domain of fibronectin. Defilippi et al. (62) demonstrated that cells can bind to integrin antibodies but require exogenous activation of both PKC and Rho for stress fiber formation, suggesting that integrins cooperate with another molecule to activate PKC and Rho. Intriguingly, syndecan-4 overexpression in our present study led to activation of all three signaling molecules, PKCα, RhoA, integrins, and PKC function in separate pathways.

In our study, overexpression of syndecan-4 or Myr-PKCα resulted in β1 integrin-dependent cell spreading, indicating that the cross-talk between syndecan-4 and ADAM12 leads to activation of β1 integrin. This observation was confirmed by the finding that activated β1 integrin accumulated on cell surfaces of these cells. On the other hand, a mutant form of syndecan-4 lacking the PKCα binding site failed to induce activation of β1 integrin. To our knowledge, this is the first report demonstrating activation of β1 integrins by a proteoglycan, syndecan-4. PKCα and ε isoforms have been implicated in the trafficking of β1 integrins (37, 46), supporting a role for PKCα downstream of syndecan-4 in the activation and accumulation of β1 integrins at the cell membrane. The exact downstream effectors activated following syndecan-β1 integrin cross-talk observed in the present study are not known. Rac has been implicated in β1 integrin-mediated cell spreading (64), and it was recently shown that β1 integrin activation is required for dissociation of GTP-Rac from Rho-GDI and its recruitment to the plasma membrane where it can interact with effectors (65). Carcinoma cell attachment to rADAM12-cys decreased the levels of GTP-Rac1 compared with their attachment to fibronectin. Co-transfection of N17Rac1 and syndecan-4 inhibited synduced cell spreading on rADAM12-cys, indicating that Rac is downstream of β1 integrin in syndecan-4-mediated cell spreading.2 The findings that dominant negative N19RhoA inhibited only stress fiber formation but not cell spreading and that RhoA activation was unable to induce cell spreading argue against the participation of RhoA in the activation of β1 integrins by syndecan-4.

Finally, we emphasize the differences and similarities between cell adhesion on fibronectin and rADAM12-cys. The cell-binding domain of fibronectin supports cell attachment via β1 integrins and requires the syndecan-4 binding-hep II domain of fibronectin for full spreading and cytoskeletal reorganization (29, 54). In contrast rADAM12-cys supports cell attachment through syndecan-4 but requires β1 integrins for cell spreading and stress fiber formation (Fig. 10). Irrespective of their choice of primary attachment receptors, both fibronectin and rADAM12-cys require cooperation between syndecan-4 and β1 integrins in controlling the integral process of cell adhesion.

In conclusion, our study provides novel insights into rADAM12-cys/syndecan-4 signaling. Our findings suggest that rADAM12-cys/syndecan-4 can promote cell spreading and stress fiber formation by activating two separate pathways at different stages of cell adhesion. One involves the sequential activation of PKCα and β1 integrins and the other involves RhoA.

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