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Hierarchy between the transmembrane and cytoplasmic domains in the regulation of syndecan-4 functions

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ABSTRACT

Syndecan-4, a transmembrane heparan sulfate proteoglycan, plays a critical role in cell adhesion. Both the transmembrane and cytoplasmic domains of syndecan-4 are known to contribute to its functions, but the regulatory mechanisms underlying the functional interplay between the two domains were previously unclear. Here, we examined the functional relationship between these two domains. Fluorescence resonance energy transfer (FRET)-based assays showed that syndecan-4 expression enhanced RhoA activation. Furthermore, rat embryonic fibroblasts (REFs) plated on fibronectin fragments lacking the heparin-binding domain that interacts with syndecan-4 showed much lower RhoA activation than that in cells plated on full-length fibronectin, indicating that RhoA is involved in syndecan-4-mediated cell adhesion signaling. Syndecan-4 mutants defective in transmembrane domain-induced oligomerization and syndecan-4 phosphorylation-mimicking cytoplasmic domain mutants showed decreases in RhoA activation and RhoA-related functions, such as adhesion, spreading and focal adhesion formation, and subsequent increase in cell migration, but the inhibitory effect was much higher in cells expressing the transmembrane domain mutants. The cytoplasmic domain mutants (but not the transmembrane domain mutants) retained the capacity to form SDS-resistant dimers, and the cytoplasmic mutants showed less inhibition of syndecan-4-mediated protein kinase C activation compared to the transmembrane domain mutants. Finally, cytoplasmic domain activation failed to overcome the inhibition conferred by mutation of the transmembrane domain. Taken together, these data suggest that the transmembrane domain plays a major role in regulating syndecan-4 functions, and further show that a domain hierarchy exists in the regulation of syndecan-4.

1. Introduction

The syndecans are cell surface heparan sulfate proteoglycans (HSPG) that form an evolutionarily conserved family of type I transmembrane proteins and profoundly influence various cell behaviors, including cell adhesion, migration, cytoskeleton organization, differentiation, growth, and adhesion-dependent signal transduction [1–4]. The syndecans act as receptors for extracellular matrix (ECM) proteins and growth factors, engaging these ligands through the large glycosaminoglycan (GAG) chains of their extracellular domain. In this way, the syndecans act as adhesion receptors and modulate cell-matrix adhesion, thereby controlling cellular phenotype (e.g., the organization of the actin cytoskeleton) and distinct signal-transduction events. For example, syndecan-4 has been detected in the focal adhesions of various cells adhering on different ECMs, and the GAG chains of the extracellular domain of syndecan-4 have been shown to collaborate with integrin for adhesion-mediated signaling [5]. In this context, integrin α5β1 interacts with the RGD-containing cell-binding domain of fibronectin [6], while the high affinity heparin-binding domain of fibronectin (HepII) binds to GAG chains of syndecan-4 [7], and these interactions cooperatively regulate focal adhesion and stress fiber formation on fibronectin with integrin [7]. The interaction of syndecan-4 with the HepII domain of fibronectin induces the oligomerization of the extracellular domain of syndecan-4, which in turn regulates the interaction of the cytoplasmic domain of syndecan-4 with phosphatidylinositol 4,5-bisphosphate (PIP2) and protein kinase Cα (PKCα). Finally, syndecan-4-mediated PKCα activation results in the formation of focal adhesions and actin stress fibers [8]. Therefore, the activity of PKCα is critical for the role of syndecan-4 during cell adhesion and spreading. Indeed, pharmacological activation of PKCα using Phorbol 12-myristate 13-acetate (PMA) induces focal adhesion formation among fibroblasts spread on a surface harboring the cell-binding domain of fibronectin [9], and activated PKCα directly activates the small GTPase, RhoA

Abbreviations: ECM, extracellular matrix; FN, fibronectin; GAG, glycosaminoglycan; HSPG, heparan sulfate proteoglycan; PIP2, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; PMA, Phorbol 12-myristate 13-acetate; REF, rat embryo fibroblasts.

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through the phosphorylation of RhoGDI [10]. Thus, syndecan-4-induced PKCα activation is a critical regulatory mechanism underlying various changes in cytoskeletal organization, including the formation of focal adhesions.

As seen for many other cell surface receptors, oligomerization is the first step in the activation of syndecan-4 signaling. The binding of ligands, such as the HepII domain of fibronectin, may facilitate the clustering of syndecan-4 receptors, leading to the syndecan-4-mediated focal adhesion formation. CHO 745 cells, which are deficient in GAG chain formation of proteoglycan including syndecan-4, lack focal adhesions; however, focal adhesions can be formed in these cells by inducing the clustering syndecan-4 with extracellular domain-specific antibodies [11]. Indeed, the antibody-mediated direct clustering of syndecan-4 triggers focal adhesion and actin stress fiber assembly in fibronectin-null fibroblasts plated on the integrin-binding domain of fibronectin [12], and syndecan-4 ligation may also regulate the dynamics of focal adhesions through the phosphorylation of focal adhesion kinase (FAK) on Tyr 397 [13].

The oligomerization of syndecan-4 appears to be mediated by two separate regulatory mechanisms: one that operates through the transmembrane domain and one that operates through the cytoplasmic domain. We previously reported that syndecan chimeras defective in transmembrane domain-induced oligomerization show decreased PKCα activation and syndecan-4-mediated focal adhesion formation [14]. Therefore, the transmembrane domain-induced oligomerization of syndecan-4 is crucial for protein function. The oligomeric status of the cytoplasmic domain of syndecan-4 also appears to be important for regulating downstream signals during the formation of focal adhesions and actin stress fibers. For example, the interaction of syndecan-4 with PIP2 induces oligomerization of the syndecan-4 cytoplasmic domain, which then directly regulates the localization, stability and activity of PKCα [15]. The oligomerization of the cytoplasmic domain of syndecan-4 has also been shown to regulate its interaction with the actin-binding protein, α-actinin, resulting in the formation of focal adhesions [16].

Although studies have shown that the oligomerizations of both domains of syndecan-4 are important and functional, it is not yet clear how these two separate oligomerizations are involved in regulating the functions of syndecan-4. Here, we demonstrate that the transmembrane domain-induced oligomerization of syndecan-4 plays a major role in its adhesion-mediated functions, and further show that there is a hierarchy between the transmembrane and cytoplasmic domains in the regulation of syndecan-4 functions.

2. Materials and methods

2.1. Cell culture, antibodies, and reagent

Rat Embryo Fibroblasts (REFs) were maintained in alpha-modified Eagle's medium (α-MEM, Gibco BRL) supplemented with 5% (v/v) fetal bovine serum (FBS), penicillin (1,000 units/ml), and streptomycin (1 mg/ml). Monoclonal anti-RhoA and -β-actin antibodies, and polyclonal anti-PKCα and -integrin β1 antibodies were purchased from Santa Cruz Biotechnology Inc. (California, USA). Monoclonal anti-paxillin was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY, USA) and monoclonal anti-α-actinin was purchased from Sigma-Aldrich (St. Louis, MO, USA). PMA was from Calbiochem (San Diego, CA, USA), and fibroblast growth factor 2 (FGF2), PKCα purified from rabbit brain and fibronectin were from Upstate Biotechnology, Inc. (Lake Placid, NY, USA). Transient transfection was carried out using Effectene (Qiagen, Hilden, Germany) as described by the provided protocol.

2.2. Confocal microscopy and FRET analysis

REF cells were transfected with indicated cDNAs and imaged on a Zeiss Axiovert 200 M inverted microscope equipped with a Zeiss 510 META confocal head. All images were analyzed with Zeiss software. FRET analysis by acceptor photobleaching was carried out as described previously [17] with some modification. Briefly, REF cells co-transfected with pRaichu 1502 (from M. Matsuda, 18) and either syndecan-4 or syndecan-4 mutants were plated onto coverslips, then fixed for 5 min in 4% (w/v) formaldehyde, washed with PBS, and visualized with a C-Apochromat 40X/1.2-W objective lens using a 458 nm argon laser light and Meta detector (462.6–516.1 nm) for Cyan Fluorescent Protein (CFP) excitation and emission and a 514 nm argon laser light and Meta detector (516–591 nm) for Yellow Fluorescent Protein (YFP) excitation and emission. FRET efficiency was measured by acceptor photobleaching; pre-bleach CFP and YFP images were collected simultaneously following excitation at 458 nm for CFP and 514 nm for YFP. A selected region was irradiated with the 514 nm laser line (100% intensity, 60 iterations, using a 458/514 nm dual dichroic mirror) to bleach YFP. Post-bleach CFP and YFP images were collected simultaneously (at 458/514 nm) immediately following photobleaching. FRET was measured as an increase in CFP fluorescence intensity following YFP photobleaching. FRET efficiency was calculated as $100\times((\text{CFP post-bleach} - \text{CFP pre-bleach})/\text{CFP post-bleach})$ using the FRET Macro in the Zeiss Aim software, correcting for CFP and YFP background in each channel.

2.3. Rho activity assay

The cells were washed with ice-cold TBS and lysed in lysis buffer (50 mM Tris, pH 7.2, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM $MgCl_2$, 10 μg/ml each of leupeptin and aprotinin, and 1 mM phenylmethylsulfonyl fluoride). Cell lysates were clarified by centrifugation at 13,000 ×*g* at 4 °C for 10 min, and Rho activity was measured in a pull-down assay using the Rho binding domain (RBD) from Rhotekin. Equal volumes of lysates were incubated with GST-RBD beads at 4 °C for 2 h. The beads were washed four times with lysis buffer. Bound RhoA proteins were detected by western blotting using a monoclonal antibody against RhoA.

2.4. Expression and purification of recombinant GST-syndecan-4 proteins

Rat sydnecan-4 cDNAs encoding full length (SDC4) and deletion mutant (4TC) were synthesized by polymerase chain reaction (PCR) and subcloned into glutathione-S-transferase (GST) expression vector pGEX-5X-1 (Amersham Biosciences). GST-tagged phosphorylation-mimicking mutant (183E) construct was provided by Dr. John Couchman (University of Copenhagen, Denmark). These constructs together with other oligomerization-defective mutants (4GL, 14) were used to transform *Escherichia coli* DH5α and expressions of fusion proteins were induced with 1 mM IPTG for 4 h. The fusion proteins were purified with glutathione-agarose beads as previously described [19].

2.5. Cellular fractionation

After washing twice with PBS, hypo-osmotic solution (20 mM Tris/HCl, pH 7.5, 2 mM 2-mercaptoethanol, 5 mM EGTA, 2 mM EDTA) containing a protease inhibitor cocktail (1 μg/ml aprotinin, 1 μg/ml antipain, 5 μg/ml leupeptin, 1 μg/ml pepstatin A, and 20 μg/ml phenylmethylsulfonyl fluoride) was added to the culture plates. Cells were subsequently scraped off the plates, and homogenized on ice. The homogenate was centrifuged at 13,000 ×*g* for 15 min at 4 °C. The membrane fraction was collected by solubilizing the remaining pellet in RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 mM NaF, and 2 mM Na_3VO_4) containing a protease inhibitor cocktail, and then RIPA lysates were centrifuged at 13,000 ×*g* for 15 min at 4 °C. Equal amounts of lysates were resolved by SDS-PAGE, transferred on to PVDF membranes and probed with the indicated antibodies.