REVIEW ARTICLE

Cytoplasmic interactions of syndecan-4 orchestrate adhesion receptor and growth factor receptor signalling

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Syndecan-4 is a ubiquitous transmembrane proteoglycan that localizes to the focal adhesions of adherent cells and binds to a range of extracellular ligands, including growth factors and extracellular-matrix proteins. Engagement of syndecan-4 is essential for adhesion formation in cells adhering via certain integrins, and for cell proliferation and migration in response to growth factors. The cytoplasmic domain of syndecan-4 interacts with a number of signalling and structural proteins, and both extracellular and cytoplasmic domains are necessary for regulated activation of associated transmembrane receptors. PDZ domain-containing scaffold proteins (syntenin and CASK) bind to the C-terminus of the syndecan-4 cytoplasmic domain and co-ordinate clustering of receptors and connection to the actin cytoskeleton.

Syndecan-4 also binds and activates protein kinase C α in the presence of phosphatidylinositol 4,5-bisphosphate, and regulates signalling by Rho-family GTPases and focal adhesion kinase. This review discusses the cytoplasmic interactions of syndecan-4 and how they affect cell behaviour as a consequence of the interaction with extracellular ligands. These conclusions also offer an insight into the role of syndecan-4 *in vivo*, and are consistent with phenotypes generated as a consequence of abnormal syndecan-4 expression in pathologies and gene disruption studies.

Key words: CASK, integrin, PDZ domain, protein kinase C, syntenin.

THE HEPARAN SULPHATE CHAINS OF SYNDECAN-4 BIND TO THE EXTRACELLULAR MATRIX AND PROMOTE CELL ADHESION

Cell adhesion to the extracellular-matrix protein fibronectin is mediated by a range of transmembrane receptors from the integrin and transmembrane proteoglycan superfamilies. Fibronectin contains several receptor-binding sites that have been expressed as recombinant polypeptides, and confer different morphological properties on the cell. All matrix adhesion complexes include members of the integrin family of heterodimeric receptors, and the most ubiquitous fibronectin-binding integrins are $\alpha 4 \beta 1$, $\alpha 5 \beta 1$ and $\alpha v \beta 3$. Integrins $\alpha 5 \beta 1$ and $\alpha v \beta 3$ bind to the RGD (Arg-Gly-Asp) tripeptide and the PHSRN (Pro-His-Ser-Arg-Asn) synergy site of fibronectin type III repeats 9–10, which are referred to as the central cell-binding domain [1,2]. Cells plated on to a polypeptide encompassing these repeats spread and form integrin-containing adhesions, but fail to form stress fibres or to recruit cytoskeletal proteins (including vinculin, talin and paxillin) to the nascent adhesions [3,4]. In order to form mature focal adhesions, cells require stimulation with one of the heparin-binding domains of fibronectin. Addition of a soluble heparin-binding polypeptide after spreading on the cell-binding domain of fibronectin is sufficient for adhesion formation, and the heparin-binding polypeptide is not required as an adhesive substrate (Figure 1) [3]. Adhesion formation, which is dependent on the interaction between the polypeptide and cell surface heparan sulphate proteoglycans, can be inhibited by an excess of soluble heparin competitor or treatment of the cells with heparatinase, but not chondroitinase ABC [5]. Fibronectin

contains up to three heparin-binding sites at its C-terminus, two within type III repeats 13 and 14, and one within the alternatively spliced IIICS region [6,7]. The heparin-binding site within the 13th type III repeat is sufficient for focal adhesion formation in cells spread on the cell-binding fragment of fibronectin [4]. The motif is rich in arginine residues, and both heparin-binding and adhesion-stimulatory activities are abrogated by substitution of these basic residues [4,8]. Fibronectin contains further heparin-binding sites within the N-terminal type I repeats, and proteolytic fragments mapping to this region stimulate adhesion formation in cells spread on the cell-binding domain [3]. This region has been studied less extensively than the C-terminal heparin-binding domain because the interaction with proteoglycans is of lower affinity [9].

Heparan sulphate proteoglycans that are candidate receptors for the heparan-binding fragment of fibronectin are found on the surface of all adherent mammalian cells [10]. Among these proteoglycans is the syndecan family of transmembrane receptors that comprises four members, syndecans-1–4, each of which is substituted with heparan sulphate chains close to the N-terminus [11,12]. Syndecans-1–3 exhibit tissue-specific expression, and are most abundant in epithelial cells, fibroblasts and neuronal tissue respectively [13]. Syndecan-4 is expressed ubiquitously and is enriched in the focal adhesions of a range of adherent cell types [14], and has proven to be the proteoglycan responsible for focal adhesion formation in response to fibronectin. Syndecan-4 is recruited to focal adhesions when cells spread on the cell-binding domain of fibronectin are stimulated with a heparin-binding polypeptide, causing similar recruitment of the cytoskeletal proteins

Abbreviations used: EGF, epidermal growth factor; GAP, GTPase-activating protein; GDI, guanine nucleotide dissociation inhibitor; GEF, guanine nucleotide exchange factor; FAK, focal adhesion kinase; (b)FGF, (basic) fibroblast growth factor; LD4, leucine-rich domain 4; MAGUK, membrane-associated guanylate kinase; PIP₂, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; PKL, paxillin kinase linker; SH2(3), Src homology 2(3).

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Figure 1 Engagement of syndecan-4 is necessary for adhesion formation in cells adhering via integrin $\alpha 5 \beta 1$

Fibroblasts plated on to a ligand of integrin $\alpha 5 \beta 1$ adhere and spread (A), but fail to form vinculin-containing focal adhesions or stress fibres until stimulated with a syndecan-4 ligand (B).

vinculin, talin and α -actinin [15]. Conclusive evidence that syndecan-4 is the proteoglycan responsible for focal adhesion formation was achieved by clustering the receptor with a specific antibody. Treatment of fibroblasts spread on the cell-binding domain of fibronectin with an anti-syndecan-4 antibody restores focal adhesion and stress-fibre formation to the level seen in cells plated on to whole fibronectin [16]. Furthermore, overexpression of syndecan-4 causes the cortical actin to rearrange into stress fibres, and results in an increase in the number of focal adhesions [17].

The generation of syndecan-4-deficient mice has made it possible to demonstrate that syndecan-4 is absolutely required for focal adhesion formation in cells adhering to the cell-binding domain of fibronectin. Syndecan-4 (–/–) fibroblasts fail to form vinculin-containing adhesions when stimulated with a soluble heparin-binding fragment of fibronectin [18]. Surprisingly, cells spread for 4 h on coverslips pre-coated with both the cell-binding and heparin-binding domains of fibronectin form focal adhesions, despite lacking syndecan-4. This suggests that cells are able to compensate for the lack of syndecan-4, to a certain extent, and form adhesions when stimulated at the ventral, but not the apical, surface. The proteoglycans that substitute for syndecan-4 have not been identified, but might include the other syndecans, which are all expressed in fibroblasts

but are not overexpressed in the absence of syndecan-4. A similar effect on adhesion formation has been reported when syndecan-4 expression is down-regulated using antisense technology [17]. Transfected CHO cells are unable to form focal adhesions or stress fibres, even when cultured for 24 h, suggesting that these cells might also lack the proteoglycan required to compensate for the loss of syndecan-4.

Fibronectin also includes binding sites for integrin $\alpha 4 \beta 1$ within the alternatively spliced regions between type III repeats 14 and 15 (IIICS) and 7 and 8 (EIIIB) [19,20]. Integrin $\alpha 4 \beta 1$ binds to the LDV (Leu-Asp-Val) motif within the IIICS region and, surprisingly, focal adhesion formation does not depend on clustering of syndecan-4. Cells plated on to a recombinant polypeptide encompassing repeats 12–15 and the IIICS region, but in which the heparin-binding motifs have been mutated, spread and form vinculin-containing focal adhesions (Z. Mostafavi-Pour and M. J. Humphries, unpublished work). Likewise, cells form vinculin-containing adhesions when plated on the IIICS polypeptide in the presence of an excess of a soluble heparin competitor. Thus it would seem that clustering of syndecan-4 is essential for focal adhesion formation in cells adhering via integrin $\alpha 5 \beta 1$, but not those adhering via $\alpha 4 \beta 1$.

SYNDECAN-4 LOCALIZATION AND FUNCTION IN ADHERENT CELLS

Syndecan-4 co-localizes with vinculin in the focal adhesions of fibroblasts spread on fibronectin, vitronectin, laminin and type I collagen [14]. Syndecan-4 is recruited to focal contacts beneath the cell body of migrating fibroblasts [21], whereas most syndecan-4 is associated with the peripheral focal adhesions of static fibroblasts spread on fibronectin [15,17]. A model of molecular heterogeneity among adhesion complexes is now emerging, and there are reports of adhesions containing various complements of cytoskeletal proteins, depending on the position of the adhesion and the behaviour of the cell [22–24]. Syndecan-4 may be one of the proteins that is recruited to specific adhesions, in which case the properties of the extracellular matrix and the migratory status of the cell are likely to contribute to the localization. Syndecan-4 is also localized to ruffled membranes at the periphery of spreading cells (Z. Mostafavi-Pour and M. J. Humphries, unpublished work), and may form the focal point for the formation of new adhesions. Although syndecan-4 regulates vinculin recruitment during adhesion to the cell-binding domain of fibronectin, there is no direct evidence of syndecan-4 nucleating adhesion formation in lamellipodia. Indeed, the central localization of syndecan-4-containing adhesions in migrating cells suggests that syndecan-4 is not included in nascent peripheral adhesions, although the composition of newly formed adhesions is likely to differ in spreading compared with migrating cells.

Recruitment of syndecan-4 to adhesions is not dependent solely on the interaction with the extracellular matrix. Glycosylation-deficient CHO cells, overexpressing syndecan-4, form vinculin- and syndecan-4-containing adhesions, albeit at lower efficiency than wild-type cells [25]. Furthermore, overexpression of syndecan-4 mutants containing a truncated cytoplasmic domain prevents the formation of stress fibres and focal adhesions [17]. These studies demonstrate that the localization and function of syndecan-4 depend on interactions of the cytoplasmic domain, in addition to the interaction between the extracellular matrix and the substituted glycosaminoglycan chains.

The influence of syndecan-4 clustering can be seen during the earliest stages of adhesion formation. Cells plated on to recom-

binant polypeptides encompassing the cell-binding and heparin-binding domains of fibronectin form filopodia within 2 min, lamellipodia within 5 min and start to assemble stress fibres by 30 min [4]. Cells plated on to a polypeptide in which the heparin-binding site has been mutated failed to form filopodia and spread slowly, taking 60 min to spread fully. As might be expected, these cells never formed organized stress fibres. These experiments indicate that syndecan-4 stimulates processes that are generally associated with the Rho-family GTPases, including RhoA, Rac1 and Cdc42, and also the protein kinase C (PKC) family of serine/threonine kinases [26–28]. An involvement of syndecan-4 in GTPase and PKC signalling is supported by a putative role in cell migration. Invasion of squamous carcinoma cells into a collagen matrix, and migration of fibrosarcoma cells on the cell-binding domain of fibronectin, can be stimulated by a polypeptide encompassing the 13th fibronectin type III repeat [29,30]. These effects are abolished by pretreatment of the cells with heparinase or substitution of the arginine residues within the heparin-binding polypeptide. The antithrombin-stimulated migration of monocytes and lymphocytes is also abolished by removal of heparan sulphate chains or addition of anti-syndecan-4 antibodies [31], and disruption of the syndecan-4 gene in primary fibroblasts abolishes migration during *in vitro* wound healing assays [32]. It appears that cell migration directed by either extracellular-matrix or soluble factors is dependent on the heparan sulphate chains of syndecan-4, and this effect might be mediated by GTPases or PKC. The relative contributions of Rac and PKC may differ between cell types, as specific PKC inhibitors block the antithrombin-induced migration of lymphocytes, but not monocytes [31]. It will be interesting to see whether the variation in mechanisms is due to differences in the syndecan-4 interactions of different cell types, or whether it depends on the contribution of other receptors.

Syndecan-4-dependent signalling contributes to the survival of cells adhering via integrin $\alpha 5 \beta 1$. Cells plated on to a ligand of $\alpha 5 \beta 1$ detach from the matrix and enter apoptosis after 20 h unless stimulated with a heparin-binding fragment of fibronectin [33]. Once detached, the cells are committed to apoptosis and cannot be rescued by replating on to whole fibronectin. Cells adhering via integrin $\alpha 4 \beta 1$ to a modified IIICS region in which the heparin-binding residues have been substituted form vinculin-containing adhesions and do not enter apoptosis, regardless of syndecan-4 clustering. This suggests that syndecan-4 promotes survival by stimulating adhesion formation, rather than mediating a direct survival signal; whatever the mechanism, it appears that syndecan-4 plays an important role in the suppression of apoptosis.

DOMAIN STRUCTURE OF SYNDECAN-4

Each of the four members of the syndecan family is composed of a variable extracellular domain, and single-span transmembrane and cytoplasmic domains that are highly conserved between syndecans (Figure 2) [39]. The extracellular domains of each syndecan are substituted with heparan sulphate glycosaminoglycan chains at between three and five positions close to the N-terminus, although a small proportion of syndecans are substituted with chondroitin sulphate in certain tissues [11,12]. Conserved serine residues are substituted with four sugar moieties that act as a primer for polymerization of a GlcNAc-GlcA polysaccharide. The polysaccharide chains are extensively modified and sulphated, resulting in flexible heparan sulphate chains that can adopt a range of conformations [40], and interact with a number of ligands, including growth factors, extracellular-matrix proteins, chemokines and protease inhibitors [10]. The

heparan sulphate chains are essential for the normal function of proteoglycans [41] and mediate all known extracellular interactions of syndecan-4, although fibroblasts can adhere specifically to the immobilized syndecan-4 core protein [42]. The syndecan extracellular domains also include a membrane-proximal proteolytic cleavage site that is required for shedding of the ectodomain during wound healing [43]. The cleavage site probably comprises a cluster of basic residues that are conserved between mammalian syndecans, but are not conserved in *Drosophila* syndecan, which is nevertheless shed constitutively [38].

The syndecan transmembrane and cytoplasmic domains are highly conserved both between species and between isoforms [39]. The cytoplasmic and transmembrane domains of syndecan-4 share identity between human, mouse and chicken, suggesting that the cytoplasmic interactions of syndecans are of utmost importance. The transmembrane domain comprises a single-span hydrophobic helix, anchored at either end by charged residues. The cytoplasmic domain is 28 residues long and comprises a membrane-proximal conserved region (C1), a central variable region (V), and a short distal conserved region (C2). The conserved regions are almost identical with those in the other mammalian syndecans, and the syndecans of *Drosophila* and *Caenorhabditis elegans* (Figure 2B). The variable region is unique to syndecan-4, but shares greater similarity with syndecan-2 than with syndecans-1 and -3. Noteworthy features of the cytoplasmic tail are the high proportion of basic residues, which have a significant effect on the structure of the domain, and the conserved EFYA (Glu-Phe-Tyr-Ala) motif at the C-terminus, which conforms to the consensus PDZ ligand motif as described below.

SYNDECAN-4 EXISTS AS A HOMODIMER

Syndecan-4 exists as a dimer *in vivo*, regardless of clustering by extracellular ligand. Immunoblots of whole-cell extracts consistently show that the syndecan-4 core proteins exist as homodimers or higher-order multimers that are resistant to SDS and can only be separated into monomers by a single freeze-thaw step [44]. Chimaeric receptors, composed of the syndecan-4 transmembrane and cytoplasmic domains plus the Fc receptor extracellular domain, co-immunoprecipitate with endogenous syndecan-4 both before and after syndecan clustering with extracellular ligand [45]. The dimerization motif has been mapped to the transmembrane domain and four membrane-proximal residues of the ectodomain that are conserved in each of the syndecans (Figure 2B) [46–48]. Unlike the other syndecans, the cytoplasmic domain of syndecan-4 also forms SDS-resistant dimers, and the dimerization motif has been mapped to the central variable region [49]. The solution structure of the complete cytoplasmic domain confirms that a pair of syndecan-4 tails dimerize and that the variable regions form a twisted clamp structure [49]. Both the C1 and C2 conserved regions exhibit extensive flexibility in solution, although the C1 region would be constrained by the transmembrane domain in the intact syndecan-4. Owing to its highly basic nature, it is probable that the C1 region contributes to the structure of the syndecan-4 dimer. Unless neutralized by a negatively charged molecule such as acidic phospholipid, the mutual repulsion of the C1 domains might overcome the peptide-peptide interaction of the twisted clamp and disrupt the structure of the syndecan-4 cytoplasmic tails.

Phosphatidylinositol 4,5-bisphosphate (PIP₂) binds to the cytoplasmic domain of syndecan-4 and induces a molecular rearrangement of the dimeric peptide, as demonstrated by NMR

Figure 2 Domain structure of syndecan-4

(A) Syndecan-4 is composed of a large extracellular domain substituted with heparan sulphate at three positions, a single-span transmembrane domain, and a short cytoplasmic domain. The cytoplasmic domain can be subdivided into two conserved regions (C) and one variable region (V), based upon sequence conservation between syndecans, and binds a number of cytoplasmic proteins. (B) The C1 and C2 regions of the cytoplasmic domain are conserved between *Homo sapiens* syndecans-1–4 and the syndecans of *D. melanogaster* (D.m) and *C. elegans* (C.e) [34–38]. The first four residues of the extracellular domain are also conserved between mammalian syndecans, and are necessary for dimerization.

and CD [50]. The NMR structure shows that PIP_2 associates closely with lysine residues in the centre of the syndecan-4 twisted clamp, stabilizing the dimeric structure. The structural data suggest that interaction with PIP_2 regulates folding of the cytoplasmic domains of dimeric syndecan-4, and could influence other cytoplasmic interactions. There is also evidence from gel filtration studies that syndecan-4 cytoplasmic tails might form higher-order multimers in the presence of PIP_2 [51]. Although formation of these complexes has been observed *in vitro*, the existence or role of these structures *in vivo* have yet to be confirmed. Certainly, the interaction with PIP_2 is essential for the normal localization of syndecan-4. Substitution of the PIP_2 -binding lysine residues abolishes recruitment of the protein to the plasma membrane, and instead it remains sequestered in the Golgi [52].

PHOSPHORYLATION OF THE SYNDECAN-4 CYTOPLASMIC DOMAIN

The cytoplasmic domain of syndecan-4 includes one serine, one threonine and three tyrosine residues (Figure 2B) that are candidates for phosphorylation, and could regulate the cytoplasmic interactions of syndecan-4. In confluent fibroblasts, serine-183 is the only residue of the syndecan-4 cytoplasmic domain that is phosphorylated. The proportion of syndecan-4 that is phosphorylated in serum-starved cells rises from 30 % to

80 % upon PKC stimulation with phorbol ester [53], leading to enrichment of syndecan-4 in focal adhesions [21]. Phosphorylation can be inhibited by a generic PKC inhibitor, although not by an inhibitor that is specific to the conventional calcium-dependent PKCs. Peptides encompassing the syndecan-4 cytoplasmic domain are not phosphorylated by $\text{PKC}\alpha$, β or γ *in vitro*, and the serine residue does not fall within an RXXS consensus PKC phosphorylation motif [46]. This suggests that syndecan-4 might be phosphorylated by one of the calcium-independent PKC isoenzymes, and the KKDEGSY sequence of syndecan-4 bears most similarity to the consensus phosphorylation motif of $\text{PKC}\delta$ [54].

In vitro assays confirm that serine-183 is indeed a substrate for $\text{PKC}\delta$, and expression of dominant negative $\text{PKC}\delta$ in endothelial cells causes a 2.5-fold decrease in syndecan phosphorylation [55]. Serine-183 lies adjacent to the positively charged lysine residues that form the PIP_2 -binding site in syndecan-4 (Figure 2) [50], and phosphorylation of this residue reduces the affinity for PIP_2 by two orders of magnitude [51,56]. Given that PIP_2 binding influences the conformation of the syndecan-4 cytoplasmic domain, it appears that $\text{PKC}\delta$ has a significant effect and may regulate the cytoplasmic interactions of syndecan-4.

Two of the tyrosine residues within the syndecan-4 cytoplasmic domain fall within the C1 and C2 conserved regions (Figure 2B). Each of the mammalian syndecans, and the syndecans of *Drosophila* and *C. elegans*, contain a third tyrosine residue within

the variable region, but the residues surrounding this tyrosine vary between the syndecans. Substitution of the tyrosine residues of the conserved regions of syndecan-1 with phenylalanine has no effect on syndecan association with the actin cytoskeleton, suggesting that phosphorylation of these residues is not essential for normal syndecan function [57]. However, substitution of the tyrosine residue that falls within the variable region of syndecan-1 impairs association with the actin cytoskeleton and prevents normal localization of the syndecan. This suggests that the activity and cytoplasmic interactions of syndecan-1 are regulated by tyrosine phosphorylation, and it is conceivable that the tyrosine residues within the variable regions of the other syndecans play a similar role. A small proportion of syndecans-1 and -4 are constitutively tyrosine phosphorylated in adherent fibroblasts, and tyrosine phosphorylation can be increased to nearly 100% by treatment with the phosphatase inhibitor pervanadate [58]. Phosphorylation can be blocked with herbimycin A or PP1, specific inhibitors of Src-related kinases, in addition to broad-specificity kinase inhibitors such as staurosporine. The cytoplasmic domain of syndecan-3 binds to a protein complex including Src from cell lysates, and can be competed off by a polypeptide encompassing the C1 domain that shares 100% identity between syndecans-3 and -4. Gel overlay assays demonstrate that Src does not bind directly to the syndecan cytoplasmic domain, but via an unidentified 30 kDa protein, yet Src is still a strong candidate for tyrosine phosphorylation of syndecan-4 [59]. Herbimycin A also blocks focal adhesion formation in cells spread on fibronectin [60], which may be due, in part, to regulation of syndecan-4. However, little is known about the effect of tyrosine phosphorylation on the cytoplasmic interactions of syndecan-4, and it is difficult to draw conclusions at this time.

It has been noted that treatment of cells with pervanadate, which enhances syndecan phosphorylation, also triggers shedding of the syndecan extracellular domains [58]. The extracellular domains of syndecans can be cleaved at a membrane-proximal, protease-sensitive site [61], and the soluble ectodomains are found in the fluids surrounding wounded tissue, but not in normal human plasma, suggesting a role in wound healing [43]. Tyrosine kinase inhibitors block shedding induced by phorbol ester, cellular stress due to ceramide or a 42 °C heat shock [61]. Although tyrosine phosphorylation immediately regulates cleavage of the extracellular domain, activation of PKC or ligation of the thrombin or epidermal growth factor (EGF) transmembrane receptors also stimulates ectodomain shedding by regulating tyrosine kinase activity [43]. PKC inhibitors block shedding induced by phorbol esters, whereas EGF-stimulated shedding is blocked by inhibitors of ERK (extracellular-signal-regulated kinase) kinase, and there is minimal cross-talk between pathways. Although the cytoplasmic domains of syndecan-4 are substrates for tyrosine phosphorylation, it seems more likely that shedding is regulated by phosphorylation of the transmembrane metalloproteinase responsible for cleaving the extracellular domain of syndecan-4. Stimulated cells are capable of cleaving the syndecan-4 of adjacent cells in which the tyrosine kinases remain quiescent, precluding any dependence on the phosphorylation state of the syndecan-4 cytoplasmic tail [61]. This means that the effect of tyrosine phosphorylation of syndecan-4 on cell signalling remains unresolved; such phosphorylation is more likely to influence cytoplasmic interactions than to regulate extracellular activity.

SYNDECAN-4 BINDS AND STIMULATES PKC α

PKC isoforms are one family among a number of kinases that are activated upon adhesion to an extracellular matrix, and

evidence has emerged that activation of PKC α depends on the clustering and phosphorylation state of syndecan-4. Cells spread on ligands of integrin $\alpha 5 \beta 1$ form integrin-containing adhesions, but fail to activate PKC α or recruit cytoskeletal proteins to adhesions until stimulated with a syndecan-4 ligand [3]. Recruitment of cytoskeletal proteins to nascent focal adhesions can be driven by activation of PKC with phorbol ester, or blocked by PKC inhibitors [27], suggesting that syndecan-mediated activation of PKC α is required for recruitment of cytoskeletal proteins to focal adhesions.

PKC α and syndecan-4 co-immunoprecipitate from phorbol ester-stimulated cells and co-localize in the adhesions of embryonic fibroblasts [47]. Activated PKC α binds directly to the variable region of the syndecan-4 cytoplasmic tail in *in vitro* assays. Syndecan-4 binds to the catalytic domain of PKC α , although it is not an *in vivo* substrate for this particular PKC isoenzyme [46], and the interaction enhances the activity of PIP₂-bound PKC α above that of phospholipid-activated PKC [62]. PKC α activation by syndecan-4 is absolutely dependent upon association with PIP₂, and is abolished by substitution of lysine residues within the PIP₂-binding motif of the syndecan-4 cytoplasmic tail [52,56,62]. Thus it would appear that the variable region of syndecan-4 forms a ternary complex with PIP₂ and PKC α , leading to the hyperstimulation of PKC α (Figure 3). Phosphorylation of serine-183 within the syndecan-4 variable region prevents PIP₂ binding and inhibits PKC α activation, although it has no effect on the binding of PKC α to syndecan-4 [51,56]. Expression of a dominant negative PKC δ , the isoenzyme responsible for syndecan-4 phosphorylation, enhances PIP₂-dependent PKC α activity, but has no effect on phospholipid/Ca²⁺-stimulated PKC α [55]. This confirms that PKC δ regulates PKC α in a syndecan/PIP₂-dependent manner. Furthermore, overexpression of PKC δ in endothelial cells inhibits growth factor-stimulated proliferation to the same extent as a dominant negative PKC α , and expression of dominant negative PKC δ drives proliferation in the absence of growth factor. Based on these experiments, it has been proposed that the phosphorylation state of syndecan-4 regulates PKC α activation, which in turn mediates growth factor-stimulated proliferation.

PKC α and PKC δ are both activated in muscle cells adhering to fibronectin [63], but PKC α is localized to the focal adhesions of adherent cells, while PKC δ remains distributed diffusely throughout the cytoplasm [64]. PKC α becomes closely associated with active $\beta 1$ integrin, via the PKC regulatory domain, upon stimulation with phorbol ester, and drives recruitment of activated integrin to the membrane [65]. Cells adhere and spread more rapidly on fibronectin when stimulated with phorbol ester, and this effect can be reversed by a generic PKC inhibitor [66]. Phorbol esters also induce a 3–4-fold increase in the rate of migration on fibronectin, and it is possible that syndecan-4-mediated PKC α activation has a similar effect. Overexpression of PKC α in endothelial cells triggers an increase in migration, while overexpression of PKC δ causes the cells to become more adherent [28]. Although the effects of PKC α and PKC δ overexpression on migration have not been proven to be syndecan-4-dependent, in the light of the other experiments involving these isoenzymes, it seems highly probable. Overexpression of syndecan-4 suppresses migration, and causes cells to become more flattened and to form more focal adhesions [17]. This observation appears to contradict a model of stimulated cell migration due to syndecan-4-mediated PKC α activation. However, regulation will be dependent on both the phosphorylation state of syndecan-4 and localized PKC α activation, making it difficult to draw conclusions regarding the role of syndecan-4 in migration from these experiments. It does seem clear that syndecan-4 is important in

Figure 3 Syndecan-4 binds and activates PKC α in a PIP₂-dependent manner

The central variable region of the syndecan-4 cytoplasmic domain is rich in basic residues that bind PIP₂ and the kinase domain of PKC α , causing hyperactivation. PKC α activity is regulated by phosphorylation of Ser-183 of the syndecan-4 cytoplasmic domain, which prevents the association with PIP₂ and as a consequence causes deactivation of PKC α . Syndecan-4 is phosphorylated by PKC δ and dephosphorylated by a Ser/Thr phosphatase in response to activation of the fibroblast growth factor (FGF) receptor; PKC α activity is dependent on the balance between the two.

regulating the balance between static adhesion, migration and proliferation through a PKC α -dependent pathway.

SYNDECAN-4 IS A PUTATIVE PDZ DOMAIN LIGAND

PDZ domains are small protein-binding domains that specifically recognize a four-residue peptide motif that is usually located at the extreme C-terminus of the protein ligand [67]. In some additional cases, PDZ domains bind to internal peptide sequences; this involves the ligand folding into a tight β -hairpin that mimics the C-terminus [68]. In both cases, the PDZ domain binds the ligand through hydrogen bonds between the polypeptide backbones of both the PDZ domain and the ligand (Figure 4B). There are further hydrogen bonds between the PDZ domain and the free C-terminus of the ligand, and it is for this reason that PDZ domains rarely recognize internal sequences. The specificity of an interaction is determined by the amino acid side chains of the ligand, which fit into either charged or hydrophobic pockets of the PDZ domain. The majority of PDZ ligands have a non-polar residue at the C-terminus (termed P₀) that is accommodated by a hydrophobic pocket in the PDZ domain. However, it is the residue at P₋₂ that is the major determinant of PDZ specificity. This position is frequently occupied by a polar residue (serine or threonine) that can be co-ordinated by a histidine, or by a large hydrophobic residue (tyrosine or phenylalanine) that sits in a hydrophobic pocket of the PDZ domain (Figure 4) [69]. On this basis, most PDZ ligands have been categorized as either class I or class II respectively, although there are additional ligand motifs that do not fall into either of these broad classes. There are further determinants of PDZ domain specificity beyond the four C-terminal residues, and PDZ domains do not tend to be promiscuous, even among a single class of ligand [69]. There are reports of syndecans interacting with a number of PDZ domains, and the C-terminal

EFYA motif is conserved in all four syndecans, not only in mammalian species, but also in the syndecans of *C. elegans* and *Drosophila* (Figure 2B). The C-terminal alanine residue is consistent with a PDZ ligand motif, and the phenylalanine residue at P₋₂ marks syndecan as a class II ligand.

Syntenin is an example of a PDZ domain-containing protein that recognizes the cytoplasmic domains of syndecans [70]. Syntenin was originally isolated from a yeast two-hybrid screen using the cytoplasmic tails of each of the four mammalian syndecans, and the C-terminal EFYA motif was found to be sufficient for binding. Syntenin is composed of two PDZ domains, an N-terminal domain and a short C-terminal domain. Both PDZ domains are required for interaction with clustered syndecan tails, although mutagenesis has shown that each PDZ domain specifically recognizes the syndecan C-terminal motif [71]. A second PDZ domain-containing protein reported to bind to syndecans is the LIN-2 homologue CASK [72]. As for syntenin, the interaction between CASK and syndecan was identified through a yeast two-hybrid screen, and competition binding experiments have confirmed that CASK probably interacts with the EFYA motif of all four syndecans. The PDZ domain of CASK has been crystallized [73] and includes a hydrophobic pocket that would accommodate a hydrophobic residue in the ligand at P₋₂. The C-terminus of syndecan can be modelled on to the crystal structure (Figure 4A) and, significantly, the histidine residue that confers specificity for class I ligands is absent from both the CASK and syntenin PDZ domains (Figure 5). Substitution of the serine and aspartate residues in the syntenin PDZ domains with histidine abolishes the interaction with syndecan, confirming that these residues are required for a specific interaction [71].

Synectin [also referred to as GIPC (GAIP-interacting protein C-terminus)] is another putative syndecan-4-binding protein that was identified through a two-hybrid screen [75]. Sequence align-

Figure 4 The C-terminus of syndecan-4 can be modelled on to the ligand-binding pocket of the CASK PDZ domain

(A) The PDZ domain of CASK has been crystallized, complexed to the C-terminus of an adjacent PDZ domain [73], and the C-terminal EFYA motif of syndecan-4 can be modelled on to this structure. The large hydrophobic pocket on the surface of CASK, composed of Val-159 and the surrounding non-polar residues, could accommodate the phenylalanine residue at P₋₂ of the syndecan-4 cytoplasmic tail, conferring specificity for the class II ligand. (B) Schematic representation of the interaction between the CASK PDZ domain and syndecan-4. The C-terminus of syndecan-4 is co-ordinated by electrostatic interactions between the peptide backbones of the PDZ domain and the ligand. Specificity is determined by the hydrophobic pocket that binds the phenylalanine residue at P₋₂ of the syndecan-4 tail.

ments reveal that the ligand-binding pocket of the syndectin PDZ domain contains a histidine residue (Figure 5) that should confer specificity for class I ligands. Furthermore, there is evidence that syndectin binds to the melanosomal membrane protein gp75 [76]

and to integrins $\alpha 5$ and $\alpha 6$ [77], each of which includes a serine or threonine residue at P₋₂. These data raise the possibility that syndectin does not in fact interact with syndecan-4 *in vivo*, or at least not via the PDZ domain, which is not sufficient to support binding to syndecan-4 when other residues are deleted [75]. The absence of such an interaction would not exclude the recruitment of syndectin to focal adhesions. Syndectin may be recruited to an integrin cytoplasmic tail, allowing co-immunoprecipitation with syndecan-4 as part of a large protein complex. Indeed, there is evidence of syndectin co-localizing with $\alpha 6$ integrin at the ends of retraction fibres in colon carcinoma cells, although syndectin is also reported to localize to lamellipodia, where the integrin is not found [77].

ORGANIZATION OF SYNDECANS BY MOLECULAR SCAFFOLD PROTEINS

PDZ domain proteins commonly act as molecular scaffolds, linking a number of binding partners through multiple PDZ domains and other protein-binding motifs [78]. Of human PDZ domain-containing proteins, 18% include three or more PDZ domains and 72% contain additional protein recognition motifs [79]. PDZ domain proteins are frequently involved in clustering transmembrane receptors via their C-terminal motifs and linking them to cytoskeletal or signalling proteins [80]. The two known syndecan-4-binding proteins, syntenin and CASK, have been implicated in this type of role, although they have relatively simple domain structures compared with other scaffold proteins. Syntenin contains a pair of PDZ domains, but no additional protein-binding motifs [70], and the close proximity of the PDZ domains means that a pair of protein ligands linked by syntenin would need to be very closely associated. Given that syndecans exist as homodimers [45], it is probable that the two PDZ domains of syntenin bind to a pair of linked syndecan cytoplasmic tails to form a stable protein complex. This model is supported by the observation that syntenin binds to syndecan tails in two-hybrid experiments in which the syndecan cytoplasmic domains are presented as dimers, but fails to bind monomeric syndecan tails in *in vitro* binding assays [70].

The *in vivo* association of syntenin with syndecan has been confirmed by immunoprecipitation [81], and immunofluorescence studies have demonstrated extensive co-localization of the proteins both at the plasma membrane and in vesicular membranes of syndecan-4-transfected CHO cells [70]. Syntenin is distributed diffusely in untransfected CHO cells, demonstrating that the interaction with syndecan is essential for the normal distribution of syntenin in subconfluent cells. Blot overlay assays using whole-cell extracts and syntenin fused to glutathione S-transferase have confirmed that syndecans are the major syntenin ligand in human epithelial cells [71].

No additional protein recognition motifs have been identified in syntenin, although the N-terminal domain includes tyrosine and proline motifs that could form ligands for Src homology 2 (SH2) and SH3 domains. However, there are several examples of PDZ domains forming dimers that could allow the formation of higher-order protein complexes [82]. It is possible that part of syntenin could fold into a β -hairpin finger that might act as a ligand for the PDZ domain of a second syntenin molecule. Due to differences in the consensus motifs of C-terminal ligands and β -fingers that bind to the same PDZ domain [82], it is impossible to search for a putative β -finger sequence in syntenin that might support this type of dimerization mechanism. Syntenin forms homodimers *in vivo*, and the PDZ domains are sufficient for dimerization, although the N-terminal domain increases the avidity of the interaction [83]. Point mutations within the carboxylate-

Figure 5 Sequence conservation of class I and class II PDZ domains

The PDZ domains of syntenin, CASK, 55K, hDlg and syntectin were aligned by Clustal W [74]. The positions of α -helices and β -strands, assigned according to the crystal structure of the CASK PDZ domain, are shown above the alignment, and residues that line the ligand-binding pocket are marked underneath. The residue that acts as the key determinant of ligand specificity is marked with a star, and is usually a histidine residue in class I PDZ domains such as those of hDlg and syntectin. This residue is more poorly conserved in class II PDZ domains and contributes to the hydrophobic pocket. Residues that are identical are shaded in black and conservative substitutions are shaded grey.

binding loops of the syntenin PDZ domains reduce or abolish dimerization, suggesting that the interaction involves PDZ domain occupancy. Although isolated syntenin PDZ domains fail to bind to clustered syndecan tails, recombinant tandem repeats of the second (but not the first) syntenin PDZ domain bind to syndecan in two-hybrid and *in vitro* assays [71]. These experiments suggest that there is an allosteric relationship between a pair of PDZ domains that is required for ligand recognition, and that the PDZ domains of syntenin differ in their ligand specificity. The two PDZ domains of syntenin might bind to a heterologous pair of ligands *in vivo*, allowing syntenin to either dimerize or bind a second protein ligand while associated with syndecan. Indeed, dimerization of syntenin could prove necessary for a stable interaction between syndecan and syntenin, since surface plasmon resonance experiments have shown that the PDZ domains of syntenin dissociate rapidly from immobilized syndecan cytoplasmic domains [70].

In addition to clustering syndecans, syntenin may have a role in linking syndecan-4 to other transmembrane receptors or cytoplasmic factors (Figure 6). There are reports of syntenin binding to the C-terminal motifs of a number of other transmembrane receptors and signalling proteins, including neurofascin, protein tyrosine phosphatase η , B ephrins and neuroligin [71,83–85]. Syntenin also co-fractionates and co-immunoprecipitates with syndecan-1 and the E-cadherin complex in fibroblastic and epithelial cells [81]. Several of these receptors, including B ephrin and E-cadherin, are localized exclusively to cell–cell junctions, whereas syndecan-4 is localized to the basal membrane of cells [15], precluding the simultaneous association of syntenin with syndecan-4 and cell–cell adhesion receptors. These observations suggest that syntenin might associate with different syndecans or alternative transmembrane ligands under different circumstances. Immunofluorescence studies have shown that, in primary fibroblasts, syntenin localizes to focal adhesions and decorates stress fibres, in a similar pattern to syndecan-4 [81]. In comparison, syntenin co-localizes with syndecan-1 along the length of the cell–cell contact in confluent MDCK and MCF-7 epithelial cells. The spatial constraints of syndecan-4 limit the number of multi-molecular protein complexes that could be formed, and the alternative localization of syntenin suggests that association of syndecan-4 with cytoplasmic proteins is a regulated process. It has been reported recently that the PDZ domains of syntenin and CASK bind to

PIP₂, and that the paired PDZ domains of syntenin can be recruited to PIP₂-rich membranes [86]. PDZ domains cannot associate simultaneously with PIP₂ and a peptide ligand, which means that PIP₂ might regulate the association between syndecan-4 and PDZ domain-containing proteins *in vivo*. It is interesting to note that PDZ1 of syntenin has higher affinity for PIP₂ than PDZ2, the opposite arrangement to the interaction between syntenin and syndecan-4. This could mean that syntenin interacts with PIP₂ and syndecan-4 simultaneously through the paired PDZ domains, and stabilizes the association of syndecan-4 with lipid rafts.

CASK is the human homologue of the *C. elegans* PDZ domain protein LIN-2, which interacts with transmembrane receptors as part of the LIN-2–LIN-7–LIN-10 PDZ domain protein heterotrimer [87]. Immunoprecipitation studies have revealed that the mammalian homologues of LIN-2, LIN-7 and LIN-10 (CASK, Veli and Mint1 respectively) bind to the cadherin complex through a direct interaction between Veli and β -catenin [88]. In addition to the Veli- and Mint1-binding sites, CASK contains several other protein-binding motifs that might indirectly link transmembrane receptors to the actin cytoskeleton. CASK is a member of the membrane-associated guanylate kinase (MAGUK) protein family [89], and contains an SH3 domain, a guanylate kinase domain and a calmodulin-dependent kinase domain in addition to a single PDZ domain [72]. The PDZ domain of CASK binds to the cytoplasmic tails of syndecans [72,90], preferentially recognizing syndecans-2 and -4 over syndecans-1 and -3 [71]. CASK co-localizes with syndecans-1 and -2 in the basolateral membranes of epithelial cells, and the two proteins co-immunoprecipitate from COS cells transfected with CASK and syndecan-2 [72,90]. Site-directed mutagenesis of the guanylate kinase and calmodulin-dependent kinase domains of LIN-2 has no effect on LIN-2 function in *C. elegans* [89]. This suggests that, rather than possessing intrinsic catalytic activity, CASK probably acts as a scaffolding protein, like other MAGUK family members. The PDZ domain of CASK binds to additional transmembrane receptors, including neuroligin in rat neurons [91] and junctional adhesion molecule in epithelial cells [92]. Like syntenin, CASK might link a number of transmembrane receptors in an adhesion complex, although the absence of multiple PDZ domains in CASK would necessitate the formation of a large multi-molecular protein complex. As with syntenin-containing complexes, the basal localization of syndecan-4 will

Figure 6 Syndecan-4 acts as an organizing centre for transmembrane receptors and is anchored to the actin cytoskeleton

The cytoplasmic domains of syndecan-4 interact with scaffold proteins, such as syntenin and CASK, that might in turn recruit additional transmembrane receptors (such as integrins, phosphatases and growth factor receptors) to adhesions. Syndecan-4 is also linked to the actin cytoskeleton through CASK and the ERM family of actin-binding proteins, which includes protein 4.1 and ezrin. Clustering of syndecan-4 into focal adhesions is essential for adhesion formation in cells adhering via integrin $\alpha5\beta1$, and may depend on both a mechanical link between receptors and activation of signalling pathways. PTP, protein tyrosine phosphatase; 1 and 2 represent PDZ domains 1 and 2 respectively.

limit the connections that can be mediated by CASK. Although CASK can be recruited to cell–cell junctions through interaction with junctional adhesion molecule or the cadherin complex, it is not possible to mediate a link between these proteins and syndecan-4.

SYNDECAN-4 PROVIDES A MECHANICAL LINK BETWEEN EXTRACELLULAR LIGANDS AND THE ACTIN CYTOSKELETON

Integrins are believed to be the transmembrane receptor responsible for linking the extracellular matrix to the acto-myosin contractile apparatus of the cell, but they are not the only receptor to associate with the actin cytoskeleton [93]. Syndecan-4 is localized to focal adhesions and aligns with actin stress fibres of subconfluent fibroblasts [14], and associates directly with the Triton-insoluble cytoskeleton of mesangial cells [94]. Syndecan-4 and α -actinin remain associated with focal adhesions and microfilament bundles in the presence of detergent concentrations that disrupt the localization of the actin-associated proteins talin, vinculin and paxillin, demonstrating that syndecan-4 is anchored securely to the actin cytoskeleton. Clustering of syndecan-4 stimulates the rearrangement of the actin cytoskeleton into ordered stress fibres, yet recruitment of syndecans into focal complexes is dependent on the actin cytoskeleton and can be disrupted by cytochalasin D [95]. This suggests that a two-way relationship exists between syndecan-4 and the actin cytoskeleton, and that syndecan-4 associates with actin through proteins bound to its cytoplasmic domain (Figure 6). The PDZ domain protein CASK contains a Hook domain that binds to the actin/spectrin-binding protein 4.1 [72]. Protein 4.1 is a member

of the ERM protein family, so called because it includes ezrin, radixin and moesin. Each of the ERM family members includes a C-terminal actin-binding site and a binding site for a transmembrane receptor that links actin filaments to a membrane-bound protein complex. The CASK–protein 4.1 complex promotes assembly of actin microfilaments in brain extract, and can link the microfilaments to the cytoplasmic tail of neuexin, an alternative CASK PDZ domain ligand [96]. Although there is not, as yet, any evidence of an interaction between syndecan-4 and protein 4.1, it is possible that a protein complex including syndecan-4, CASK and protein 4.1 might direct the arrangement of actin filaments in non-neuronal cells, where syndecan-4 is likely to act as the ligand of the CASK PDZ domain.

The link between syndecan-4 and the cytoskeleton might be reinforced by a direct interaction between the cytoplasmic domain of syndecan-4 and members of the ERM family. Ezrin co-immunoprecipitates with syndecan-2 from COS-1 cells due to an interaction between the N-terminus of ezrin and the cytoplasmic domain of syndecan-2 [97]. The actin-binding domain of ezrin is essential for the association of syndecan-2 with the Triton-insoluble cytoskeleton, revealing a mechanism by which syndecans are anchored securely to the actin cytoskeleton. Ezrin does not bind to syndecan-1, but may bind to syndecan-4, which shares greater identity with syndecan-2. The N-terminal domains of the ERM family members are highly conserved between proteins, so it is possible that syndecan-4 could bind to another family member, such as protein 4.1, re-inforcing the hypothetical link between syndecan-4 and actin through CASK and protein 4.1. It is interesting to note that, in erythrocytes, the MAGUK protein p55, which is closely related to CASK, binds to the

transmembrane receptor glycoporphin C, and that both p55 and glycoporphin C bind to protein 4.1 [98]. Similarly, the intrinsic protein 4.1-binding activity of neuexin enhances the CASK-dependent recruitment of protein 4.1 to the cytoplasmic domains of neuexin [96]. These examples set precedents for ternary complex formation involving a transmembrane receptor, a MAGUK protein such as CASK, and an ERM-family protein. The interaction between glycoporphin C and protein 4.1 is dependent on a basic motif within the cytoplasmic tail of glycoporphin C [98]. The cytoplasmic tail of syndecan-4 includes a similar abundance of basic residues, and could bind protein 4.1 by a similar mechanism. Although there is no direct evidence for the formation of a ternary complex including syndecan-4, CASK and protein 4.1 or ezrin, it is an attractive model that cannot be discounted.

Evidence that syndecan-4 might be involved in the transmission of tension between matrix and cytoskeleton comes from a study using cultured vascular smooth muscle cells. Application of mechanical tension to cultured cells induced the transcription and translation of syndecan-4 within 30 min, followed by redistribution of the membrane-bound pool [99]. When cells were exposed to cyclic strain for longer than 1 h, the dorsal pool of membrane-associated syndecan-4 diminished, while total membrane-associated syndecan-4 actually increased, indicating significant recruitment to the ventral membrane. Immunofluorescence studies confirmed that syndecan-4 was redistributed from the leading lamellae to the ventral membrane, beneath the body of the cell, accompanied by simultaneous dissociation of vinculin from focal adhesions and the ventral surface. It is interesting to note that cells overexpressing syndecan-4 are unable to show specific redistribution of syndecan-4 to the ventral membrane; consequently, vinculin does not dissociate from adhesions in response to tension. This experiment provides further evidence that syndecan-4 regulates the recruitment of cytoskeletal proteins, such as vinculin, to focal adhesions, and does so as a consequence of tension between the extracellular matrix and the actin cytoskeleton.

RECRUITMENT OF ADAPTER PROTEINS BY SYNDECAN-4

Certain cytoplasmic proteins that lack catalytic activity, yet do not mediate a direct structural link between transmembrane receptors and the actin cytoskeleton, are commonly regarded as adapter proteins, involved in the organization of the focal adhesion. There is evidence that syndecan-4 might recruit such proteins to the focal adhesion indirectly through further interactions of the syndecan cytoplasmic tail. Syndesmos is a small cytoplasmic protein that binds to the variable and conserved membrane-proximal regions of syndecan-4, but does not bind to the cytoplasmic domain of other syndecans in *in vitro* assays [100]. Syndesmos is myristoylated, allowing it to anchor directly to the membrane and co-localize with syndecan-4 in ventral adhesions. Overexpression of syndesmos in fibroblasts augments cell spreading and filopodia formation, suggesting that syndesmos might be one of the mediators of syndecan-stimulated spreading [100]. Immunoprecipitation experiments have identified an *in vivo* interaction between syndesmos and tyrosine-phosphorylated paxillin, and *in vitro* assays have confirmed that syndesmos binds both paxillin and the homologue Hic-5 [101]. This observation is particularly significant in the context of the differential requirement for syndecan-4 during adhesion to ligands of $\alpha 4$ and $\alpha 5$ integrins. Unlike cells adhering to fibronectin via integrin $\alpha 4 \beta 1$, those adhering via $\alpha 5 \beta 1$ require clustering of syndecan-4 in order to form vinculin-containing focal adhesions (Z. Mostafavi-Pour and M. J. Humphries, unpublished work). It

has been suggested that paxillin binds directly to the integrin $\alpha 4$ subunit and is required for $\alpha 4$ -mediated cell migration [102]. Syndecan-4 and syndesmos may be responsible for the indirect recruitment of paxillin to integrin $\alpha 5$ -containing adhesions during cell spreading.

The *in vivo* interaction between paxillin and syndesmos is dependent on PKC activation [101], which might be stimulated by syndecan-4. PKC α is activated in cells adhering via integrin $\alpha 5 \beta 1$ upon syndecan stimulation, but remains inactive in cells adhering via $\alpha 4 \beta 1$ (Z. Mostafavi-Pour and M. J. Humphries, unpublished work). Paxillin is serine phosphorylated by PKC during adhesion to fibronectin and vitronectin [103,104], and this may be the signal that regulates the association of paxillin and syndesmos.

SYNDECAN-4 SIGNALS VIA RHO-FAMILY GTPases

As already discussed, there is a great deal of circumstantial evidence to suggest that syndecan-4 might regulate signalling by Rho-family GTPases. Based upon the dramatic induction of stress-fibre formation in cells stimulated with a heparin-binding polypeptide or antibodies against syndecan-4, it has been proposed that syndecan-4 regulates RhoA activation directly [16]. The Rho inhibitor C3 exotransferase blocks focal adhesion and stress-fibre formation in response to syndecan-4 clustering. Conversely, stimulation of Rho with lysophosphatidic acid, in the absence of syndecan-4 clustering, drives adhesion and stress-fibre formation to a level that is comparable with that in cells plated on to whole fibronectin. These experiments suggest that Rho is involved in syndecan-4-stimulated adhesion formation, but there remains a lack of evidence that RhoA is activated as a direct consequence of clustering of the syndecan-4 cytoplasmic tails. For this to occur, it would probably be necessary for syndecan-4 to recruit a guanine nucleotide exchange factor (GEF) that could activate one or more of the GTPases. It has been reported that members of the ERM family stimulate dissociation of RhoA from the inhibitor Rho guanine nucleotide dissociation inhibitor (GDI), allowing GTPase activation [105]. Paxillin recruits the putative ADP-ribosylation factor/GTPase-activating protein (GAP) paxillin kinase linker (PKL) to focal adhesions through interaction of the leucine-rich domain 4 (LD4) motif, and regulates Rac activation [106]. Mutation of the LD4 motif of paxillin or the paxillin-binding site of PKL causes uncontrolled activation of Rac, leading to random migration. Both paxillin and ERM proteins are associated with syndecan-4 protein complexes, and could provide a link to GTPase signalling. Tiam-1 is a specific Rac GEF containing a class II PDZ domain that might bind to the cytoplasmic domain of syndecan-4 [70]. Tiam-1 would provide a more direct mechanism for GTPase activation by syndecan-4, but, like all the models discussed here, the validity of this hypothesis remains unclear.

Neurofibromin is a Ras GAP that binds to the membrane-proximal domain of all four syndecans *in vitro*, and co-immunoprecipitates with syndecan-3 and CASK from rat brain [107]. It is expressed primarily in neuronal tissue, where syndecan-3 is the most abundant syndecan, but is also co-expressed with syndecan-4 in glial cells. Neurofibromin includes two distinct syndecan-binding polypeptides that might interact with the cytoplasmic domains of a pair of clustered syndecans or form a single binding pocket in the folded protein. Neurofibromin acts as a tumour suppressor, and down-regulates Ras by stimulating intrinsic GTPase activity [108]. The interaction with syndecans might be responsible for localized Ras inactivation at the membrane, and could influence cell proliferation. Although Ras is not a member of the Rho GTPase family, neurofibromin remains

the only example of direct regulation of GTPases by syndecan-4, and over the next few years we can expect a drive to identify other GAPs, GEFs and GDIs that interact with the cytoplasmic domains of syndecans.

SYNDECAN-4 INTERACTS WITH RECEPTOR TYROSINE KINASES

Heparan sulphate proteoglycans, and syndecan-4 in particular, play an important role in growth factor signalling, by modulating both extracellular and cytoplasmic interactions of the receptors. The heparan sulphate chains of the extracellular domain of syndecan-4 recruit soluble growth factors, including fibroblast growth factor (FGF) and transforming growth factor β 1, to the membrane, and enable binding to the high-affinity receptor [12,109]. The mechanism by which syndecan-4 induces association between growth factor and receptor remains unresolved; possibilities include presentation of the growth factor to the receptor by raising the local concentration, induced dimerization of the growth factor, or appropriate orientation of the growth factor for receptor binding [40]. However, the interaction between syndecan-4 and growth factor is not merely a passive mechanism for immobilizing the growth factor at the membrane. Upon clustering with basic FGF (bFGF), syndecan-4 redistributes to non-caveolae membrane rafts on the apical surface of the cell [45], which may bring syndecan-4 into close association with the FGF receptor. The cytoplasmic domain of syndecan-4 also contributes to growth factor signalling: while chimaeric receptors composed of the syndecan-4 cytoplasmic domain and heparan sulphate-substituted extracellular domains enhance the cellular response to bFGF, chimaeras lacking the cytoplasmic domain do not [110]. Glycosylation-deficient chimaeras also fail to enhance the response to bFGF, demonstrating that both the cytoplasmic domain and the glycosaminoglycan chains of syndecan-4 regulate growth factor signalling. Substitution of the PIP₂- or PDZ domain-binding sites within the syndecan-4 cytoplasmic tail results in a dominant negative phenotype that blocks migratory and proliferative responses to bFGF [52]. This is achieved by inhibition of bFGF-dependent PKC α activation, which appears to be essential for growth factor-stimulated signalling. Stimulating cells with bFGF decreases serine phosphorylation of the syndecan-4 cytoplasmic domain, and can be blocked by type I or type 2A serine/threonine phosphatase inhibitors (Figure 3) [53]. A syndecan-4 mutant containing a truncated PDZ domain-binding motif becomes hyperphosphorylated, particularly after stimulation with bFGF [52]. These data suggest that the FGF receptor regulates syndecan-4 by activating a serine phosphatase that normally associates with the C-terminus of syndecan-4 via a PDZ domain. Dephosphorylation permits recruitment of PIP₂, which in turn activates PKC α , and forms an integral part of the FGF-stimulated signalling cascade.

There is genetic evidence that mammalian CASK may play a role in growth factor receptor signalling. The LIN-2–LIN-7–LIN-10 complex, which is the *C. elegans* homologue of the mammalian CASK–Veli–Mint1 complex, binds the LET-23 EGF receptor tyrosine kinase through the PDZ domain of LIN-7 [111]. It is possible that the CASK complex clusters the growth factor receptors of mammalian cells in the same manner, although there is no evidence for a direct interaction between the CASK–Veli–Mint1 complex and mammalian EGF or FGF receptors. The PDZ domain of LIN-7 recognizes the C-terminal ETCL (Glu-Thr-Cys-Leu) sequence of the LET-23 receptor, which forms a consensus class I PDZ ligand. Only the C-terminus of ErbB-4, out of the mammalian EGF and FGF receptors, contains the consensus sequence of a class I PDZ ligand [112], but this does not rule out growth factor receptor clustering by the CASK

complex. Veli and Mint1 may bind to transmembrane receptors through alternative protein-binding motifs, and promote clustering of receptors around the syndecan complex. It also seems likely that other syndecan-associated scaffolding proteins, such as syntenin, could recruit transmembrane receptors (as discussed above), and syndecan-4 might in fact act as an organizing centre for adhesion and growth factor-dependent signalling.

Receptor tyrosine kinases are not the only tyrosine kinases that are activated in response to adhesion to fibronectin. Focal adhesion kinase (FAK) becomes tyrosine phosphorylated and activated within 30 s of plating on to fibronectin. Fibroblasts spread on the cell-binding domain of fibronectin exhibit limited FAK phosphorylation until stimulated with the heparin-binding fragment of fibronectin, and syndecan (–/–) fibroblasts exhibit a similar decrease in FAK activation [33,113]. FAK phosphorylation can be restored by activation of Rho, but not PKC, demonstrating that clustering of syndecan-4 is necessary for FAK activation and that the signal is mediated by Rho, but not PKC. Previous experiments have shown that integrin clustering with specific antibodies induces FAK phosphorylation [114], and it appears that FAK activation is regulated by co-operative signals from both integrins and syndecans. It is interesting that disruption of the FAK gene in mice results in a similar phenotype to disruption of the fibronectin gene, suggesting that FAK mediates signalling by fibronectin receptors [115]. FAK-null fibroblasts exhibit defects in migration and spreading that resemble the behaviour of fibroblasts in the absence of syndecan-4 clustering, although they do form focal adhesions. Given that FAK propagates signals required for migration and adhesion formation, a model of syndecan-4-dependent FAK activation is consistent with the role of syndecan-4 that has already been described.

Syndecan clustering may also regulate tyrosine phosphorylation by association with tyrosine phosphatases. Syntenin binds to the C-terminus of protein tyrosine phosphatase η , via its paired PDZ domains, and could recruit the transmembrane phosphatase into a syndecan-containing protein complex [84]. Protein tyrosine phosphatase η is negatively regulated by PKC, so that recruitment into a syndecan-containing adhesion might result in localized inactivation of the phosphatase. This phosphatase has not been co-localized with syndecan-4 by immunofluorescence, but the model depicts an alternative mechanism whereby syndecans could regulate phosphorylation-dependent signalling.

SYNDECAN-DEPENDENT VESICLE TRAFFICKING

The cytoplasmic domains of syndecans-2 and -4 are reported to bind to the novel cytoplasmic protein synbindin [116]. The C-terminal EFYA motif of the syndecan tail is essential for this interaction, and synbindin and syndecan-2 co-localize in and co-immunoprecipitate from transfected cells in an EFYA-dependent manner. Synbindin lacks an intact PDZ domain; although the polypeptide that encompasses the syndecan-binding site resembles the C-terminal part of a number of PDZ domains, it lacks the peptide loop that hydrogen-bonds the backbone of the EFYA ligand. This means that the syndecan-4-binding site of synbindin is either a novel variant of the classical PDZ domain or an entirely different peptide-binding motif altogether.

Synbindin resembles a yeast protein (p23) that is a component of the multimeric complex TRAPP (transport protein particle), which is involved in vesicle fusion with the membrane. The central domain of synbindin resembles other proteins involved in

vesicle transport, including the yeast protein BET 5. Subcellular localization in neurons confirms that synbindin associates with transport vesicles, and suggests that it may be involved in membrane trafficking. Whether synbindin, which is widely expressed, can play a similar role in non-neuronal cells in association with syndecan-4 has yet to be addressed. Syntenin is also localized to early endocytic and recycling endosomes in MDCK cells [117]. The vesicular co-localization of syndecan-binding proteins suggests that syndecan-4 itself could be involved in endocytosis and membrane trafficking.

WOUND HEALING *IN VIVO*

It is now generally accepted that syndecan-4 plays a key role in wound healing in mature animals, and a number of examples of syndecan-4 expression in response to wounding have been documented. There is a transient increase in the expression of syndecan-4 in granulation tissue, along the edges of wounds in human or murine neonatal skin. Expression is induced throughout the dermis within 12 h of wounding and persists for approx. 10 days, by which stage the wound is almost healed [118]. A similar response is seen upon wounding of the arteries of mice or rats, where syndecan-4 expression is induced rapidly throughout the vessel wall around the site of injury [119,120]. The increase in syndecan-4 expression correlates with an increase in proliferation that could be explained by PKC α activation by syndecan-4. Expression of syndecan-4 appears to be essential for maintenance of the proliferative capacity of cells. For example, myogenic muscle cells are the only adult skeletal muscle cells to retain expression of syndecans-3 and -4 [121]. These cells also retain expression of FGF and hepatocyte growth factor receptors that are activated during muscle regeneration after wounding, in a heparan sulphate-dependent manner. Thus it would appear that syndecan-4 potentiates growth factor-stimulated proliferation after wounding in adult tissue. Expression of syndecan-4 is elevated in the highly proliferative keratinocytes and endothelial cells around the edge of the wound, and is not restricted to the migratory keratinocytes within the fibrin clot [118]. This suggests that overexpression of syndecan-4 during wound healing is primarily responsible for the up-regulation of proliferation, rather than migration, to close the wound.

Syndecan-4-null mice develop normally and are indistinguishable from their wild-type littermates at birth, indicating that syndecan-4 is not essential for embryonic development. However, wound healing in the adult mice is delayed due to failure of the dermis to form granulation tissue within 3 days, and a subsequent failure to revascularize the healing wound [32]. The decrease in the formation of granulation tissue and angiogenesis are both indicative of a loss of proliferative capacity of cells surrounding the wound. *In vitro* data suggest that syndecan-4 regulates both migration and proliferation, and the role of syndecan-4 in wound healing is likely to depend on both of these processes, despite the recent emphasis on the regulation of proliferation.

Ectodomain shedding of syndecan-4 is stimulated after wounding by a proline-rich antimicrobial peptide present in the fluid surrounding the wound [122]. Hence elevated expression is necessary to maintain a stable pool of membrane-associated syndecan-4 in cells surrounding the wound [43]. The net result of the increase in syndecan-4 expression and shedding is that the tissue fluid surrounding the wound contains an unusually high concentration of soluble heparan sulphate proteoglycan that probably regulates growth factor-stimulated signalling [123]. Quite what the increase in turnover means to the cytoplasmic interactions of syndecan-4 is unclear, but it is bound to have a

dramatic effect on signalling cascades and cytoskeletal arrangement.

MANIFESTATION OF SYNDECAN-4 ABNORMALITIES IN DISEASE

Changes in syndecan-4 expression have been described in a number of pathological disorders. Overexpression of syndecan-4 is often associated with an increase in cell proliferation and migration that is easily reconciled with our understanding of the interaction between syndecan-4 and PKC *in vitro*. Biopsies from patients suffering from the proliferative kidney disease IgA nephropathy exhibit overexpression of syndecan-4 and cytoskeletal proteins that include α -actinin, vinculin and paxillin [94]. Likewise, liver carcinomas exhibit overexpression and an enriched cytoplasmic pool of syndecans-1 and -4 [124]. The increase in cell proliferation in both of these pathologies has been attributed to the enhanced response to soluble growth factors.

Syndecan-4 activity can also be altered indirectly and result in a pathological phenotype. Tenascin-C is an adhesion-modulating matrix molecule that binds to the heparin-binding domain of fibronectin and prevents syndecan-4 clustering [125]. It is frequently expressed in tumours, and leads to an increase in cell proliferation by blocking the interaction of syndecan-4 with fibronectin, but not with growth factors. In contrast, disruption of the gene encoding heparan sulphate 2-sulphotransferase, which is required for the production of mature heparan sulphate in mice, results in embryonic lethality during late gestation [126]. This is due to failure in ureteric bud branching in the kidney, although eye and skeleton defects are also seen. The effect of this mutation is not restricted to syndecan-4, as all syndecans and glypicans are substituted with heparan sulphate, but demonstrates that collectively the extracellular interactions of heparan sulphate proteoglycans are necessary for normal development during late embryogenesis. It seems that syndecan-4 deficiencies are frequently linked with kidney disorders, although the phenotype is not normally manifested until the kidney is placed under stress. Syndecan-4-null mice demonstrated normal renal function until challenged with the nephrotoxin κ -carrageenan; seven out of 24 null mice died within 7 days of treatment, whereas all of the wild-type mice survived [127]. This phenotype resembles the effect of syndecan-4 gene disruption on wound healing, in that the mice are healthy until exposed to stress. These observations suggest that syndecan-4 is involved primarily in tissue repair under adverse conditions, rather than the maintenance of tissue morphology in a healthy animal.

In placental tumours, syndecan-4 expression is lowered, and the strict localization to specific cell types is lost [128]. This results in abnormal interaction between cell receptors and extracellular growth factors, leading to an increase in the invasive behaviour of placental cells. These observations are at odds with data from the syndecan-4 knockout mouse, which suggest that syndecan-4 is not necessary for embryogenesis. It is probable that other proteoglycans, such as syndecan-1 and glypican-1, can compensate for the loss of syndecan-4 in the knockout mouse, but that in tumours where several proteoglycans are down-regulated the resultant abnormalities become apparent. This means that, in wild-type animals, syndecan-4 may contribute to the behaviour of a multitude of cell types, and that on disrupting the syndecan-4 gene we are only looking at a phenotype that cannot be compensated by other proteoglycans. The full extent of syndecan-4 function is likely to extend well beyond our current understanding and, by co-ordinating other transmembrane receptors, syndecan-4 may prove to be one of the most influential receptors in the cell.

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