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COMMENTARY

Syndecan-4 and integrins: combinatorial signaling in cell adhesion

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SUMMARY

It is now becoming clear that additional transmembrane components can modify integrin-mediated adhesion. Syndecan-4 is a transmembrane heparan sulfate proteoglycan whose external glycosaminoglycan chains can bind extracellular matrix ligands and whose core protein cytoplasmic domain can signal during adhesion. Two papers in this issue of JCS demonstrate, through transfection studies, that syndecan-4 plays roles in the formation of focal adhesions and stress fibers. Overexpression of syndecan-4 increases focal adhesion formation, whereas a partially truncated core protein that lacks the binding site for protein kinase Cα and phosphatidylinositol 4,5-bisphosphate acts as a dominant negative inhibitor of focal adhesion formation. Focal adhesion induction does not require interaction between heparan sulfate glycosaminoglycan and ligand but can occur when non-glycanated core protein is overexpressed; this suggests that oligomerization of syndecan-4 plays a major role in signaling from the extracellular matrix in adhesion.

Key words: Syndecan, Proteoglycan, Heparan sulfate, Cytoskeleton, Focal adhesion

INTRODUCTION

Three very recent publications, including two published in this issue of JCS, have placed a cell surface heparan sulfate proteoglycan in the spotlight (Echtenmeyer et al., 1999; Longley et al., 1999; Saoncella et al., 1999). The focus of these papers is the potential role of the syndecan-4 heparan sulfate proteoglycan in the adhesion of cultured cells to extracellular matrix (ECM) substrates. For many years, we have known that the integrin receptors are a major component of adhesion processes (reviewed by Hynes, 1996; Schwartz et al., 1995; Yamada and Miyamoto, 1995). Many members of this family are implicated not only in cell adhesion and migration but also in signaling at the cell surface. The details of the signaling processes are still being determined, but clearly they involve communication with the actin-associated cytoskeleton and promotion of cell survival (Clark and Brugge, 1995: Damsky and Werb, 1992; Howe et al., 1998; Hynes, 1996; Schwartz et al., 1995; Yamada and Miyamoto, 1995). Indeed, signals from integrins might be fundamental to the phenomenon of anchorage-dependent growth. However, other receptors involved in cell adhesion have also been identified, for example, the syndecans that interact with a range of ECM components, and CD44 glycoprotein, which is a part-time proteoglycan (i.e. sometimes glycanated) that has potential roles in interaction with hyaluronan (Skelton et al., 1998).

Attention is now focusing on integrin-associated proteins that might regulate adhesion by lateral associations and thereby modify the primary cellular responses mediated through integrin-ligand interactions. For example, the TM4SF or tetraspanin proteins can regulate the adhesion of some integrins, such as α3β1 (Hemler, 1998). Integrin-mediated responses can also be modified by members of the syndecan family of cell surface proteoglycans (Woods and Couchman, 1998, 1999). These molecules have for some years (reviewed by Bernfield et al., 1999; Carey, 1997; Gallagher, 1997; Fuki et al., 1997; Zimmerman and David, 1999) been known to interact with ECM molecules, in addition to many other ligands, such as growth factors, lipases and even pathogens, although their role(s) has remained obscure. Four mammalian members of the syndecan family have been identified and cloned, whereas one only has been identified in *Drosophila* or *Caenorhabditis elegans*. All share some properties. They are type 1 membrane glycoproteins that are usually substituted with heparan sulfate chains, although some also bear chondroitin or dermatan sulfate. Each has a short cytoplasmic domain that has two regions of high homology (C1 and C2) flanking a central (V) region of variable sequence (Woods and Couchman, 1998). Attention is now strongly focused on the cytoplasmic domains of syndecans because they participate in adhesion processes through transmembrane signaling (reviewed by Bernfield et al., 1999; Rapraeger and Ott, 1998; Woods and Couchman, 1998).

The last of the four mammalian syndecans to be identified was syndecan-4, and this molecule is providing interesting insight into syndecan biology. Syndecan-4 is widely expressed,

in contrast to the other three syndecans, which exhibit rather tissue-specific distributions (Kim et al., 1994). Several years ago, we demonstrated that syndecan-4, but not the other syndecans, is a widespread constituent of focal adhesions (Woods and Couchman, 1994). These adhesive structures represent points of firm anchorage between the cell and the substratum, and are at the termini of actin-containing microfilament bundles (reviewed by Burridge and Chrzanowska-Wodnicka, 1996; Jockusch et al., 1995). Although the presence of integrins in these structures was described many years ago, and, indeed, their ligation is obligatory for focal adhesion formation, only more recently has it become apparent that syndecan-4 is another transmembrane partner in their formation (Fig. 1; Echtenmeyer et al., 1999; Longley et al., 1999; Saoncella et al., 1999). Soon after we localized syndecan-4 to focal adhesions, Baciu and Goetinck (1995) showed that its presence was governed by protein kinase C activity. However, until more recently, the precise manner in which syndecan-4 regulates focal adhesion architecture and assembly remained obscure.

SYNDECAN-4 AND INTEGRINS, PARTNERS IN FOCAL ADHESION FORMATION

Nearly fifteen years ago, we used model systems with fibronectin and its isolated domains as substrates to show that the high-affinity heparin-binding domain (HepII), in addition to the more central region of the fibronectin molecule that is now known to interact with several integrins, is required for focal adhesion formation (Woods et al., 1986). This provided early evidence that integrin ligation may not be sufficient to promote focal adhesion formation. Similar work has emerged from several other laboratories (Bloom et al., 1999; Huhtala et al., 1995; Izzard et al., 1986). These studies implicated a cell-surface heparan sulfate proteoglycan in focal adhesion assembly and, when syndecan-4 proteoglycan was found subsequently in focal adhesions, suggested that this is the required partner of integrins. Now, Saoncella et al. (1999), using an elegant system of fibronectin-null fibroblasts, demonstrate how important syndecan-4 proteoglycan can be in focal adhesion assembly. Fibronectin-null fibroblasts spread when seeded on the integrin-binding 120 kDa fragment of fibronectin, but could not organize their cytoskeleton or form focal adhesions (Saoncella et al., 1999): these observations confirm earlier findings (Woods et al., 1986). Addition of bivalent antibodies that cluster syndecan-4 on the cell surface provided the extra signal required for focal adhesion assembly (Saoncella et al., 1999). Appropriate control antibodies failed to do this. Therefore, although integrins are essential, cell-surface proteoglycan is also needed, and the combination is sufficient to drive focal adhesion assembly.

Our new findings provide further evidence for a role for syndecan-4 proteoglycan in regulation of focal adhesion assembly (Longley et al., 1999). Wild-type Chinese hamster ovary (CHO) fibroblasts establish small peripheral focal contacts when seeded on ECM substrates. The formation of these small adhesions does not abrogate the ability of these cells to migrate in scratch wound or chemotaxis assays. Stable transfection of full-length syndecan-4 in these cells led to elevated levels of the proteoglycan on the cell surface, which were accompanied by a sharp increase in both the size and the number of focal adhesions. In addition, the distribution of the focal adhesions altered: in the transfected cells, they were present not only peripherally but also more centrally under the cell. This resulted in decreased migratory ability, which is consistent with the idea that focal adhesions are points of stable anchorage to the ECM. These two studies (Longley et al., 1999; Saoncella et al., 1999) demonstrate that focal adhesion assembly can be directly related to the availability and clustering of syndecan-4 on the cell surface, in addition to integrin-mediated signaling.

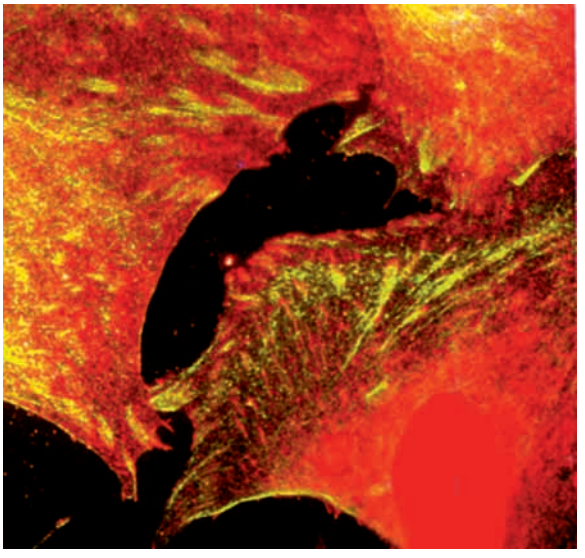

Fig. 1. Double immunofluorescence labeling for syndecan-4 (FITC) and integrin $\alpha5\beta1$ (Texas Red) in rat embryo fibroblasts spread for 4 hours on fibronectin.

STRUCTURAL REQUIREMENTS OF THE SYNDECAN-4 PROTEOGLYCAN

Now that a role for syndecan-4 in focal adhesion assembly has been established, the underlying molecular mechanisms are being determined. The new studies yield some expected, and some unexpected, results (Echtermeyer et al., 1999; Longley et al., 1999). Given that earlier work showed that the heparin-binding domain of fibronectin promotes focal adhesion formation, we expected that the heparan sulfate chains on syndecan-4 serve as the ligand-binding site and, therefore, are essential. Echtermeyer et al., show that, by contrast, overexpression of syndecan-4 core protein in a CHO cell mutant incapable of heparan sulfate synthesis is, nevertheless, sufficient to promote focal adhesion formation. These mutant cells failed to make focal adhesions when seeded on fibronectin substrates, unless they overexpressed syndecan-4; this confirms previous observations (LeBaron et al., 1988). All syndecans oligomerize (reviewed by Carey, 1997); once clustering reaches a critical level in the membrane, oligomerization might be spontaneous and independent of ligand binding. Therefore, oligomerization of the syndecan-4 core protein on the cell surface might provide a major signal for focal adhesion assembly, which can even bypass any requirement for the heparan sulfate chains. We therefore

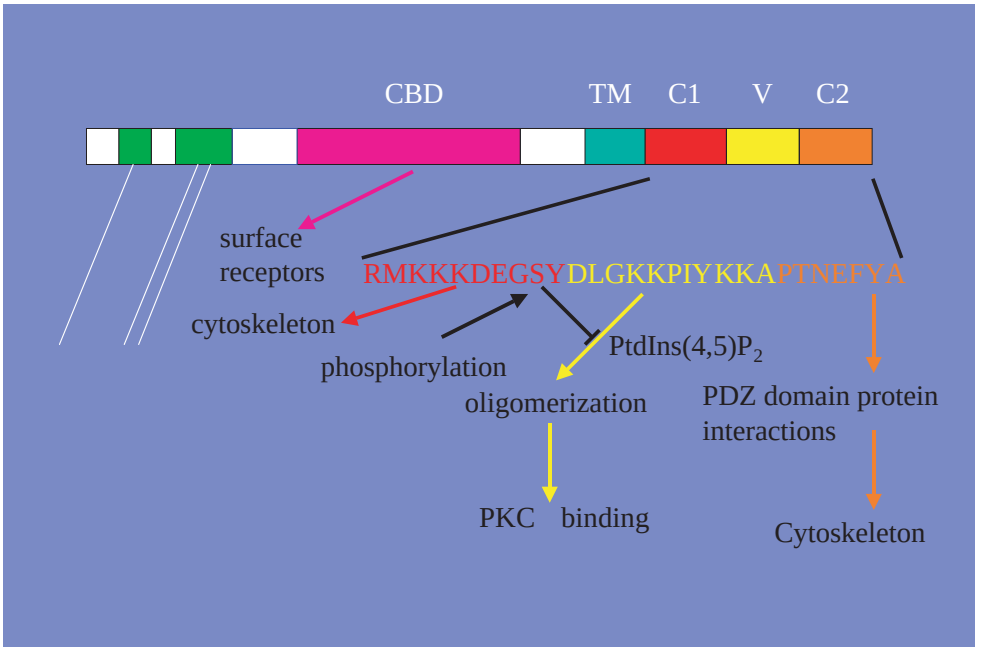

Fig. 2. Schematic of the interactions of syndecan-4 core protein that are involved in adhesion. The core protein domain interactions are indicated by colored arrows. Serine phosphorylation blocks interaction with PtdIns(4,5)P_2, oligomerization, and PKCα binding and superactivation. CBD, cell binding domain; TM, transmembrane domain, C1 and C2, constant regions 1 and 2; V, variable region. The C1 region is highly conserved between all syndecans, whereas the C2 region has more limited homology except for the terminal EFYA. White lines represent glycosaminoglycan chains.

speculate that the role of heparin-binding ligands is to regulate the organization of heparan sulfate in such a way as to allow controlled clustering of the syndecan-4 core protein. Additional interactions between the heparin-binding domain of fibronectin and the core protein of syndecan-4 cannot be ruled out. Indeed, McFall and Rapraeger (1997, 1998) suggest that a site on the ectodomain of the syndecan-4 core protein interacts with another cell-surface, but as yet uncharacterized, receptor(s) (Fig. 2).

If we consider the inside of the cell, given that signaling appears to be through the unique V region of syndecan-4, it was perhaps not surprising that overexpression of a truncated form of the syndecan-4 proteoglycan did not promote focal adhesion formation. Indeed, a syndecan-4 construct that has a truncated central V region acts as a dominant negative mutant. We show that, in cells expressing this mutant, focal adhesion formation is diminished. There is also some decrease in cell spreading. We draw two conclusions from these experiments. First, the cytoplasmic domain of syndecan-4 is essential for promotion of focal adhesions. Second, truncated forms of the core protein oligomerize with endogenous wild-type proteoglycan and somehow abrogate appropriate signaling responses that normally play a major role in focal adhesion formation. A syndecan-4 construct that entirely lacks the cytoplasmic domain also acts dominant negatively when expressed at high levels. Somewhat surprisingly, after transfection of cells with partially or fully cytoplasmically truncated syndecan-4, the ability of those expressing few focal adhesions to migrate was also compromised. This may be due to the fact that incomplete spreading does not allow the cytoskeleton to be developed sufficiently to respond to chemotactic gradients, or to such decreased adhesion that the traction needed for migration cannot be generated (reviewed by Huttenlocher et al., 1995). In control experiments, overexpression of wild-type syndecan-2 or equivalent truncated forms of syndecan-2 did not produce these dramatic effects on focal adhesion assembly and migration (Longley et al., 1999).

HOW DOES SYNDECAN-4 SIGNAL?

Growing evidence suggests that members of the syndecan family directly influence intracellular events through transmembrane signaling pathways. Although other reports imply that members of the syndecan family have co-receptor roles that do not depend on intrinsic signaling ability (e.g. in the binding and interaction of fibroblast growth factor 2 with cell surface proteoglycans and high-affinity tyrosine kinase receptors), syndecans might also have intrinsic signaling capacity. Perhaps, of all the syndecan molecules, the best understood is syndecan-4, whose properties were revealed in a series of papers in 1997 and 1998. The central V region of the syndecan-4 cytoplasmic domain possesses an unusual sequence: two pairs of lysine residues flank a proline-isoleucine-tyrosine tripeptide (Fig. 2). Oh et al. (1997a,b, 1998) showed that this cytoplasmic domain appears to bind two signaling molecules: phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) and protein kinase Cα (PKCα).

Interaction with PtdIns(4,5)P_2 promotes the oligomerization of syndecan-4 cytoplasmic domains (Oh et al., 1997b, 1998). NMR studies show that the dimer formed by the syndecan-4 V region can bind PtdIns(4,5)P_2 and is stabilized by that interaction (Lee et al., 1998). The V region of syndecan-4 is unusual: it forms twisted-clamp dimers in which the two strands cross each other at either end (Lee et al., 1998). How many dimers and PtdIns(4,5)P_2 molecules are involved in a single signaling complex remains to be evaluated, but clearly oligomerization of the syndecan-4 proteoglycan not only drives focal adhesion formation but also provides a platform for the binding and activation of PKCα.

Work with fusion proteins and synthetic peptides shows that oligomerized, but not monomeric, cytoplasmic domains of syndecan-4 can bind PKCα directly, through the PKCα catalytic domain (Oh et al., 1997a). This results in direct activation of PKCα in the absence of other mediators and superactivation in their presence. The interaction is almost

completely prevented by mutation of any of the key residues of the syndecan-4 V region. These include the lysine, proline, isoleucine and tyrosine residues. In fact, changes in this peptide sequence dramatically reduce the ability of syndecan-4 to dimerize, which might explain why subsequent binding and activation of PKCα does not occur (Oh et al., 1997b). The combination of the syndecan-4 cytoplasmic domain, PtdIns(4,5)P_2 and PKCα yields a potent signaling complex, in which levels of PKCα activity are as high as can be achieved in other in vitro assays using the more conventional activators phosphatidylserine and diolein (Oh et al., 1998). There is one other interesting twist to this mechanism of activation of PKCα. Although PKCα is thought to be a calcium-requiring conventional isoform, activation through syndecan-4 proteoglycan and PtdIns(4,5)P_2 renders the enzyme insensitive to changing calcium levels, and PKCα activity is uncompromised even in the presence of EDTA (Oh et al., 1998).

Horowitz and Simons (1998a,b) have also shown that oligomers of the syndecan-4 cytoplasmic domain are required for PKCα activation. They demonstrated in 3T3 cells that a single serine residue within the conserved (C1) region of syndecan-4 cytoplasmic domain that is proximal to the membrane can be phosphorylated in the absence of growth factor stimulation (Fig. 2). Dephosphorylation occurs after addition of FGF-2 (Horowitz and Simons, 1998a), which presumably binds to the syndecan proteoglycan through its heparan sulfate chains. Moreover, phosphorylation of this serine residue blocks oligomerization of the syndecan-4 cytoplasmic domain, along with its ability to activate PKCα (Horowitz and Simons, 1998b).

Activation of PKCα or any other PKC isoform by direct interaction with a cell-surface receptor is rare, and regulation of activity is mostly through regulatory domain interactions. Interestingly, the catalytic domain of the kinase appears to be the site that interacts with syndecan-4 (Oh et al., 1997a), whereas PtdIns(4,5)P_2 activates PKCα through interactions with the PKCα regulatory domain (Lee and Bell, 1991). These data suggest that the proteoglycan cytoplasmic domain, inositol phospholipid and kinase form a ternary complex. It will be interesting to understand this mode of activation of PKC at the molecular level.

Whereas in vitro experiments showed that a ternary complex could activate PKCα strongly, further work with fibroblasts showed that PKCα and syndecan-4 associate together in a complex that can be immunoprecipitated from fibroblasts (Oh et al., 1997a). Complex formation depends on prior activation of PKCα by treatment of the cells with phorbol ester. Moreover, the presence of synthetic peptides corresponding to the syndecan-4 V-region competitively inhibits this association, which confirms that the interaction is through this key region of the syndecan-4 cytoplasmic domain. Double immunofluorescence microscopy also showed that PKCα and syndecan-4 proteoglycan could co-distribute in cells (Oh et al., 1997a). Hyatt et al., have shown previously that PKCα can be a focal adhesion component, along with syndecan-4, but that it is not detectable in the small focal adhesions or focal contacts of transformed cells (Hyatt et al., 1990). This suggests either that it is not required for assembly or, more likely, that its presence is subject to flux and may not be needed to maintain focal contacts. The fact that PKC inhibitors prevent focal adhesion assembly in fibroblasts but do not cause focal adhesion disassembly once these structures are formed is consistent with the latter suggestion (Woods and Couchman, 1992).

RECEPTOR PARTICIPATION IN FOCAL ADHESION ASSEMBLY

Given the clear involvement of two divergent receptors, namely the integrins and the syndecan-4 proteoglycan, we must now ascertain how each receptor contributes to the overall process of focal adhesion formation. Whereas the integration of individual integrin subunits into focal adhesions is dependent on the matrix substrate, syndecan-4 appears to be a rather more universal component (Woods and Couchman, 1994). Nevertheless, interactions between integrins and matrix glycoprotein or collagen clearly are a prerequisite for focal adhesion formation, and signaling processes that involve this component of the system are under intense scrutiny. Previous work has shown that signaling through tyrosine kinases occurs, given that some inhibitors block focal adhesion formation (Burridge et al., 1992; Romer et al., 1994). For many years, focal adhesion kinase ($pp120^{FAK}$) was believed to be an essential component of this signaling system (Richardson and Parsons, 1995), but recent data clearly indicate that this might not be so. Not only do FAK-null fibroblasts make normal numbers, or even an excess, of focal adhesions (Ilic et al., 1995), evidence now indicates that FAK signaling plays more of a role in cell survival and mitogenic pathways (Gilmore and Romer, 1996; Hungerford et al., 1996). It therefore remains to be evaluated exactly which, and how, tyrosine kinases are involved in focal adhesion promotion.

One possible scenario for focal adhesion assembly involving both the integrins and syndecan-4 proteoglycans is as follows. Integrin-ligand interaction leads to non-receptor tyrosine kinase activation, and association with adaptor proteins and cytoskeletal elements (see Burridge and Chrzanowska-Wodnicka, 1996; Clark and Brugge, 1995; Howe et al., 1998; Schwartz et al., 1995; Yamada and Miyamoto, 1995). Integrin ligation also elevates the activity of PIP 5-kinase (McNamee et al., 1993). This should lead to increased levels of PtdIns(4,5)P_2, which not only binds cytoskeletal elements such as α-actinin and profilin and can regulate further cytoskeletal interactions (Fukami et al., 1994; Goldschmidt-Clermont et al., 1990; Weekes et al., 1996), but also binds syndecan-4 proteoglycan (Lee et al., 1998). Elevated levels of PtdIns(4,5)P_2 in the vicinity of integrins might then provide a platform for syndecan-4 dimerization, which could be aided by interactions between syndecan-4 and its ligands (e.g. heparin-binding domains of glycoproteins). Indeed, PtdIns(4,5)P_2 is needed for focal adhesion formation (Gilmore and Burridge, 1996). Data suggest that integrin ligation and syndecan-4 ligation need not be temporally coincident, but integrin ligation must occur as an early event (Woods et al., 1993). At some point PKCα must become activated and translocate from the cytosol to the membrane. The stimulus for this is still not clear, but PKC activation is needed for cell spreading and focal adhesion formation (De Nichilo and Yamada, 1996; Lewis et al., 1996; Vuori and Ruoslahti, 1993; Woods and Couchman, 1992). Co-immunoprecipitation experiments show that syndecan-4 and PKCα associate only

after activation of PKC (Oh et al., 1997a). When PKCα translocates to the membrane, it can then bind to oligomers of the syndecan-4 cytoplasmic domain, in association with PtdIns(4,5)P_2, which could lead to subsequent strong localized activation of the serine/threonine kinase. Many focal adhesion components are PKC substrates, at least in in vitro experiments, and Baciu and Goetinck (1995) showed that PKC activity is necessary to draw syndecan-4 into the forming focal adhesions.

Any involvement in adhesion of other proteins that interact with syndecan-4 is not clear, but is distinctly possible. The C-terminal FYA motif of each syndecan can interact with PDZ domain proteins (Fanning and Anderson, 1996). Syntenin (Grootjans et al., 1997), CASK (Cohen et al., 1998) and synectin (Gao and Simons, 1998) have been identified thus far, although none has been shown to interact with syndecan-4 in living cells or to be present in focal adhesions. Whether PDZ domains are involved in focal adhesion assembly is, therefore, unknown but under investigation. There are other suggestions, based on studies with syndecan-3, that the membrane proximal C1 domain of syndecans interacts with cytoskeletal proteins. In the case of syndecan-3, association of a Src-cortactin complex with the conserved C1 region has been demonstrated (Kinnunen et al., 1998). Given that Src might be very much involved in adhesion processes, this is an avenue worthy of future exploration. Other possibilities are interactions with protein 4.1 or its relatives (reviewed by Rapraeger and Ott, 1998), and it is of note that the ezrin/radixin/moesin group of proteins and talin share some structural relationship with protein 4.1.

Ultimately the focal adhesion becomes the terminus of the microfilament bundle, and many studies (reviewed by Burridge and Chrzanowska-Wodnicka, 1996; Mackay and Hall, 1998) have demonstrated that focal adhesion formation results from the activation of the G protein Rho. Rho activation causes stress fiber and focal adhesion formation, possibly through activation of downstream kinases that, ultimately, phosphorylate myosin-light-chain phosphatase and myosin light chain, which results in myosin-filament assembly. How Rho is activated following integrin ligation is not clear, but Saoncella et al. (1999) provide provocative evidence for involvement of syndecan-4. In their studies, signaling through clustering of syndecan-4 with antibodies to form focal adhesions could be bypassed by treatment with lysophosphatidic acid (LPA), a known activator of Rho. Moreover, the ability of clustering antibodies against syndecan-4 core protein to promote focal adhesion formation was inhibited by the C3 exotransferase. These two pieces of evidence suggest that the syndecan-4 proteoglycan, in some manner, leads to the activation of Rho. It will be interesting to determine whether this is a widespread phenomenon applicable to many different cell types that form focal adhesions in response to ECM substrates. Interestingly, activation of Rho also results in increased PtdIns(4,5)P_2 levels. This should potentiate syndecan-4 oligomerization and superactivation of PKC, in addition to having known effects on microfilament assembly. Defilipi has suggested that the combined activities of PKC and Rho function in focal adhesion formation (Defilipi et al., 1997). Of course, complementary signaling mechanisms activated by integrins, such as tyrosine kinase cascades and docking of adapter proteins, might also be affected by syndecan-4.

In conclusion, focal adhesion formation appears to be a consequence of the summation of contributions of two distinct classes of receptor, the integrins and the syndecan-4 proteoglycan. Many of the details remain to be resolved, and combinatorial signaling of this type presents many challenges. However, focal adhesion formation is not alone in requiring multiple signals. Many immune responses, such as antigen presentation and lymphocyte activation, require combined signaling events. Combinatorial signaling might explain how integrins not only can promote migratory responses, in which focal adhesions are either transient or reduced in number, but, if accompanied by additional signaling through syndecan-4 proteoglycan, might then promote anchorage to ECM substrates. If this is the case, levels of syndecan-4 might rise during tissue repair. This does occur**:** syndecan-4 is upregulated in response to skin (Gallo et al., 1994) and vascular (Cizmeci-Smith et al., 1997) wounding. Indeed, the proteoglycan might represent an early response gene (Cizmeci-Smith et al., 1997). Among the promoters of syndecan-4 synthesis is the antibacterial PR-39 peptide found in wound fluid (Gallo et al., 1994). Thus, in a wound environment, increased expression of syndecan-4 might allow cells to respond to matrix and growth factors by increased adhesion (retaining cells in the wounded area and contracting the wound) and division (to repopulate the area).

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