Syndecan-1 Transmembrane and Extracellular Domains Have Unique and Distinct Roles in Cell Spreading

Kyle J. McQuade and Alan C. Rapraeger†

From the Department of Pathology and Laboratory Medicine and Graduate Program in Cellular and Molecular Biology, University of Wisconsin-Madison, Madison, Wisconsin 53706

Raji cells expressing syndecan-1 (Raji-S1) adhere and spread when plated on heparan sulfate-binding extracellular matrix ligands or monoclonal antibody 281.2, an antibody directed against the syndecan-1 extracellular domain. Cells plated on monoclonal antibody 281.2 initially extend a broad lamellipodium, a response accompanied by membrane ruffling at the cell margin. Membrane ruffling then becomes polarized, leading to an elongated cell morphology. Previous work demonstrated that the syndecan-1 cytoplasmic domain is not required for these activities, suggesting important roles for the syndecan-1 transmembrane and/or extracellular domains in the assembly of a signaling complex necessary for spreading. Work described here demonstrates that truncation of the syndecan-1 extracellular domain does not affect the initial lamellipodial extension in the Raji-S1 cells but does inhibit the active membrane ruffling that is necessary for cell polarization. Replacement of the entire syndecan-1 transmembrane domain with leucine residues completely blocks the cell spreading. These data demonstrate that the syndecan-1 transmembrane and extracellular domains have important but distinct roles in Raji-S1 cell spreading; the extracellular domain mediates an interaction that is necessary for dynamic cytoskeletal rearrangements whereas an interaction of the transmembrane domain is required for the initial spreading response.

The syndecans, a family of four cell surface heparan sulfate proteoglycans, are expressed on all adherent cells with roles including growth factor signaling, lipoprotein catabolism, and cell-cell and cell-matrix adhesion (1). Via their heparan sulfate glycosaminoglycan chains, the syndecans bind growth factors and their receptors, microbes, viruses, lipoproteins, proteases, and extracellular matrix proteins (2, 3). The syndecans are thought to act as matrix co-receptors with integrins to mediate cell binding and signaling on ligands that contain both heparan sulfate and integrin-binding motifs (3, 4). For example, fibroblasts plated on fibronectin adhere and spread via integrins, but binding of syndecan-4 is required for focal adhesion assembly. Syndecans, however, are also able to mediate adhesion and spreading when acting as the sole adhesion receptor, suggesting that the syndecan core proteins alone are able to mediate the assembly of signaling complexes that regulate adhesion and cell spreading (5–8). Here, the respective contributions of the syndecan cytoplasmic, transmembrane, and extracellular domains in the assembly of these signaling complexes remain unclear.

The prime candidate to transduce syndecan-mediated signals is the cytoplasmic domain. Two regions of the cytoplasmic domain, C1 and C2, are highly conserved across species and across the four family members. The membrane proximal C1 region has been suggested to bind members of the FERM family of adaptor molecules, shown by the binding of syndecans-1 and -2 with ezrin (9). Ezrin bridges syndecan-2 with the cytoskeleton and regulates membrane protrusive activity, suggesting one mechanism by which the syndecans may link the extracellular matrix to dynamic cytoskeletal rearrangements. The C2 region consists of a C-terminal EFYA amino acid motif that interacts with type II PDZ domains in CASK, syntenin, and synectin, proteins that are proposed to link the syndecans to intracellular signaling cascades and the cytoskeleton. The C1 and C2 regions flank a central variable domain (V) that differs among the four family members. The V region of syndecan-4 plays an important role in focal adhesion formation through its binding of phosphatidylinositol-4,5-bisphosphate and activation of protein kinase Cα (10). The V region of syndecan-2 interacts with and is phosphorylated by EphB2 (11), an event that induces oligomerization of syndecan-2 and stimulates the maturation of dendritic spines. Although interactions have not been described for the V regions of the cytoplasmic domains of syndecans-1 and -3, novel interactions of these regions are likely to impart specific signaling capabilities.

Compared with the cytoplasmic domains, little is known about the syndecan transmembrane and extracellular domains. The transmembrane domains are highly conserved across family members, suggesting that this region may impart overlapping, if not identical, functions on each syndecan. One role, best defined for syndecan-3, may be to mediate oligomerization (12). This depends on glycine residues within the membrane and four charged extracellular juxtamembrane residues. Another proposed role for the transmembrane domain is to target syndecan family members to lipid rafts, specialized membrane signaling structures into which syndecans-1 and -4 associate (13, 14).

The syndecan extracellular domains are divergent, sharing only attachment sites for glycosaminoglycan chains, but there is emerging evidence that they have binding partners. The syndecan-1 and -4 extracellular domains, when provided as a ligand for cell adhesion, bind to fibroblast cell surfaces (15). An example of an extracellular domain interaction that regulates cell invasion is the inhibition of myeloma cell migration into collagen gels by cell surface syndecan-1 (16). This inhibition appears to trace to the core protein rather than the attached
glycosaminoglycan chains, and syndecan-1 extracellular domain tethered to the membrane with a GPI anchor is sufficient for these activities. Similarly, MDA-MB-231 breast carcinoma cells bind and spread when plated on syndecan-1-specific ligand only when α5β1 integrins are activated; this spreading can be blocked by addition of recombinant syndecan-1 extracellular domain (17). The activities of the extracellular domain also extend to other syndecans. Colon carcinoma cells plated in serum are induced to round up and detach when treated with soluble extracellular domain of syndecan-2, presumably because the soluble ectodomain disrupts an adhesion complex at the cell surface (8). Additionally, fibroblast growth factor 2-stimulated proliferation in the developing wing bud and cultured chondrocytes is blocked by antibodies against the syndecan-3 extracellular domain, suggesting a role for this protein domain in the assembly of the fibroblast growth factor 2 signaling complex (18, 19).

Raji cells are a useful system to investigate the potential signaling roles of the syndecan-1 core protein. These lymphoid cells grow in suspension and are normally devoid of syndecans but gain the ability to bind to heparan sulfate-binding extracellular matrix ligands or syndecan antibody when transfected but gain the ability to bind to heparan sulfate-binding extracellular matrix ligands or syndecan antibody when transfected with the syndecan-1 cytoplasmic domain. Raji-S1 cells exhibit a motile response as early as 1 h after plating on mAb 281.2 (20). Upon binding syndecan ligand, Raji-S1 cells generate signals resulting in cell spreading, presumably via the assembly of a signaling complex in the plasma membrane. Raji-S1 cell spreading on mouse syndecan-1-specific mAb 281.2 is a multi-step process. Within 15–20 min of plating, cells radially extend a broad lamellipodium. This is accomplished by a high degree of membrane ruffling at the spreading margin. Active membrane ruffling polarizes over the next 1–2 h, giving rise to two or more lamellipodia that continue to extend and elongate the cells into a bi- or multi-polar shape. These steps require different signals as tyrophostin 25 blocks the initial lamellipodial extension and, by default, subsequent spreading events, whereas the polarized spreading that follows is blocked by genistein (21). Importantly, none of the steps requires the syndecan-1 cytoplasmic domain, suggesting that the syndecan acts through a signaling mechanism that is assembled in trans by the syndecan transmembrane and/or extracellular domains. Work described here demonstrates that sequences within the syndecan-1 transmembrane domain mediate interactions that are required for initial lamellipodial extension. In addition, sequences within the syndecan-1 extracellular domain are required for active membrane ruffling and cell polarization. Thus, the syndecan-1 transmembrane and extracellular domains play important and distinct roles in Raji cell spreading.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Molecular Biology—**Raji lymphoid cells were grown in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum (HyClone Laboratories), 4 mM L-glutamine (Sigma), and antibiotics. Raji cells expressing syndecan-1 in which the cytoplasmic domain is truncated to remove the 33 C-terminal amino acids (Raji S1 ARCy) were described previously (20) and grown in medium containing 300 μg/ml hygromycin-B (Roche Applied Science). Full-length mouse syndecan-1 in pcDNA3 was kindly provided by Dr. Ralph Sanderson (University of Arkansas, Little Rock, AR) as were S187syn/glyp, a mutant in which these 87 N-terminal amino acids of the syndecan-1 extracellular domain were deleted, and S170C/70 Glyp, a mutant in which these 87 N-terminal amino acids were fused to the Gpi linkage of rat glypican-1.2 S1FcyR-S1, a chimera comprised of the extracellular domain of human IgG Fc receptor 1a (CD64) fused to the transmembrane and cytoplasmic domains of human syndecan-1, was a generous gift of Dr. Kevin Williams (Thomas Jefferson University) (13). The coding region of S1FcyR-S1 was excised from pEUK-C1 with Xbal and XhoI and subcloned into the single vector LITMUS29 (Invitrogen) to generate LT29 S1 FcyR-S1. LT29 S1 FcyR-S1 was cut with XbaI, and the resulting overhang was filled using T4 polymerase. S1FcyR-S1 was excised using XhoI, and the resulting fragment was ligated into the EcoRV and XhoI sites of pcDNA3. S1 FcyR-S1, lacking the cytoplasmic domain of S1 FcyR-S1, was constructed by PCR mutagenesis. The coding region correspondingly to the transmembrane and extracellular domains of S1 FcyR-S1 were amplified using a primer complementary to the cytomegalovirus promoter (5′-ATGGCGGTCCGTGGTACCGG) and a primer that inserts a premature stop codon after the first arginine in the syndecan cytoplasmic domain (5′-CTCGAGTCATGCACTGGAGAACCAACCAAG). A syndecan-1 cDNA in which the transmembrane domain was replaced with residues (S1TM–S161) was constructed using site-directed mutagenesis by overlap extension (22). First, the S1TM–S161 coding region spanning the extracellular domain and two-thirds of the poly-leucine transmembrane domain was amplified using the mouse syndecan-1 DNA in pcDNA3 as a template, the cytomegalovirus primer described above, and a mutagenic primer that changes the transmembrane domain to leucine residues (5′-GAGCGGAGGAGCAGAGTGAAGAAGGAGCAGAACATAACAGGATTTCTTTGCTTCCAAAAAGGCTTGT). Second, the DNA spanning the cytoplasmic and transmembrane domains was amplified using a mutagenic primer spanning the junction of the transmembrane and cytoplasmic domains (5′-CTCGAGTCATGCACTGGAGAACCAACCAAG) and a primer corresponding to the pcDNA3 S6 promoter (5′-ATTAGGTGACACTTAG). The resulting products, which have overlapping extensions, were combined and amplified using the cytomegalovirus and Sp6 primers. This product was cloned into the KpnI and XbaI sites of pcDNA3 and confirmed by sequencing. Constructs were introduced into Raji parental cells by electroporation using a BTX ECM 830 square wave electroporator. Transfected cells were labeled using mAb 281.2 against mouse syndecan-1 or mAb 10.1 (Santa Cruz Biotechnology, Inc.) against CD64 and Alexa-488 conjugated secondary antibodies (Molecular Probes) and sorted by flow cytometry. Immuno-reactive cells were maintained in medium containing 1.5 μg/ml Genetin (Invitrogen).

**Cell Spreading Mediated by Distinct Domains of Syndecan-1**

**Lipid Raft Isolation and Western Blotting—**Lipid rafts were isolated and Western blots were performed as described previously (20). Brieﬂy, ligands (mAb 281.2–10 μg/ml, heparin-binding domain of ﬁbronecin-200 μg/ml in calcium- and magnesium-free phosphate-buffered saline) were applied to acid-etched or nitrocellulose-coated 10-well slides (Erie Scientific) and incubated for 1–2 h at 37 °C. Cells expressing S1FcyR-S1 or S1FcyR-S1 were plated on an antibody sandwich in which wells were ﬁrst coated with goat anti-mouse antibody (200 μg/ml; Jackson Laboratories), rinsed, and then coated with mAb 10.1 (100 μg/ml). Slides were blocked with 1% heat-denatured bovine serum albumin in calcium- and magnesium-free phosphate-buffered saline for a minimum of 30 min at 37 °C. Cells were rinsed and plated in HEPES-buffered RPMI containing 0.1% heat-denatured bovine serum albumin at a density of 15,000 cells per well. After rinsing, cells were allowed to attach and spread for 2 h prior to live observation using an inverted microscope or ﬁxed via the periodate-llysine-paraformaldehyde method (23). Cells processed for ﬂuorescence microscopy were permeabilized in 0.2% Triton X-100, labeled with rhodamine-conjugated phalloidin (Molecular Probes), and analyzed on a Nikon Microphot-FX microscope. Spread cells were scored as having a maximum cell diameter of greater than 25 μm. Approximately 50–80% of cells spread when plated on mAb 281.2, depending on the experiment. For cholesterol-depletion experiments, cells were pre-treated with 5 μM methyl-β-cyclodextrin (Sigma) 30 min prior to plating and plated in the presence of the drug until ﬁxation. For recovery experiments, 0.5 μM cholesteryl ester was added to cells 30 min prior to plating and 10 min after plating. For staurosporine-induced motility assays cells were plated on mAb 281.2-coated coverslips for 1 h before the addition of 1 μM staurosporine (Sigma). The response to staurosporine was documented with time lapse microscopy using MetaVue Image analysis software. Cells that extended a lamellipodium that migrated in response to inhibitor were scored as inhibited, cells that extended a lamellipodium that responded to staurosporine by extending short, dynamic filopodia were not counted as positive cells.

**Lipid Raft Isolation and Western Blotting—**Lipid rafts were isolated with modifications to standard procedures (24). Raji cells (5 × 10⁶) were washed with and resuspended in 1 ml of HEPES-buffered RPMI containing 0.1% heat-denatured bovine serum albumin at a concentration of 1 μg/ml for 10 min at 37 °C. After rinsing, goat anti-rabbit antibody (Jackson Laboratories) was added at 5 μg/ml for an additional 10 min to cluster the syndecan. For cells expressing S1FcyR-S1 or S1FcyR-S1, human IgG (5 μg/ml) (Jackson Laboratories)-
RESULTS

The Syndecan-1 Extracellular Domain Is Required for Bipolar Raji Cell Spreading—A panel of syndecan-1 mutants was screened to identify determinants within the syndecan-1 core protein that are required to signal spreading in the Raji cells (Fig. 1A). Populations of Raji cells expressing high levels of these syndecan-1 mutant proteins were sorted by flow cytometry and plated on a substratum comprised of mAb 281.2, which specifically engages the mouse syndecan-1 protein. As described previously (20, 21), removal of all but the first arginine residue of the syndecan-1 cytoplasmic tail (Raji-S1\(^{\text{A58}}\)) does not impair syndecan-1 mediated spreading (Fig. 1, C and G). Raji-S1\(^{\text{A58}}\) cells bind and spread when plated on mAb 281.2, with a morphology indistinguishable from that of cells expressing full-length syndecan-1 (Fig. 1B).

To test the role of the syndecan-1 extracellular domain, two syndecan-1 extracellular domain mutants, S1\(^{\text{A88--252}}\) and S1\(^{\text{A77a/syn/glyp}}\), were assayed for the ability to signal spreading. S1\(^{\text{A88--252}}\) is a deletion mutant in which a majority of the extracellular domain has been removed. After signal peptide cleavage, S1\(^{\text{A88--252}}\) contains the N-terminal 70 amino acids of the syndecan extracellular domain along with the syndecan-1 transmembrane and cytoplasmic domains. These 70 N-terminal amino acids include three glycosaminoglycan attachment sites and the epitope recognized by mAb 281.2. When plated on mAb 281.2, Raji-S1\(^{\text{A88--252}}\) cells bind and extend lamellipodia, but they are unable to polarize (Fig. 1, D and G). A second mutant that was tested was the S1\(^{\text{A77a/syn/glyp}}\) chimera that contains the same extracellular domain truncation but is anchored to the membrane by a GPI linkage of rat glypican-1. Raji-S1\(^{\text{A77a/syn/glyp}}\) cells bind to the substratum comprised of mAb 281.2 at levels equal to that of Raji-S1 cells but are deficient in their ability to spread. When using the criterion of a spread cell having a maximum cell diameter of 25 \(\mu\)m (Fig. 1, E and G), Raji-S1\(^{\text{A77a/syn/glyp}}\) cell spreading is less than 20% compared with cells expressing full-length syndecan-1, but even that 20% are cells that extend only a few short filopodia. Flow cytometry

![Fig. 1. Mutation of the syndecan-1 extracellular domain alters Raji cell spreading. A, schematic of syndecan-1 mutants. Raji cells expressing S1 (B), S1\(^{\text{A58}}\) (C), S1\(^{\text{A88--252}}\) (D), and S1\(^{\text{A77a/syn/glyp}}\) (E) were plated on glass coated with 10 \(\mu\)g/ml mAb 281.2 directed against the syndecan-1 extracellular domain. F, Raji-S1\(^{\text{FcR}}\) cells were plated on glass coated first with anti-mouse IgG (200 \(\mu\)g/ml) and then coated with 100 \(\mu\)g/ml mAb 10.1 against CD86. Cells were incubated for 2 h at 37 °C prior to fixation and staining with rhodamine-conjugated phallolidin. G, cell spreading in B-F was quantified as described under “Experimental Procedures.” 60% of adherent cells are spread in B. Bar, 50 \(\mu\)m.](http://www.jbc.org/content/early/2018/07/18/jbc.M117.786364/F1.large.jpg)
analysis confirms that expression of S1^87syn/glyp is equivalent to that of syndecan-1 in cells that do spread (data not shown). This suggests that the replacement of the transmembrane domain with the GPI linkage further reduces the ability of the syndecan to trigger spreading signals.

As a further test of the extracellular domain, the S1^PcR-S1 chimera was used in which all but the juxtamembrane glutamic acid residue of the syndecan-1 extracellular domain has been replaced with the extracellular domain of CD64, the high affinity IgG receptor. Raji cells sorted to express high levels of S1^PcR-S1 also spread when plated on ligand, namely mAb 10.1 directed against the CD64 extracellular domain (Fig. 1F) or on human IgG, a natural ligand for CD64 (data not shown). Like cells expressing S1^88–252, Raji-S1^PcR-S1 cells are stalled following the initial lamellipodial extension, demonstrating again that sequences in the syndecan-1 extracellular domain mediate association with signaling molecules that are required to trigger Raji cell polarity.

The Syndecan-1 Extracellular Domain Mediates Membrane Ruffling and Staurosporine-induced Raji Cell Motility—To further examine differences between cells expressing wild-type syndecan-1 and syndecan-1 extracellular domain mutants, cells were observed using videomicroscopy. Raji-S1 cells plated on mAb 281.2 exhibit a high degree of membrane ruffling, continually extending and retracting lamellipodia and filopodia along the substratum and extending processes out of the plane of the substratum as they spread (Fig. 2A and Supplemental Material). Raji cells expressing S1^cyto also exhibit membrane ruffling, but both Raji-S1^A88–252 cells plated on mAb 281.2 and Raji-S1^PcR-S1 cells plated on mAb 10.1 exhibit decreased membrane activity when compared with Raji-S1 cells (data not shown), indicating that the syndecan-1 extracellular domain plays an important role in regulating cytoskeletal dynamics.

To emphasize the differences between wild-type syndecan-1 and the syndecan-1 mutants, cells were treated with staurosporine, a broad spectrum serine/threonine and tyrosine kinase inhibitor that has been shown to stimulate membrane activity (27). Raji-S1 cells that are treated with 1 μM staurosporine exhibit greatly increased membrane activity. This increased activity leads to the formation of small dynamic lamellipodia at discrete locations at the margins of spread Raji cells. These lamellipodia often form at regions of the membrane that are irregular or at the ends of bipolar cells, thereby enhancing cell polarity. The lamellipodia are motile, as they migrate as far as two cell diameters away from the cell body within minutes of treatment, remaining connected by a stalk (see Fig. 2, B (arrows) and C and see Supplemental Material). Some of these motile lamellipodia split into two or more smaller lamellae that migrate from the original stalk (Fig. 2B, asterisk). In some experiments, entire cells migrate along the mAb 281.2-coated substratum in response to inhibitor treatment. This motility is mediated specifically through syndecan-1 as cells are plated on syndecan-1-specific ligand. Spreading on this ligand occurs independently of β1 integrins, the matrix-binding integrins expressed on Raji cells (21), and mAb 13, a β1 integrin-blocking antibody, has no effect on staurosporine-mediated motility (data not shown). Staurosporine enhances polarity and the formation of motile lamellipodia when added to Raji-S1^cyto cells, indicating that these responses do not depend on the syndecan-1 cytoplasmic domain (Fig. 2E). Raji-S1^A88–252 cells, which exhibit minimal membrane ruffling in the absence of staurosporine, respond poorly when treated with the drug (see Fig. 2D and Supplemental Material). Some cells display a slight increase in membrane activity, but motile lamellipodia are reduced by nearly 3-fold when compared with Raji-S1 cells (Fig. 2E). Raji-S1^PcR-S1 cells are completely refractory to staurosporine treatment (Fig. 2E). These data indicate that although the syndecan-1 extracellular domain is not necessary for the initial lamellipodial extension, it is required for active membrane ruffling and acquisition of cell polarity.

The Syndecan-1 Transmembrane Domain Is Required To Signal Lamellipodial Extension—Truncation of neither the syndecan-1 ectodomain nor the syndecan-1 cytoplasmic domain inhibits lamellipodial extension of Raji cells plated on mAb 281.2, suggesting a role for the transmembrane domain in mediating signals required for spreading. To assess the role of the transmembrane domain in spreading, Raji cells were transfected to express S1^PcR-S1^cyto in which the syndecan transmembrane domain is fused to the extracellular domain of CD64. Cells sorted to express high levels of S1^PcR-S1^cyto and plated on mAb 10.1 exhibit lamellipodia (Fig. 3C), suggesting these sequences alone are sufficient to mediate spreading in the Raji cells. These cells fail to polarize, an expected result, as they lack the syndecan-1 ectodomain.

To further examine the requirement for the syndecan-1 transmembrane domain in Raji cell spreading, the transmembrane sequence of wild-type syndecan-1 was replaced with a 25-amino acid poly-leucine sequence (Fig. 3A). The S1^TM → pl mutant is expressed by the cells and displayed on the cell surface. Populations of Raji-S1 and Raji-S1^TM → pl were sorted to ensure equal cell surface expression (Fig. 3F) and plated on a substratum comprised of mAb 281.2. Cells expressing S1^TM → pl bind syndecan-1 antibody at levels equal to wild-type syndecan-1 (Fig. 3D), but spreading is reduced over 4-fold when compared with cells expressing wild-type syndecan-1 (Fig. 3, D and E). Thus, the syndecan-1 transmembrane domain is necessary for the initial signals required for lamellipodial extension in the Raji cells, in agreement with the findings described earlier for the S1^87syn/glyp chimera (Fig. 1E).

Intact Lipid Rafts Are Required For Syndecan-1-mediated Raji Cell Spreading—Recent studies (13, 14) have demonstrated that syndecans-1 and -4 are targeted to lipid rafts, discrete regions of the plasma membrane that act as scaffolds for molecules involved in cell adhesion and other signaling cascades (28, 29). To determine whether syndecan-1-mediated spreading requires intact lipid rafts, Raji-S1 cells were treated with methyl-β-cyclodextrin (MβCD), a compound that disrupts lipid raft structure by removing cholesterol from the plasma membrane (29). Raji-S1 cells were pretreated for 30 min with 5 mM MβCD and plated on mAb 281.2 (Fig. 4, A, C, and E) or the heparin-binding domain of fibronectin (HBD-FN) (Fig. 4, B, D, and F), in the continued presence of the drug. For Raji cells plated on HBD-FN, nitrocellulose-coated slides were used to enhance ligand binding to the substrate. When viewed with phase contrast optics, cells that spread on HBD-FN send out a broad lamellipodium and blend into the nitrocellulose substratum (Fig. 4B, black arrows) whereas cells that do not spread have clear and rounded cell margins (Fig. 4B, white arrows). Cells treated with MβCD are able to bind mAb 281.2 and HBD-FN, but treatment completely inhibits the ability of cells to spread (Fig. 4, C and D), suggesting that intact lipid rafts are required. MβCD does not alter syndecan-1 expression on the surface of Raji cells as monitored by flow cytometry (Fig. 4H). In addition, when added to cells immediately before plating, 0.5 μM cholesterol completely rescues MβCD inhibition of Raji cell spreading and slightly enhances spreading on HBD-FN (Fig. 4G). Cells to which cholesterol has been added spread with the same kinetics and morphology as untreated cells (Fig. 4, E and F). This indicates that MβCD inhibition of Raji-S1 cell spreading is specific to the ability of the drug to remove cholesterol from the membrane.
Syndecan-1 Associates into Lipid Rafts When Expressed in Raji Cells—To examine whether syndecan-1 associates into lipid rafts, Raji-S1 cells were extracted in 1.0% Triton X-100 on ice, and the resulting cell lysates were separated on OptiPrep density gradients to quantify syndecan-1 in the rafts that float to the top of these gradients. Nearly all of the syndecan-1 protein expressed on the surface of suspended Raji-S1 cells is found in fractions 4 through 10 (Fig. 5A). These fractions include the densest regions of the gradient, as well as intermediate fractions found between the dense fractions and buoyant lipid raft fractions. When syndecan-1 is clustered with mAb 281.2 and anti-rat antibodies, more than 20% of the total syndecan-1 associates into lipid rafts.

**Fig. 2.** The syndecan-1 extracellular domain is required for membrane ruffling and staurosporine-induced motility. A, Raji-S1 cells were plated on mAb 281.2 for 1 h and then recorded by time-lapse videomicroscopy for an additional hour. Arrows indicate irregular cell edges that correspond to regions of membrane ruffling. See Supplemental Material (S1 no treatment). B, Raji-S1 cells were plated for 1 h on mAb 281.2, treated with 1 mM staurosporine for an additional 1 h, and then fixed and stained with rhodamine-conjugated phalloidin. Lamellipodia that migrate away from the cell body are marked with arrows. Lamellipodia that split into two or more lamellae and migrate from the original stalk are highlighted with asterisks. C, phase contrast images of a Raji-S1 cell plated on mAb 281.2 before and after staurosporine treatment. Refer to Supplemental Material (S1 + staurosporine). D, Raji-S1<sup>Δ148–252</sup> cells were treated with staurosporine. See Supplemental Material (S1<sup>Δ148–252</sup> + staurosporine). E, quantification of staurosporine-induced motility in Raji cells expressing syndecan-1 mutants. Cells that extended lamellipodia that migrated away from the cell body were scored as cells exhibiting a motility response. Arrows indicate areas of membrane ruffling and lamellipodial outgrowth. Bars, 50 μm.
decan-1 protein shifts to the two most buoyant fractions (Fig. 5, A and B). Association into these buoyant fractions is reduced to less than 2% by the addition of saponin and to less than 5% by the addition of MβCD (Fig. 5, A and B), both of which disrupt lipid rafts by removing cholesterol from the membrane. A pan-Src antibody, which recognizes a single 60-kDa band in the Raji cells, shows that over 30% of pan-Src immunoreactivity is present in the lipid rafts in the Raji cells, as would be expected (30), and this is completely abrogated by addition of saponin to the extraction medium (Fig. 5A). The transferrin receptor, which does not associate into lipid rafts (31), segregates into the densest fractions (fractions 8–10) (Fig. 5A). No transferrin receptor is seen in more buoyant fractions even when clustered with anti-transferrin receptor and secondary clustering antibodies (data not shown), indicating that antibody-induced clustering alone is insufficient for association into buoyant density gradient fractions.

Association of syndecan-1 into lipid rafts in Chinese hamster ovary cells is mediated by the transmembrane and/or cytoplasmic domains (13). To identify regions in the syndecan-1 protein that are important for mediating lipid raft association in the Raji cells, lysates from cells expressing syndecan-1 mutant proteins were examined on OptiPrep density gradients (Fig. 5C). S1Δcyto associates into Triton X-100-resistant domains when clustered with antibodies, indicating that this domain is not required for raft association. The syndecan-1 extracellular domain mutants S1Δ88–252 and S1ΔC also associate into rafts when clustered with antibodies, indicating that the syndecan-1 extracellular domain is not absolutely required for raft association. Interestingly, S1ΔFcyR associates into rafts less efficiently than S1Δ88–252. It is possible that the antibodies used to cluster S1ΔFcyR (human IgG- and F(ab′)2-specific anti-human IgG) do not work as well as mAb 281.2 used to cluster the other syndecan-1 proteins or that sequences at the N terminus of the syndecan-1 core protein play a role in raft association. The S1Δcyto/glyp chimera, which does not signal spreading, associates into lipid rafts via its GPI tail, which is a constitutive raft targeting signal.

The observation that either removal of the cytoplasmic domain or truncation of the extracellular domain does not inhibit association into rafts suggests a role for the transmembrane domain in raft targeting. However, S1ΔM also associates into rafts when clustered, indicating that mutation of the syndecan transmembrane domain is insufficient to inhibit raft association and suggesting that multiple raft targeting sequences may be present in the syndecan-1 core protein. To determine whether the transmembrane domain is sufficient for raft association, S1Δcyto was analyzed on density gradients. Importantly, S1ΔFcyR does not associate into lipid rafts when clustered (Fig. 5, C and D). This suggests that the extracellular and cytoplasmic domains may both harbor sequences that direct the proteoglycan to lipid rafts as only removal of both of these domains inhibits raft association. Importantly, S1Δcyto mediates lamellipodial spreading in the Raji cells without associating into rafts. This demonstrates that raft association is not required for spreading and suggests that downstream effectors, but not the syndecan itself, require association into lipid rafts to mediate signaling leading to cell spreading.
Interestingly, syndecan-1 associates into OptiPrep gradient fractions that are intermediary between raft and non-raft domains even without clustering with antibodies or when treated with saponin (Fig. 5, A and E, lanes 3–6). This suggests that syndecan-1 may associate into lipid domains that are resistant to extraction with both Triton X-100 and saponin. Association into these intermediate fractions is not affected by removal of the syndecan-1 cytoplasmic domain. The S1<sup>87V/S1<sup>88A–252</sup> mutants also associate into these intermediate fractions, but S1<sup>FR/R-S1</sup> does not, suggesting that sequences near the N terminus may be important for this association (Fig. 5E).

**DISCUSSION**

Spreading of Raji-S1 cells on syndecan-1-specific ligands can be divided into two distinct steps, namely (i) the initial formation of a broad lamellipodium, which is blocked by tyrphostin 25 and requires the syndecan-1 transmembrane domain, and (ii) the subsequent polarization of cells, which is blocked by genistein, depends on active membrane ruffling, and requires the syndecan-1 extracellular domain. This suggests that interactions of the syndecan-1 transmembrane and extracellular domains direct the assembly of a signaling complex that mediates Raji cell spreading.

The syndecan transmembrane domains are highly conserved across family members, suggesting conserved biological activities. Work described here demonstrates that Raji cell spreading is blocked when the syndecan-1 transmembrane domain is replaced by leucine residues, providing the first evidence of a direct role for this domain in signaling. One role that has been suggested for the syndecan transmembrane domains is to direct the association of the proteoglycan into lipid rafts.

Lipid rafts are membrane scaffolds where signaling molecules direct a number of cellular activities including endocytosis, growth factor signaling, immune cell activation, and cell adhesion. Clustering stimulates association of a number of transmembrane receptors including the T-cell receptor, growth factor receptors, GPI-linked proteins, and integrins into lipid rafts (28–31). The mechanism by which clustering results in raft association is not clear, although several models have been proposed (32, 33). In rafts, these receptors come into contact with signaling molecules such as kinases, phosphatases, and GTPases, thereby stimulating the propagation of signaling cascades that regulate cellular processes. Intact lipid rafts are required for spreading as Raji-S1 cell spreading is blocked by MβCD, which disrupts lipid rafts. Furthermore, clustering of syndecan-1 directs the proteoglycan to cold Triton X-100-resistant membrane fractions, indicative of association into lipid rafts. Truncation of either the syndecan-1 cytoplasmic or extracellular domain has no effect on raft association, but removal of both does block raft targeting. This indicates that the syndecan transmembrane domains are highly conserved across family members, suggesting conserved biological activities. Work described here demonstrates that Raji cell spreading is blocked when the syndecan-1 transmembrane domain is replaced by leucine residues, providing the first evidence of a direct role for this domain in signaling. One role that has been suggested for the syndecan transmembrane domains is to direct the association of the proteoglycan into lipid rafts.

Fig. 4. Methyl-β cyclodextrin inhibits Raji-S1 cell spreading. Raji-S1 cells were plated on glass coated with mAb 281.2 (A, C, and E) or nitrocellulose coated with the HBD-FN (200 μg/ml) (B, D, and F). C and D, cells were pre-treated with 5 mM MβCD for 30 min and plated in the presence of the drug. E and F, cholesterol (0.5 mM) was added to MβCD-treated cells 5 min prior to plating. All cells were incubated for 2 h at 37 °C before observation. G, quantification of cell spreading in A–F. H, Raji-S1 cells pretreated with 5 mM MβCD for 30 min were labeled with mAb 281.2 and alexa-488 conjugated secondary antibodies in the presence of the drug and analyzed by flow cytometry. Black arrows indicate spread cells whereas white arrows indicate unspread cells. Bar, 50 μm.
tors such as ErbB family members, adhesion molecules such as the β3 integrin subunit, and the transmembrane domains of various polytopic membrane proteins (34, 35). These GXXGX sequences are thought to mediate dimerization (36), a function best described for the erythrocyte membrane glycoprotein glycophorin A in which the GXXGX motif mediates interaction between the polypeptide backbones of opposing transmembrane domains. This interaction is made possible because of the small size of the glycine side groups and is stabilized by van der Waals forces along the length of the GXXGX sequence (37). The syndecan-1 GXXGX motifs may facilitate interaction with another GXXGX motif-containing protein that cooperates to mediate spreading. Another possibility is that these GXXGX motifs mediate oligomerization of the syndecan, forming a new

![Image A](image1.png)

**Fig. 5.** **Syndecan-1 associates into lipid rafts when clustered on the surface of Raji cells.** A, suspended Raji-S1 cells were incubated with mAb 281.2 and anti-rat antibodies to cluster the syndecan and then lysed in 4 °C Triton X-100 or MβCD. Lysates were separated by Optiprep density gradient centrifugation and processed for SDS-PAGE and Western blotting. Western blots were probed with mAb 281.2 and then stripped and reprobed with polyclonal antibody SRC-2 against Src family kinases and mAb H68.4 against the transferrin receptor. B, quantification of syndecan-1 association into lipid rafts from three independent experiments. C, raft association of syndecan-1 mutants compared with wild-type protein. D, syndecan-1, S1<sup>178-225</sup>, and S1<sup>178-225,131-153</sup> were clustered with mAb281.2 or human IgG and Cy5-conjugated secondary antibodies before lysis and separation on an Optiprep gradient. The positions of these antigens were monitored by detecting the position of the antibody directly on the gel. E, Western blot comparing the distribution of syndecan-1 on the density gradient with S1 FcR-S1.
surface with which a signaling co-receptor may interact. In support of this hypothesis, mutation of the second GXXG motif, which is conserved in all of the syndecans, blocks oligomerization of the syndecan-3 transmembrane domain (12). In addition to the roles of the syndecan-1 transmembrane domain, the syndecan-1 extracellular domain is required for membrane ruffling and polarized spreading, suggesting the syndecan-1 extracellular domain interacts with a signaling partner that generates a set of distinct signals resulting in highly dynamic cytoskeletal rearrangements. Staurosporine-treated cells exhibit increased membrane ruffling and extend motile lamellipodia, providing the first evidence that a syndecan family member can mediate motility when acting as the sole adhesion receptor. This parallels recent reports that document signaling functions for the syndecan-1 extracellular domain. When expressed on human ARH-77 myeloma cells, syndecan-1 blocks the ability to migrate through collagen gels, an activity that also requires regulation of cytoskeletal dynamics and maps to the syndecan-1 extracellular domain (16). The syndecan-1 extracellular domain also plays an important role in syndecan-1-mediated spreading of human breast carcinoma cells. MDA-MB-231 cells that are plated on syndecan-1-specific antibody spread via a mechanism involving α5β1 integrins (17). This syndecan-1-mediated signaling is inhibited by the addition of soluble fusion proteins containing sequences from the syndecan-1 extracellular domain, indicating that an interaction with another cell surface protein is important for cell spreading. Signaling co-receptors that interact with the syndecan-1 extracellular domain on ARH-77 and MDA-MB-231 cells have not been identified but could include a number of proteins that regulate cytoskeletal dynamics such as integrins or tetrarospanins. It will prove interesting to determine whether syndecan-1-mediated signaling in these cell types, along with polarization in the Raji-S1 cells, relies on the same or different co-receptors and downstream signaling pathways.

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Kyle J. McQuade and Alan C. Rapraeger

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