Trans-regulation of Syndecan Functions by Hetero-oligomerization*

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Background: Syndecans form non-covalently linked homodimers through their highly conserved transmembrane domains.

Results: Syndecan-2 and -4 exhibit heteromolecular interaction, and this interaction regulates syndecan-mediated cellular functions.

Conclusion: Hetero-oligomerization produces distinct syndecan functions.

Significance: Our finding offers new insights into the underlying signaling mechanisms of syndecans.

Syndecans, a family of transmembrane heparansulfate proteoglycans, are known to interact through their transmembrane domains to form non-covalently linked homodimers, a process essential for their individual functions. Because all syndecan transmembrane domains are highly conserved and thus might mediate interactions between different members of the syndecan family, we investigated syndecan interactions in detail. All recombinant syndecan-2 and -4 protein variants containing the transmembrane domain formed not only sodium dodecyl sulfate (SDS)-resistant homodimers but also SDS-resistant heterodimers. Biochemical and structural data revealed that recombinant syndecan-2 and -4 formed intermolecular interactions in vitro, and the GXXXG motif in transmembrane domain mediated this interaction. When exogenously expressed in rat embryonic fibroblasts, syndecan-2 interacted with syndecan-4 and vice versa. Furthermore, bimolecular fluorescence complementation-based assay demonstrated specific hetero-molecular interactions between syndecan-2 and -4, supporting hetero-oligomer formation of syndecans in vivo. Interestingly, hetero-oligomerization significantly reduced syndecan-4-mediated cellular processes such as protein kinase Cα activation and protein kinase Cα-mediated cell adhesion as well as syndecan-2-mediated tumorigenic activities in colon cancer cells such as migration and anchorage-independent growth. Taken together, these data provide evidence that hetero-oligomerization produces distinct syndecan functions and offer insights into the underlying signaling mechanisms of syndecans.

Syndecans are a family of evolutionarily conserved transmembrane heparan sulfate proteoglycans that participate in the regulation of a broad range of adhesion-mediated cell functions (1). Structurally, they are composed of an extracellular domain, a single transmembrane domain, and a short cytoplasmic domain. The extracellular domain of syndecans interacts with a number of ligands in the extracellular matrix through heparan sulfate chains and interactions that activate syndecans and lead to their regulation of receptor signaling events (1–3). The transmembrane domain is composed of 25 hydrophobic amino acid residues that are responsible for the molecular interaction that causes homo-oligomerization of syndecan core proteins (4, 5). The strictly conserved GXXXG motif is involved in these interactions and induces homodimer formation of syndecans, even in the presence of the strong anionic detergent sodium dodecyl sulfate (SDS), a property known as SDS-resistant dimerization (4). The cytoplasmic domain contains two highly conserved C1 and C2 regions separated by a variable region. The intervening variable region, which is distinct for each syndecan member yet conserved across species, promotes syndecan-specific intracellular functions.

Four members of the syndecan family have been identified in vertebrates (6), and their expression is highly regulated in complex patterns in individual cell types and tissues and at different developmental stages (7). Syndecan-1 is the predominant family member in epithelial cells, syndecan-2 is abundantly expressed in fibroblasts and mesenchymal cells, syndecan-3 abounds in neuronal cells, and syndecan-4 is widely expressed (7). Notably, the expression of syndecans is altered in pathological situations such as wound healing and neoplastic transformation. In particular, altered expression of syndecan family members has been reported in various human tumors, including lung, breast, and colorectal cancers (8, 9).

Transduction of signals through non-covalent dimerization/oligomerization of cell surface receptor proteins containing a single transmembrane domain in response to ligand binding is a well-recognized signaling paradigm (10). As is the case for many other cell surface receptors, homodimerization/oligomerization is the first step in the activation of syndecan signaling, and the syndecan transmembrane domain plays a major role in this process (5). Given the high pairwise sequence identity of syndecan transmembrane domains (48–72%) and the conservation of the GXXXG dimerization motif, all syndecan transmembrane domains might be expected to mediate heteromeric oligomerization as well as homomeric interactions. A previous study using synthetic peptides corresponding to the transmem-

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brane domain of each syndecan paralog has measured the association tendencies of the syndecan transmembrane domain (11). This study found that the transmembrane domains of the four syndecan paralogs self-associate to very different degrees and are capable of a distinct set of heteromeric interactions, independent of extracellular and cytoplasmic domains (11). However, whether full-length syndecans form heterodimers and, if so, how this heterodimerization is involved in regulating syndecan functions, have not been investigated. Here, we demonstrate that transmembrane domain-mediated hetero-oligomerization of syndecan-2 with syndecan-4 occurs in vitro and in intact cells and regulates syndecan-mediated signal transduction, producing unique functional outcomes.

Experimental Procedures

Antibodies and Materials—Monoclonal antibodies against glutathione S-transferase (GST), His, HA, β-actin, RhoA, integrin β1, extracellular signal-regulated kinase (ERK) and phospho-ERK, and polyclonal antibodies against Myc and protein kinase Ca (PKCa) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). A monoclonal antibody to α-actinin was purchased from Sigma, and a polyclonal antibody to syndecan-4 was purchased from Santa Cruz Biotechnology. Monoclonal antibodies to paxillin and Rac1 were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY), and a monoclonal anti-syndecan-2 antibody was produced by AdipoGen Inc. (Incheon, Korea). Fibronectin was from Upstate Biotechnology, Inc. (Lake Placid, NY). Transient transfection was carried out using Effectene (Qiagen, Hilden, Germany) as described by the provided protocol.

Cell Culture—Rat embryonic fibroblasts (REFs)2 were maintained in α-modified Eagle’s medium (α-MEM; Gibco) supplemented with 5% (v/v) fetal bovine serum (FBS), penicillin (1000 units/ml), and streptomycin (1 mg/ml). HT29 cells were maintained with 5% (v/v) fetal bovine serum (FBS), penicillin (1000 units/ml), and streptomycin (1 mg/ml). HEK293T and B16F10 cells were maintained in Dulbecco’s modified Eagle’s medium (Welgene, Daegu, Korea); HEK293T and B16F10 cells were maintained in McCoy’s 5a medium (Welgene, Daegu, Korea); and 293T and Rac1 were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY), and a monoclonal anti-syndecan-2 antibody was produced by AdipoGen Inc. (Incheon, Korea). Fibronectin was from Upstate Biotechnology, Inc. (Lake Placid, NY). Transient transfection was carried out using Effectene (Qiagen, Hilden, Germany) as described by the provided protocol.

Expression and Purification of Recombinant GST-Syndecan Core Proteins—cDNAs of rat syndecan-1, -2, and -4 encoding full-length proteins (SDC1, SDC2, SDC4), the transmembrane domain and cytoplasmic domain (2TC, 4TC), the extracellular domain (2E), the transmembrane domain (2T), the cytoplasmic domain (2C), or the transmembrane domain containing four additional flanking extracellular amino acid residues (KRTE and ERTE) in the extracellular domain and the cytoplasmic domain (2E2TC, 4E2TC) were synthesized by polymerase chain reaction (PCR) and subcloned into the GST expression vector pGEX-5X-1 (Amersham Biosciences). Mutations were generated by PCR-based site-directed mutagenesis (Stratagene, La Jolla, CA) and confirmed by DNA sequence analysis of the resulting plasmids. These constructs were used to transform Escherichia coli DH5α, and the expression of GST fusion proteins was induced by incubation with 1 mM isopropyl-β-D-thiogalactopyranoside for 4 h at 37 °C. The fusion proteins were purified with glutathione-agarose beads (GE Healthcare) as described previously (5).

Purification of Recombinant His-syndecan Proteins by GST-Syndecan-bound Glutathione-agarose Bead Affinity Chromatography—cDNA encoding the entire rat syndecan-2 or -4 core protein was subcloned into the His expression vector pET32a+ (Novagen, Madison, WI), and the expression of fusion proteins in E. coli BL21 was induced by incubating with 0.3 mM isopropyl-β-D-thiogalactopyranoside at 25 °C for 16 h. Proteins were released by lysing E. coli cells with lysis buffer (20 mM Na2HPO4, pH 8.0, 150 mM NaCl, 5 mM β-mercaptoethanol, 1% Triton X-100) and sonicating on ice for 1 h. After removing insoluble material by centrifugation at 13,000 × g for 30 min at 4 °C, the supernatants containing His-syndecan fusion proteins were applied to a glutathione-agarose column containing prebound GST-syndecans. The column was washed 3 times, and bound proteins were eluted with elution buffer (50 mM Tris-HCl, pH 8.0, 5 mM reduced glutathione). Fractions were analyzed by SDS-PAGE followed by Coomassie Blue staining and Western blotting using antibodies against GST, His, syndecan-2, and syndecan-4.

Sample Preparation for the Nuclear Magnetic Resonance (NMR) Experiment—cDNA encoding the rat transmembrane domain of syndecan-2 and syndecan-4 were subcloned into the His-thioredoxin expression vector pET32a+, and the enterokinase enzyme recognition site, DDDDK, was inserted between His-thioredoxin tag and target protein. Fusion protein expression was induced in E. coli BL21(DE3) cells with 1 mM isopropyl-β-D-thiogalactopyranoside in optical density values of 0.55 at 600 nm and overexpressed at 25 °C for 18 h. Harvested cell pellet was lyzed with lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM β-mercaptoethanol) and sonicated on ice for 1 h. After centrifugation at 13,000 × g for 30 min, supernatant was removed, and insoluble precipitant was used for the resolubilization step using refolding buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM β-mercaptoethanol, 1% n-dodecyl β-D-maltoside (Affymetrix)). Resolubilized fraction was applied to Ni-NTA affinity column. The column was washed twice times using 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM β-mercaptoethanol, 20 mM imidazole, and 0.1% n-dodecyl β-D-maltoside, and target protein was eluted in 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM β-mercaptoethanol, 250 mM imidazole, 0.05% n-dodecyl β-D-maltoside. After desalting column work using PD-10 (GE Healthcare), enterokinase enzyme was treated to remove the tag at 37 °C for 12 h. Nonspecific impurity and His-thioredoxin tag was removed using additional Ni-NTA affinity column work. Final target protein was dialyzed in 20 mM ammonium bicarbonate and lyophilized. For the preparation of NMR samples, M9 minimal media and isotopes such as [13C]glucose and [15]NH4Cl were used during cell culture. All NMR samples are prepared in 10 mM Na2HPO4, pH 7.0, 4 mM DTT, 1 mM sodium azide, 100 mM dodecylphosphocholine, 10% D2O.

NMR Experiment and Data Analysis—All NMR spectra were obtained at 318 K on a Bruker DRX 900 MHz spectrometer with...
Cryoprobe™ and processed and analyzed using NMRPipe/ NMRDraw and the Sparky program. Sequential resonance assignment was executed using $^{1}H, ^{13}N$ heteronuclear single quantum coherence, three-dimensional HNCA, CBCA- CONH, and HNCO. NMR titration experiment was performed using $^{15}N$-labeled syndecan-4 and unlabeled syndecan-2 membrane domain using different molar ratios (1:1, 1:2, and 1:3). Chemical shift change values were calculated using the equation $\Delta \delta_{XY} = \left(\Delta \delta_{13N}^{2} + \Delta \delta_{15N}^{2}/5\right)^{1/2}$.

**Immunoprecipitation**—For the immunoprecipitation of HA-tagged syndecans, REs transfected with the indicated cDNAs were lysed with 1% Nonidet P-40 lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 2 mM Na$_3$VO$_4$, and 10 mM NaF) containing a protease inhibitor mixture. Individual cell lysates were mixed for 30 min on ice, and protein mixtures were incubated with anti-HA affinity Matrix (Roche Applied Science) for 2 h at 4 °C. Immunocomplexes were collected by centrifugation, washed 3 times with PBS, and incubated with protein G-Sepharose beads (GE Healthcare) for 2 h at 4 °C. Immune complexes were incubated with anti-syndecan antibodies for 2 h at 4 °C followed by incubation with protein G-Sepharose beads for 1 h. Immunocomplexes were collected by centrifugation, washed 3 times with PBS, and eluted with 1 M NaCl. To remove the IgG of eluted proteins and extract the syndecan 8.0, 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 2 mM PMSF, washed with 30 mM sodium acetate, pH 4.0, containing 4 M guanidine HCl and 0.1% Tween-20, and then analyzed by slot blotting using the indicated antibodies.

For Western blotting of immunoprecipitates, cells were lysed with 1% radioimmune precipitation assay lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, 10 mM NaF, 2 mM Na$_3$VO$_4$) containing a protease inhibitor mixture. Cell lysates were incubated with anti-syndecan-4 antibody for 2 h at 4 °C followed by incubation with protein A-Sepharose beads for 1 h. Immunocomplexes were collected by centrifugation, washed 3 times with PBS, and eluted with 1 M NaCl. Glycosaminoglycan chains were removed by incubating samples with a pH 1.5 nitrous acid solution as previously described (12) and analyzed by Western blotting using an anti-syndecan-2 antibody.

**Bimolecular Fluorescence Complementation**—The vector constructs encoding fragments of pECFP were kindly provided by Dr. Minsoo Kim (University of Rochester Medical Center). In the current study the sequences encoding pECFP amino acid residues 1–154 (CN) or 155–238 (CC) were fused to the 3’ ends of the coding regions of syndecans by conventional restriction enzyme-based cloning; the resulting constructs generated are depicted schematically in Fig. 4E. For bimolecular fluorescence complementation assays, HT29 cells were transiently co-transfected with the indicated cDNAs then fixed for 5 min in 4% paraformaldehyde, washed with PBS, and imaged on a Zeiss Axiosvert 200 M inverted microscope equipped with a Zeiss 510 META confocal head using a C-ApoChromat 40×/1.2-W objective lens and a 458-nm argon laser light and Meta detector (462.6–516.1 nm) for pECFP excitation and emission.

**In Vitro PKC Assay**—For nonradioactive Peptag assays, Peptag C1 peptide (Promega, Madison, WI) was incubated in reaction buffer (20 mM HEPES, pH 7.4, 1 mM DTT, 10 mM MgCl$_2$, 1 mM ATP) containing phosphatidylinositol 4,5-bisphosphate (50 μm) in a final volume of 25 μl for 30 min at 30 °C. The reactions were stopped by heating to 95 °C for 10 min. The samples were separated on a 0.8% agarose gel at 100 V for 15 min. Phosphorylated peptides migrated toward the cathode (+), whereas non-phosphorylated peptides migrated toward the anode (−). The gel was photographed on a transilluminator.

**Centrifugal Detachment Assay**—Cell-substratum adhesiveness was quantified using an inverted centrifugal detachment assay. Fibronectin was diluted in serum-free medium (10 μg/ml), added to the plates, and incubated at 25 °C for 1 h. The plates were then washed with PBS and blocked with 0.2% heat-inactivated BSA for 1 h. After washing with PBS, cells were incubated for additional 2 h at 37 °C in 5% CO$_2$. After removing unattached cells, plates were then filled with serum-free medium, sealed with Paraflim, and centrifuged inverted for 5 min at 300 × g at room temperature using a large capacity tabletop centrifuge (Hanil science industrial, Korea). After the centrifugation, retained cells were counted using a hemocytometer.

**Cellular Fractionation**—After washing twice with PBS, a hypo-osmotic solution (20 mM Tris/HCl, pH 7.5, 2 mM 2-mercaptoethanol, 5 mM EGTA, 2 mM EDTA) containing a protease inhibitor mixture 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 radioimmune precipitation assay buffer containing a protease inhibitor mixture and then centrifuged at 13,000 × g for 15 min at 4 °C. Equal amounts of lysates were resolved by SDS-PAGE, transferred onto PVDF membranes, and probed with the indicated antibodies.

**Rac and Rho Activity Assay**—GST-PAK-PBD binding assays were performed essentially as described previously (13). Briefly, the p21 binding domain of PAK1 (PBD) was expressed in E. coli as a GST-PAK-PBD fusion protein, purified using glutathione-agarose beads, and added to cell lysates. Bound proteins were collected by centrifugation and suspended in SDS sample buffer. Proteins were fractionated by SDS-PAGE and transferred to PVDF membranes, and the amount of precipitated Rac1 was estimated by Western blotting with an anti-Rac1 antibody. Rho activity was measured in a pulldown assay using the Rho binding domain from Rhotekin. Equal volumes of lysates were incubated with GST-Rho binding domain beads at 4 °C for 2 h, after which the beads were washed 4 times with lysis buffer, and bound RhoA proteins were detected by Western blotting using a monoclonal antibody against RhoA.
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men in PBS for 1 h, and incubated with the indicated antibodies. The slides were mounted with VECTASHIELD Mounting Medium (Vector Laboratories, Burlingame, CA) and imaged using a fluorescence confocal microscope (Carl Zeiss, Gottingen, Germany).

Monitoring Cell Spreading and Migration—Cell spreading and migration were monitored using the xCELLigence system (Roche Diagnostics). For cell spreading, E-plate 16 assemblies (Roche Diagnostics) were coated with fibronectin (10 μg/ml) and seeded with cells (1.5 × 10^4 cells/well). Each plate was then assembled on the RTCA DP Analyzer, and data were gathered at 5-min intervals for 5 h at 37 °C, 5% CO2. The data were analyzed using the provided RTCA software. For cell migration, a CIM-plate 16 (Roche Diagnostics) was used. The lower chambers were filled with fresh medium (160 μl/well) containing 10 μg/ml fibroblast growth factor-2 and 10% FBS, and the upper chambers were filled with medium (30 μl/well) containing 5% FBS. Plates were incubated at 37 °C, 5% CO2 for 1 h, and background was measured using the RTCA DP Analyzer. Transfected cells were added to each well, and the plate was incubated at 25 °C. After 30 min, the CIM-plate was assembled onto the RTCA DP Analyzer, and cell migration was assessed at 15-min intervals for 24 h at 37 °C, 5% CO2. The obtained data were analyzed using the provided RTCA software.

Transwell Migration Assay—The lower surface of Transwell inserts (Costar) was coated with gelatin (10 μg/ml), and the membranes were allowed to dry for 1 h at room temperature. The Transwell inserts were assembled into a 24-well plate, and the lower chamber was filled with McCoy's 5a media containing 10% FBS and fibroblast growth factor-2 (10 μg/ml). Cells (5 × 10^5) were added to each upper chamber, and the plate was incubated at 37 °C for 30 h. Migrated cells were stained with 0.6% hematoxylin and 0.5% eosin and counted.

Anchorage-independent Growth in Soft Agarose—Each well of a 6-well culture plate was coated with 3 ml of bottom agar mixture (McCoy's 5a, 10% FBS, 0.6% agar). After the bottom layer had solidified, 2 ml of top agar mixture (McCoy's 5a, 10% fetal bovine serum, 0.3% agar) containing 2 × 10^6 cells was added to each well, and the cultures were incubated at 37 °C in a 5% CO2 atmosphere. Every 5 days, normal growth medium was gently layered over the cultures. Colony formation was monitored daily with a light microscope. Colonies in soft agar were photographed with a digital camera after incubation for 21 days.

Statistical Analysis—Data are represented as the mean from at least three independent experiments. Statistical analysis was performed using an unpaired Student's t test. A p value less than 0.01 was considered statistically significant.
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FIGURE 1. Syndecans form SDS-resistant heterodimers through their transmembrane domains. A, schematic representation of syndecan-2 (SDC2) and syndecan-4 (SDC4) core proteins. The extracellular domain (E), the transmembrane domain (T), the cytoplasmic domain (C), and four amino acid residues in the membrane flanking region (ERTE, KRTE) are shown. Two of the conserved glycine residues (*) replaced with leucine residues are indicated by arrows in 2GL and 4GL. The transmembrane domain of syndecan-2 and -4 was replaced with the single transmembrane domain of PDGF receptor to yield 2EPT2C and 4EPT4C, respectively. B, recombinant GST-syndecan core proteins were purified with glutathione-agarose beads as described under "Experimental Procedures" (left). Puriﬁed recombinant proteins (SDC2 or SDC4) were mixed with the corresponding mutants (2TC or 4TC) for 30 min on ice, separated by SDS-PAGE, and stained with Coomassie Blue (right). Molecular mass markers in kilodaltons are shown. Migration positions of SDS-resistant syndecan-2 dimers (●) and monomers (□), syndecan-4 dimers (■) and monomers (▲), syndecan-2TC dimers (○) and monomers (◇), and syndecan-4TC dimers (△) and monomers (●) are indicated. Each His-tagged thioredoxin-syndecan protein was afﬁnity-puriﬁed over Ni-NTA columns, and puriﬁed recombinant proteins (SDC2 or SDC4) were mixed with the corresponding mutants (2eTC or 4eTC) for 30 min on ice. Mixtures were separated by SDS-PAGE and stained with Coomassie Blue. Migration positions of SDS-resistant wild type syndecan dimers (●), syndecan-2TC dimers (○), and syndecan-4TC dimers (△) and monomers (●) are indicated. C, His-tagged thioredoxin-syndecan proteins including the tobacco etch virus cleavage site were incubated with tobacco etch virus protease. Each tag-free recombinant syndecan protein was mixed with His-tagged syndecan proteins, separated by SDS-PAGE, and stained with Coomassie Blue. Migration positions of His-tagged syndecan dimers (●) and monomers (□), syndecan-4TC dimers (△) and monomers (▲), and cleaved syndecan dimers (◇) are indicated. D, molecular mass markers in kilodaltons are shown. Migration positions of SDS-resistant wild type syndecan dimers (●), syndecan-2TC dimers (○), and syndecan-4TC dimers (△) and monomers (▲) are indicated. E, puriﬁed recombinant syndecan-4 protein was mixed with the indicated recombinant proteins, separated by SDS-PAGE, and stained with Coomassie Blue. Migration positions of SDS-resistant wild type syndecan dimers (●), syndecan-2TC dimers (○), and syndecan-4TC dimers (△) and monomers (▲), and syndecan-4TC dimers (△) and monomers (▲) are indicated. F, recombinant GST-syndecan core proteins (SDC2 and SDC4) and oligomerization-defective mutants (2GL, 2EPT2C, 4GL, and 4EPT4C) were puriﬁed using glutathione-agarose beads (top). Puriﬁed recombinant proteins (2TC or 4TC) were mixed with the indicated recombinant proteins, and mixtures were separated by SDS-PAGE (bottom).

TABLE 1

The extra band of GST-tagged recombinant protein mixture was identiﬁed to be heparan sulfate proteoglycan core protein (syndecan-2 and -4) by mass spectrometry

| Calculated mass | Expected mass | Delta mass | Score | Sequence |
|-----------------|---------------|------------|-------|----------|
| 978.5134        | 978.5472      | 0.0338     | 27    | RKPSAYQKA |
| 1209.5401       | 1209.5760     | 0.0360     | 23    | KPEIIEAAEQ |
| 1383.7358       | 1383.7636     | 0.0279     | 111   | KPSPLTSQFLPI |
| 1689.8859       | 1689.9096     | 0.0240     | 12    | RISLTSAAPEVFTMLKT |
| 1689.8859       | 1689.9247     | 0.0388     | 43    | RISLTSAAPEVFTMLKT |
| 1705.8808       | 1705.8818     | 0.0011     | 44    | RISLTSAAPEVFTMLKT + Oxidation (Met) |
| 1705.8808       | 1705.9086     | 0.0279     | 2     | RISLTSAAPEVFTMLKT + Oxidation (Met) |
| 1705.8808       | 1705.9092     | 0.0285     | 8     | RISLTSAAPEVFTMLKT + Oxidation (Met) |
| 3751.5367       | 3751.6066     | 0.0699     | 44    | KDMYDSSSIEEASGLYPIDDYSSASSGAGYEDKG |
| 4511.8969       | 4512.0257     | 0.1288     | 85    | RABLSDDKMYDSSSIEEASGLYPIDDYSSASSGAGYEDKG + Oxidation (Met) |

the presence of syndecan-2 transmembrane domain, the chemical shift perturbation of glycin residues of syndecan-4 transmembrane domain were observed, indicating the participation of GXXXG motif in the intermolecular interaction. In addition, evident chemical shift perturbations were observed in Ile-155, Gly-157, Gly-158, Val-159, Val-160, Val-166, and Phe-167 residues in the transmembrane domain of syndecan-4 upon syndecan-2 interaction (Fig. 38). Particularly, the most dramatic perturbations were observed in the residues, Gly-157, Val-159, and Val-160 residues (Fig. 3C). The molar intensity ratio between syndecan-4 homodimer and heterodimer of syndecan-2 and -4 was 1:3 based on peak intensities of the heteronuclear single quantum correlation spectrum (Fig. 3D), implying that the molecular interaction of syndecan-4 transmembrane domain to synde-
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TABLE 2
The extra band of GST-tagged recombinant protein mixture was identified to be heparan sulfate proteoglycan core protein (syndecan-2 and -4) by mass spectrometry.

| Calculated mass | Expected mass | Delta mass | Score | Sequence          |
|-----------------|---------------|------------|-------|-------------------|
| Mr              | Mr            |            |       |                   |
| 1612.7944       | 1612.8196     | 0.0252     | 38    | RETEVIDQDDLEBRY   |
| 2752.4548       | 2752.4986     | 0.0438     | 20    | RTPRVISSLVPLDHNHPENAQPQIRV |

FIGURE 2. Syndecans form both homomeric and heteromeric associations with partner syndecans. A, scheme for purifying His-syndecan proteins using GST-syndecans immobilized on glutathione beads. #1, cell lysates from E. coli expressing GST-syndecan fusion proteins were applied to a column of glutathione-agarose beads, and beads were washed with lysis buffer containing 1% Triton X-100. #2, cell lysates from E. coli expressing His-syndecan fusion proteins were applied to a column of glutathione-agarose beads containing bound GST-syndecan fusion proteins, and beads were washed with His lysis buffer containing 1% Triton X-100. #3, bound materials were eluted with elution buffer containing reduced glutathione. B and C, total lysates of cells expressing His-tagged syndecan-2 were passed over a column of glutathione-agarose bound to GST (B) or GST-syndecan-2 (C), washed three times, and eluted. Each fraction was analyzed by SDS-PAGE followed by Coomassie Blue staining or Western blotting using the indicated antibodies. D and E, total lysates of cells expressing His-tagged syndecan-4 (D) or His-tagged syndecan-2 (E) were passed over a column of glutathione-agarose bound to GST-syndecan-2 (D) or GST-syndecan-4 (E), washed three times, and eluted. Each fraction was analyzed by SDS-PAGE followed by Western blotting using the indicated antibodies.

Syndecan-2 is stronger than that of syndecan-4. Taken together, these data confirm that syndecan-2 and -4 form heteromeric interactions in vitro and that the transmembrane domain mediates the intermolecular interaction between syndecans.

Syndecan-2 Interacts with Syndecan-4—Because syndecans are modified with highly negatively charged glycosaminoglycan chains that may disrupt their interactions through charge repulsion, the hetero-oligomerization characteristics of endogenously expressed syndecans may differ from those of recombinant proteins. To investigate the intermolecular interactions of syndecans at the cellular level, we separately expressed HA- or Myc-tagged syndecan-2 or -4 in rat embryonic fibroblasts, and equal amounts of total cell lysate from REF-HA-syndecan-2 or REF-HA-syndecan-4 cells were mixed with those from REF-Myc-syndecan-2 or REF-Myc-syndecan-4 cells. Each mixture of total cell lysates was then immunoprecipitated with an anti-HA antibody, and the immunoprecipitate was analyzed by slot blotting using an anti-Myc antibody. As shown in Fig. 4A, HA-tagged syndecan-2 was co-immunoprecipitated with Myc-tagged syndecan-2 (Fig. 4A, bottom, left). A similar interaction of HA-tagged syndecan-4 and Myc-tagged syndecan-4 was also observed (Fig. 4A, bottom, right), supporting homo-oligomerization of both syndecan-2 and -4. Notably, HA immunocomplexes from the mixed lysates from REF-HA-syndecan-2 and REF-Myc-syndecan-4 cells or REF-HA-syndecan-4 and REF-Myc-syndecan-2 cells were immunoreactive to anti-Myc antibodies, indicating the hetero-oligomeric interactions of syndecan-2 with syndecan-4 (Fig. 4B, bottom). In addition, both slot blotting (Fig. 4C) and Western blotting (Fig. 4D) with appropriate anti-syndecan antibodies showed co-immunoprecipitation of endogenous syndecan-2 and -4, suggesting that syndecan-2 and -4 form both homomeric and heteromeric interactions in intact cells.

To further demonstrate the interaction of syndecan-2 with syndecan-4 at the cellular level, we employed a bimolecular fluorescence complementation assay. This protein-protein interaction technique relies on the ability of molecular fragments of a fluorescent protein, in this case fragments of pECFP (enhanced cyan fluorescent protein) fused to syndecan-2 and -4 (Fig. 4E), to re-associate and refold into a fluorescent structure. Cells were co-transfected with CN and CC fusion constructs of pECFP with syndecan-2 (2CN and 2CC), syndecan-4 (4CN and 4CC), and subsequently analyzed by laser-scanning confocal microscopy for fluorescence complementation. Under each co-transfection condition, fluorescence complementation of both 2CC and 2CN and of 4CC and 4CN was detected at the cell surface (Fig. 4F). Similarly, fluorescence complementation of both 2CC and 4CN and of 4CC and 2CN was detected (Fig. 4F), indicating homomeric association of both syndecan-2 and syndecan-4 and heteromeric interactions of syndecan-2 and -4. However, we failed to detect complementation of fluorescence between wild type syndecan and oligomerization-defective mutants (Fig. 4F). Collectively, these data strongly suggest that, when expressed in the same cell, syndecan-2 and -4 are capable of both homo-oligomerization and hetero-oligomerization.

Hetero-oligomerization Inhibits Syndecan-regulated Molecular Events That Depend on Homo-oligomerization—Because homo-oligomerization is crucial for syndecan functions, hetero-oligomer formation, which inevitably decreases homo-oligomerization, might result in inhibition of known syndecan functions. Previously, we and others have shown that oligomerization of the syndecan-4 cytoplasmic domain is critical for the interaction with and activation of PKCa (5, 14, 15). Solutions of purified wild type syndecan-2, purified 4eTC (a syndecan-4 deletion mutant containing the transmembrane domain with a 4-amino acid extracellular region and the cytoplasmic domain), or a mixture of 4eTC and wild type syndecan-2 were resolved by
FIGURE 3. Syndecan-4 transmembrane domain interacts with syndecan-2 transmembrane domain. A and B, 15N-labeled syndecan-4 transmembrane domain was titrated with non-labeled syndecan-2 transmembrane domain up to 1:3 molar ratio, and syndecan-4 and syndecan-4-syndecan-2 complex were displayed as red and green. C, NMR chemical shift perturbation map of the syndecan-4 transmembrane domain with syndecan-2 transmembrane domain titration. The bar diagram reveals the chemical shift changes at syndecan-4 transmembrane domain: syndecan-2 transmembrane domain molar ratio of 1:3. Chemical shift change values were calculated using the equation $\Delta \delta_{av} = \frac{(\Delta \delta_{1H})^2 + (\Delta \delta_{15N})^2}{2}$.

D, heterodimerization level of syndecan-2 with syndecan-4 was calculated as the intensity ratio between the syndecan-4 that interacted with syndecan-2 and the syndecan-4 that remained according to the intensity in NMR titration.

FIGURE 4. Syndecan-2 interacts with syndecan-4 in intact cells. A and B, REFs transiently transfected with HA- or Myc-tagged syndecans were lysed and analyzed by slot blotting (top). Individual cell lysates were mixed for 30 min on ice and protein mixtures were immunoprecipitated (IP) with HA-conjugated agarose beads. The levels of protein in each immunoprecipitate were determined by slot blotting with either anti-Myc or anti-HA antibodies (bottom). C, REF cell lysates were immunoprecipitated with the indicated antibodies as described under "Experimental Procedures." Immunocomplexes were slot-blotted with the indicated antibodies. GST-SDC2 and -SDC4 were used as positive controls for anti-syndecan antibodies. D, REF cell extracts were immunoprecipitated with anti-syndecan-2 or -4 antibodies, and immunocomplexes were treated with nitrous acid to degrade glycosaminoglycan chains. The levels of protein in each immunoprecipitate were determined by immunoblotting with anti-syndecan-4 or -2 antibodies. E, schematic representation of bimolecular fluorescence complementation plasmid constructs. F, HT29 cells were transiently co-transfected with the indicated cDNAs expressing SDC-CC and SDC-CN constructs. At 24 h post-transfection, the cells were fixed, and images were captured by confocal microscopy using a filter set specific for pECFP.

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![Diagram](https://example.com/diagram.png)

**FIGURE 5.** Disruption of homo-oligomerization inhibits the function of individual syndecans. A and B, purified recombinant syndecan-2 proteins were mixed with the syndecan-4 deletion mutant 4eTC for 30 min on ice and resolved by SDS-PAGE. Half of the gel was stained with Coomassie Blue (left). Proteins in the remaining gel half were transferred to PVDF membranes (right), after which membranes were incubated with B16F10 melanoma cell lysates, and interacting proteins were analyzed by Western blotting. Molecular mass markers, in kilodaltons, are shown. Migration positions of SDS-resistant syndecan-2 dimers (●●), 4eTC dimers (●●), and heteromeric syndecan (●●●) are indicated. C, purified recombinant syndecan-2 protein was mixed with the 4eTC, and PKC activity assays were performed (top). Molecular mass markers, in kilodaltons, are shown. Migration positions of SDS-resistant syndecan-2 dimers (●●), 4eTC dimers (●●), and heteromeric syndecan (●●●) are indicated. D, purified recombinant proteins were mixed as indicated, and gel overlay assays were performed as described in A using REF cell lysates. E, schematic representation of syndecan chimeras (PE2TPC) and deletion mutants (4ET, top). HEK293T cells were transiently co-transfected with the indicated cDNAs. After 24 h, cells were lysed, and MAPK activation was assessed using an antibody specific for phospho-ERK (pERK) followed by stripping and reprobing with an anti-ERK antibody.

SDS-PAGE. Half the gel was stained with Coomassie Blue to measure the amounts of homomeric (Fig. 5A, left, first and second lanes) and heteromeric (Fig. 5A, left, third lane) syndecans. Proteins in the other gel half were transferred to PVDF membranes (Fig. 5A, right); membranes were then incubated with B16F10 melanoma cell lysates, and interacting proteins were analyzed by Western blotting. Only homodimeric syndecan-4 mutants containing the cytoplasmic domain bound to PKCα (Fig. 5A, right); syndecan-2 homodimers and heterodimers of syndecan-2 and -4 did not. The increase in heterodimerization induced a significant decrease in syndecan-4 homodimers and led to a reduction in syndecan-4-PKCα interaction (Fig. 5B) and diminished PKCα activation (Fig. 5C, top), indicating an inhibitory effect of hetero-oligomerization on syndecan-4 function. Consistent with this, increased heterodimerization reduced the interaction of syndecan-4 with α-actinin, which is known to directly interact with the cytoplasmic domain of syndecan-4 (Fig. 5D).

Previous studies have shown that dimerization of PDGF receptor subsequently activates mitogen-activated protein kinase (MAPK) (16, 17). Moreover, a chimeric protein containing the transmembrane domain of syndecan fused to the extracellular and cytoplasmic domain of β-PDGFR receptor is sufficient to induced MAPK activation through chimera oligomerization (5). Accordingly, we explored the potential effect of transmembrane domain-mediated hetero-oligomerization on chimera-induced MAPK activation. HEK293T cells were transiently co-transfected with the chimeras PE2TPC and syndecan mutants containing extracellular and transmembrane domains of syndecan-4 (4ET), and chimera-induced MAPK activity was analyzed by Western blotting with an anti-phospho-ERK antibody. Consistent with previous data (5), phosphorylation of ERK was increased in chimera-transfected cells compared with that in vector-transfected cells (Fig. 5E). Co-transfection of the chimera with greater amounts of syndecan mutants caused a reduction in MAPK activity, implying the possibility of hetero-oligomer formation through the syndecan transmembrane domain and subsequent regulation of MAPK activation. Taken together, these results suggest that disruption of homo-oligomerization inhibits the function of individual syndecans.

**Hetero-oligomerization Negatively Regulates Syndecan-mediated Cellular Processes—**Because syndecan-4 binding to the high affinity heparin binding domain of fibronectin drives focal adhesion through PKCα and RhoA activation during cell adhesion (Fig. 6A; Refs. 18–20), we examined whether the formation of hetero-oligomers affected syndecan-4-mediated signaling. Consistent with prior studies (21), a centrifugal detachment assay showed greater cell adhesion to fibronectin by REFs over-expressing vectors encoding wild type syndecan-4 compared with those expressing the empty vector (Fig. 6B). Whereas adhesion to fibronectin increased in cells expressing individual syndecans, co-expression of syndecan-4 and -2 inhibited syndecan-4-mediated cell attachment to fibronectin. Furthermore, co-expression of syndecan-4 and -2 reduced syndecan-4-mediated PKCα membrane localization (Fig. 6C, top) and RhoA activation (Fig. 6D). In contrast, co-expression of wild type syndecan-4 and syndecan-2 dimerization-defective mutants had no effect on syndecan-4-mediated PKCα localization (Fig. 6C, bottom). As expected, induction of hetero-oligomerization reduced syndecan-4-mediated focal adhesion formation (Fig. 6E) and cell adhesion (Fig. 6F). These data suggest that hetero-oligomerization negatively regulates syndecan-4-mediated cellular processes, including PKCα activation and PKCα-mediated signaling during cell adhesion.

Because syndecan-2 regulates tumorigenic activity in an oligomerization-dependent manner (Fig. 7A; Refs. 22–24), we investigated whether hetero-oligomerization of syndecans might affect syndecan-2-mediated signal transduction involved in the regulation of tumorigenic activity. Co-localization of syndecan-2 and -4 was observed in HT29 colon adenocarcinoma cells transiently expressing syndecan-2 and -4 (Fig. 7B), suggesting that the two syndecans undergo hetero-molecular interactions in cancer cells. Similar to the observed effects on syndecan-4 signaling, our results showed that induction of hetero-oligomerization through co-overexpression of syndecan-2 and -4 significantly reduced the membrane localization of Tiam1 (T-cell lymphoma invasion and metastasis 1) (Fig. 7C) and activation of Rac (Fig. 7D), crucial regulators in syndecan-2-mediated signal transduction in colon cancer cells, compared...
with cells transfected with syndecan-2. Consistent with this, both syndecan-2-mediated cell migration and anchorage-independent growth were reduced in HT29 cells co-expressing syndecan-2 and -4 compared with those expressing syndecan-2 alone (Fig. 7, E–G), suggesting that hetero-oligomer formation inhibits syndecan-2 functions in colon cancer. Taken together, our findings indicate that hetero-oligomerization negatively regulates fundamental syndecan-mediated cellular processes.

Discussion

Syndecan core proteins are known to have the propensity to form non-covalently linked homodimers through interactions of a strictly conserved GXXXG motif in the transmembrane domain (4, 5, 25), but the potential of different syndecans to interact to form heterodimers has not been studied. Our investigation of the detailed interactions of syndecans reported here revealed that in addition to homodimer formation, all exogenously expressed syndecan-2 and -4 protein variants containing the transmembrane domain showed strong SDS-resistant heterodimer formation (Fig. 1). Consistent with this, endogenous syndecan-2 interacted with syndecan-4 (Fig. 4) notwithstanding potential repulsive forces contributed by heparan sulfate chains and steric hindrance by the large syndecan extracellular domains, both of which would tend to prevent transmembrane domain association. We also found that syndecan-1, which has a low tendency to self-associate (11), showed SDS-resistant heterodimer formation with syndecan-2 and -4. Therefore, syndecan transmembrane domains appear to be involved in both homomeric interactions of a single syndecan type and heteromeric interactions between syndecan paralogs.

At least one syndecan is expressed in virtually all normal cells, but the expression pattern is distinct in each cell type. For example, epithelial cells express syndecan-1 but not syndecan-2. Thus, opportunities for formation of syndecan hetero-oligomers may be rare in normal cells; a notable exception is vascular smooth muscle cells, which express all four syndecan family members (26). However, under pathological conditions, syndecan expression is commonly altered. During carcinogenesis, cancer cells may undergo “receptor exchange,” in which anti-tumorigenic receptors are down-regulated with the concomitant up-regulation of pro-tumorigenic receptors. For instance, the expression of syndecan-2, which is not expressed in normal epithelial cells, is increased during colorectal carcinogenesis (22). Altered syndecan-2 expression not only indicates that receptor exchange is sufficient for induction of colon tumorigenesis, it also suggests that the altered expression of syndecan-2 subsequently increases the likelihood of syndecan hetero-oligomerization. Syndecan hetero-oligomerization creates a new paradigm for integrating regulatory mechanism of syndecans in vivo. For example, syndecan-2 function has been studied independently from that of syndecan-4. However, hetero-oligomer formation between syndecan-2 and -4 raises the possibility of three cooperative regulatory mechanisms with
in cells: those involving homo-oligomers of syndecan-2, those involving homo-oligomers of syndecan-4, and those involving hetero-oligomers of syndecan-2 and -4. We hypothesized that syndecan hetero-oligomerization might reorganize the formation of functional complexes depending on the paralogs expressed and their relative abundance. Because transmembrane domain-induced homodimerization is important for the regulation of syndecan functions, the transition to heterodimers, which would necessarily constrain the formation of homo-oligomers, was expected to disrupt syndecan functions. Consistent with this, our results revealed that hetero-oligomer formation between syndecan-2 and -4 inhibited both syndecan-4-mediated cell adhesion functions (Fig. 6) and syndecan-2-mediated tumorigenic activity (Fig. 7). These data suggest that increased hetero-oligomerization negatively regulates syndecan-mediated tumorigenesis (Fig. 7). These data suggest that increased hetero-oligomerization negatively regulates syndecan-mediated tumorigenesis. The oligomeric status of the syndecan-4 cytoplasmic domain is particularly important for syndecan-4 functions, including interactions with effector molecules. Hetero-oligomerization prevents homo-association of syndecan-4 cytoplasmic domains, thereby negatively regulating syndecan-4-mediated functions. These hetero-interactions may also contribute to novel signaling specificities because, in hetero-oligomers, the identity and geometry of ligand pairs differ from that of homo-oligomers. Distinct sets of syndecans formed through hetero-oligomerization could be regulated by different ligands, resulting in differential coupling to signaling effectors and biological responses. Dissecting the cooperative signal transduction pathways of syndecan homo- and hetero-oligomers would be an interesting avenue for future research.

Transmembrane domain interactions have been shown to play a functional role in other receptors, including growth factor receptor signal transduction. Binding of ligands to the extracellular domain of the epidermal growth factor receptor (EGFR or Erb) induces the formation of receptor homo- or heterodimers and subsequent activation of the intrinsic tyrosine kinase domain (27). A hierarchy of homomeric and heteromeric association has been identified for ErbB transmembrane domain peptides (28). These interactions may contribute to unique signaling specificities because kinase/substrate pairs in a trans-phosphorylation reaction within a heterodimer differ from those of a homodimer. In NIH3T3 cells ectopically expressing EGFR and ErbB3, neuregulin stimulates anchorage-independent growth, whereas EGF does not despite the fact that EGF stimulates higher levels of receptor phosphorylation than does neuregulin (29). Similarly, EGFR is activated by HB-EGF in a breast tumor cell line, leading to tyrosine phosphorylation of the downstream signaling effector Cbl and subsequent Cbl-EGFR interactions, whereas in EGFR-expressing cells in which EGFR is activated by neuregulin through transmodulation by ErbB3 or ErbB4, Cbl is not tyrosine phosphorylated and does not complex with the EGFR (30). These data suggest that heterodimerization mediates phosphorylation of distinct sets of receptor tyrosine residues in response to differ-
ent ligands, creating an extracellular regulatory mechanism. Therefore, hetero-oligomerization through transmembrane domains might potentiate the diversification of biological functions.

This work clearly showed that syndecans form transmembrane domain-mediated heterodimers, and this dimerization regulates syndecan-mediated cellular functions. However, the effect of hetero-oligomerization might differ in various cells and tissues, perhaps due to tissue-specific differences in expression patterns and syndecan regulatory mechanisms. Further studies will be required to clarify the roles of hetero-oligomerization in specific syndecan regulatory mechanisms.

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