

# The Conserved Phenylalanine in the Transmembrane Domain Enhances Heteromeric Interactions of Syndecans\*

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The transmembrane domain (TMD) of the syndecans, a family of transmembrane heparin sulfate proteoglycans, is involved in forming homo- and heterodimers and oligomers that transmit signaling events. Recently, we reported that the unique phenylalanine in TMD positively regulates intramolecular interactions of syndecan-2. Besides the unique phenylalanine, syndecan-2 contains a conserved phenylalanine (SDC2-Phe-169) that is present in all syndecan TMDs, but its function has not been determined. We therefore investigated the structural role of SDC2-Phe-169 in syndecan TMDs. Replacement of SDC2-Phe-169 by tyrosine (S2(F169Y)) did not affect SDS-resistant homodimer formation but significantly reduced SDS-resistant heterodimer formation between syndecan-2 and -4, suggesting that SDC2-Phe-169 is involved in the heterodimerization/oligomerization of syndecans. Similarly, in an *in vitro* binding assay, a syndecan-2 mutant (S2(F169Y)) showed a significantly reduced interaction with syndecan-4. FRET assays showed that heteromolecular interactions between syndecan-2 and -4 were reduced in HEK293T cells transfected with S2(F169Y) compared with syndecan-2. Moreover, S2(F169Y) reduced downstream reactions mediated by the heterodimerization of syndecan-2 and -4, including Rac activity, cell migration, membrane localization of PKC $\alpha$ , and focal adhesion formation. The conserved phenylalanine in syndecan-1 and -3 also showed heterodimeric interaction with syndecan-2 and -4. Taken together, these findings suggest that the conserved phenylalanine in the TMD of syndecans is crucial in regulating heteromeric interactions of syndecans.

Integral membrane receptors consist of an extracellular domain that binds specific ligands, a transmembrane domain (TMD)<sup>2</sup> that transmits signals in response to ligand binding, and a cytoplasmic domain to which the signals are transmitted by the TMD and that is thereby activated, resulting in a conformational change that causes binding or induction of enzymatic activity inside the cell (1, 2). The TMD is therefore critically

important in transmitting signals from the external environment to the inside of the cell, with many recent investigations exploring the mechanism by which TMD interactions regulate cell signaling (2–6). TMDs of single-pass membrane receptors have been shown to cluster, resulting in homotypic and/or heterotypic interactions, with TMDs often forming not only homo- and/or heterodimers but higher-order oligomers in cell membranes (7–10). Most investigations of TMD interactions have analyzed homo-oligomerization *in vitro* and *in vivo*, with fewer to date assessing heterotypic TMD associations.

One of the most investigated examples of hetero-oligomerization in biology involves the family of EGF receptors, also called the ErbB family (11–13). Although only four ErbB family members have been identified to date, ErbB1 (EGF receptor), ErbB2 (HER2, Neu), ErbB3 (HER3), and ErbB4 (HER4), they have been shown to form 28 homo- and heterodimers (12). These combinations are thought to result in diverse cellular signals and to be associated with several types of diseases and cancers (12, 13). Except for ErbB3, the TMDs of ErbB family members contain two GXXXG-like motifs that are critical in the formation of homo- or hetero-oligomers of these proteins by stabilizing the interactions of transmembrane helices (14, 15).

Syndecans are a family of transmembrane heparin sulfate proteoglycans consisting of four members: syndecan-1, -2, -3, and -4. Each of these heparin sulfate proteoglycans consists of an extracellular domain, a cytoplasmic domain, and a single TMD containing the GXXXG motif (16, 17). The amino acid sequences of syndecan TMDs are highly homologous, enabling members of the syndecan family to form SDS-resistant homodimers even in the absence of ligand binding to their extracellular domains (17, 18). In addition, the GXXXG motif-mediated interactions of syndecans are important in regulating syndecan functions in cells (17). Syndecan family members can also form SDS-resistant heterodimers *in vitro* and *in vivo* through binding of their GXXXG motifs (18, 19). Furthermore, we have reported recently that the hetero-oligomerization between syndecan-2 and -4 reduces the activities mediated by these proteins (19). However, although previous findings have shown that the expression patterns of individual syndecans are regulated differently in individual cells and tissue types (20, 21) and that syndecan hetero-oligomerization in cell membranes may be important in several diseases and cancers, relatively little is known about the mechanisms underlying hetero-oligomerization of syndecan family members.

Studies have shown that interactions of the aromatic amino acids (*e.g.* phenylalanine, tyrosine, and tryptophan) are critical

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<sup>2</sup> The abbreviations used are: TMD, transmembrane domain; REF, rat embryonic fibroblast; CFP, cyan fluorescence protein; PBD, PAK1 binding domain; Ni-NTA, nickel-nitrilotriacetic acid.

not only for the assembly of soluble proteins like amyloid fibrils but also for the assembly of the TMDs of membrane proteins (22–24). Experiments using a randomized TMD library and expression in bacteria have shown that the presence of aromatic residues resulted in strong and stable self-interactions of TMDs (25, 26). We found that a unique phenylalanine present in the TMD of syndecan-2 participates in the strong and stable SDS-resistant dimerization of syndecan-2 and regulates syndecan-2-related functions (27). In addition to this unique phenylalanine, the syndecan-2 TMD contains an additional phenylalanine near the GXXXG motif, suggesting that the latter phenylalanine residue may have functional roles. This study therefore analyzed the role of the conserved phenylalanine in TMDs in the heterodimerization/oligomerization of syndecans and assessed the mechanism by which this TMD-mediated heterodimerization/oligomerization regulates syndecan functions.

### Experimental Procedures

**Antibodies and Materials**—Monoclonal anti-Erk, phospho-Erk,  $\beta$ -actin, and GST were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal anti-rac1 and monoclonal anti-paxillin were purchased from Millipore (Billerica, MA), and polyclonal anti-PKC $\alpha$ , integrin  $\beta$ 1, His, and syndecan-4 were purchased from Santa Cruz Biotechnology. Fibronectin was obtained from Upstate Biotechnology (Lake Placid, NY). The mAb to syndecan-2 was produced by AdipoGen (28).

**Cell Culture**—Rat embryonic fibroblasts (REFs) were maintained in  $\alpha$ -modified Eagle's medium (Gibco BRL) supplemented with 5% (v/v) FBS (GE Healthcare Hyclone) and gentamycin (50  $\mu$ g/ml) (Sigma-Aldrich, St. Louis, MO). HCT116 cells were maintained in McCoy's 5a medium (Welgene, Daegu, Korea). HEK293T cells were maintained in DMEM (Gibco BRL) supplemented with 10% (v/v) FBS and gentamycin (50  $\mu$ g/ml). All cell lines were maintained at 37 °C in a humidified 5% CO<sub>2</sub> atmosphere. Transient transfections were carried out using Vivamagic (Vivagen, Seongnam, Korea) as described in the provided protocol.

**Construction and Transfection of Expression Vectors**—The extracellular and transmembrane domains (amino acids 151–179) of rat syndecan-2 were linked with the cytoplasmic domain of human PDGF receptor (amino acids 557–1107) to generate the chimeric protein 2eTPC. Single point mutants in the TMDs of syndecan-2 or -4 (*i.e.* S2(F167Y), S2(F169Y), S2(F172Y), and S4(F164Y)) were constructed by commercial gene synthesis (Bioneer, Daejeon, Korea). The chimeras or point mutants were inserted into the N-terminal HA-tagging pcDNA3 expression vector (Invitrogen). HEK293T cells ( $6.0 \times 10^5$  cells/well), REFs ( $8.0 \times 10^4$  cells/well), and HCT116 cells ( $1.0 \times 10^6$  cells/well) were plated on 6-well plates, incubated at 37 °C for 24 h, and then transfected with the generated expression vectors as described in the Vivagen protocol.

**Construction of the Vector and Fluorescence Resonance Energy Transfer Assay**—The SDC3-YFP and SDC3-CFP constructs were provided by Prof. Heikki Rauvala (Neuroscience Center, University of Helsinki, Helsinki, Finland) (29). Rat syndecan-2, syndecan-4, or single point mutant cDNA was ligated

into the provided expression vector. HEK293T cells were transfected with cDNAs described earlier and imaged on a Leica TCS SP8 inverted microscope. All images were analyzed with Leica software (LAS X). Acceptor photobleaching was carried out for the evaluation of FRET efficiencies. Briefly, cells co-transfected with either syndecan-2 and -4 wild-type or mutant syndecan-2 and -4 were plated onto coverslips, fixed for 5 min in 3.5% (w/v) formaldehyde, and washed with PBS, and then the slides were mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA) and visualized with a HCX PL APO  $\times 100$  objective lens (numerical aperture, 1.40) using a 458-nm argon laser light and HyD detector (462–510 nm) for cyan fluorescent protein (CFP) excitation and emission and a 514-nm argon laser and HyD detector (518–580 nm) for YFP excitation and emission. Prebleach CFP and YFP images were collected simultaneously following excitation at 458 and 514 nm for CFP and YFP, respectively. The regions were selected automatically using Leica LAS X software (one or two bleached region(s) per cell). Selected regions were irradiated with a 514-nm laser (100% intensity, 80 iterations) to bleach YFP. Post-bleach CFP and YFP images were collected simultaneously. The FRET efficiency was calculated as  $100 \times [(D_{\text{post}} - D_{\text{pre}}) / D_{\text{post}}]$ , where  $D_{\text{post}}$  is the post-bleaching fluorescence intensity of CFP, and  $D_{\text{pre}}$  is the pre-bleaching fluorescence intensity of CFP. This calculation was performed using the FRET AB program of the Leica LAS X software package. Photobleaching was performed in 8–15 cells/experiment, 10 regions of interest were analyzed for each image, and at least five images were quantified per experiment.

**Immunoblotting**—Cultures were washed twice with PBS, and the cells were lysed in radioimmune precipitation assay buffer (50 mM Tris (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 10  $\mu$ M NaF, and 2  $\mu$ M Na<sub>3</sub>VO<sub>4</sub>) containing a protease inhibitor mixture (1  $\mu$ g/ml aprotinin, 1  $\mu$ g/ml antipain, 5  $\mu$ g/ml leupeptin, 1  $\mu$ g/ml pepstatin A, and 20  $\mu$ g/ml phenylmethylsulfonyl fluoride). Cell lysates were clarified by centrifugation at 13,000 rpm for 15 m at 4 °C, denatured with SDS-PAGE sample buffer, boiled, and analyzed by SDS-PAGE. Proteins were transferred to 0.45- $\mu$ m nitrocellulose blotting membranes (Amersham Biosciences, Piscataway, NJ) and probed with the appropriate antibodies. Signals were detected by an Odyssey CLx imager and analyzed by Image Studio Lite software (LI-COR Biosciences, Lincoln, NE).

**Expression and Purification of Recombinant His-Syndecan Core Proteins**—The cDNAs encoding the full-length rat syndecan-2 or -4 core proteins, the single point mutants (S2(F167Y), S2(F169Y), S2(F172Y), and S4(F164Y)), rat syndecan-1 core proteins, and the point mutants (S1(F269Y), S1(F269Y,C272F), and S1(C272F)), and rat syndecan-3 core proteins were synthesized by PCR and subcloned into the His-tagging expression vector pET32a+ (Novagen, Madison, WI). The expression of fusion proteins in *Escherichia coli* BL21 was induced by incubation with 0.3 mM isopropyl- $\beta$ -D-thiogalactopyranoside at 30 °C for 16 h. The *E. coli* cells were lysed with lysis buffer (20 mM Na<sub>2</sub>HPO<sub>4</sub> (pH 8.0), 150 mM NaCl, 5 mM  $\beta$ -mercaptoethanol, and 0.5% Triton X-100) containing a protease inhibitor mixture, with sonication on ice for 1 m. The insoluble material was removed by centrifugation at  $13,000 \times g$  for 30 m at 4 °C, and

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the supernatants containing His-syndecan fusion proteins were applied to Ni-NTA agarose columns (Qiagen, Hilden, Germany). Each column was washed three times with lysis buffer containing 50 mM imidazole, and the bound proteins were eluted with lysis buffer containing 500 mM imidazole.

**Expression and Purification of Recombinant GST-Syndecan Core Proteins**—The cDNAs encoding rat syndecan-2 and -4 without extracellular domain (2eTC and 4eTC) were synthesized by PCR and subcloned into the GST expression vector pGEX-5X-1 (Amersham Biosciences). These constructs were used to transform *E. coli* DH5 $\alpha$ , and the expression of GST fusion proteins was induced by incubation with 1 mM isopropyl- $\beta$ -D-thiogalactopyranoside for 4 h at 37 °C. The fusion proteins were purified with glutathione-agarose beads (GE Healthcare Life Sciences) as described previously (17).

**Cellular Fractionation**—Cultures were washed twice with PBS, and the cells were lysed in radioimmune precipitation assay buffer (50 mM Tris (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 10  $\mu$ M NaF, and 2  $\mu$ M Na<sub>3</sub>VO<sub>4</sub>) containing a protease inhibitor mixture (1  $\mu$ g/ml aprotinin, 1  $\mu$ g/ml antipain, 5  $\mu$ g/ml leupeptin, 1  $\mu$ g/ml pepstatin A, and 20  $\mu$ g/ml phenylmethylsulfonyl fluoride). Cell lysates were clarified by centrifugation at 13,000 rpm for 15 min at 4 °C, denatured with SDS-PAGE sample buffer, boiled, and analyzed by SDS-PAGE. Proteins were transferred to 0.45- $\mu$ m nitrocellulose blotting membranes (Amersham Biosciences) and probed with the appropriate antibodies. Signals were detected by an Odyssey CLx imager and analyzed by Image Studio Lite software (LI-COR Biosciences).

**Focal Adhesion Assay**—Fibronectin was diluted in serum-free medium (10  $\mu$ g/ml), added to the plates, and incubated at 37 °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 transfected with the indicated cDNAs were incubated for additional 2 h at 37 °C in 5% CO<sub>2</sub>. After washing with PBS, cells were fixed with 3.5% paraformaldehyde in PBS at room temperature for 5 min, permeabilized with 0.1% Triton X-100 in PBS for 10 min, blocked with 0.5% bovine serum albumin in PBS for 1 h, and incubated with anti-paxillin. The slides were mounted with Vectashield mounting medium (Vector Laboratories) and imaged using a fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

**Flow Cytometry**—Transfected REF cells were harvested with 5 mM EDTA and 5% FBS in PBS, washed twice with PBS, aliquoted, and incubated separately with SDC2 antibodies. After incubation for 16 h at 4 °C, cells were washed three times with PBS containing 0.05% Tween 20 and stained with FITC-conjugated secondary antibodies (Abclone, Seoul, Korea). After 1 h of incubation in the dark, cells were washed three times with PBS and resuspended in PBS. Fluorescence was then measured by flow cytometry using FACSCalibur (BD Biosciences) and analyzed with CellQuestPro software (BD Biosciences).

**Transwell Migration Assay**—The lower surface of Transwell inserts (Costar) was coated with gelatin (10  $\mu$ g/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 M medium containing 5% FBS. Cells ( $7 \times 10^5$ ) were added to each upper chamber, and the plate was incubated at 37 °C in a 5% CO<sub>2</sub> incubator for 16 h.

Migrated cells were stained with 0.6% hematoxylin and 0.5% eosin and counted.

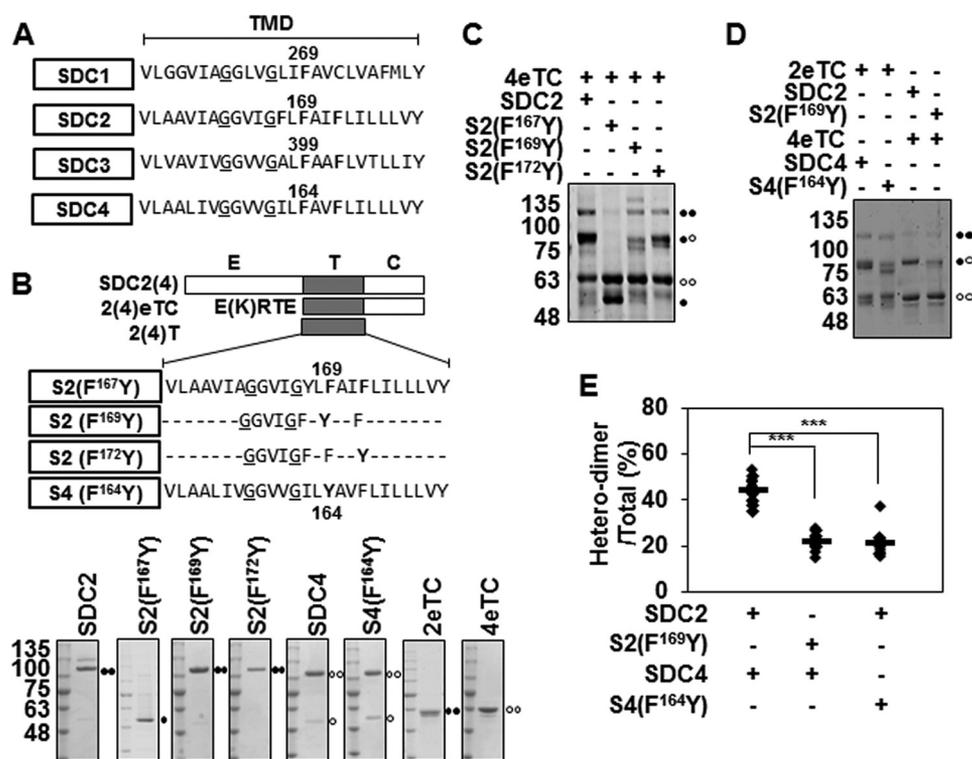
**Rac Activity Assay**—GST-PAK-PBD binding assays were performed essentially as described previously (31). Briefly, the p21-binding domain of PAK1 (PBD) was expressed in *E. coli* as a GST-PAK-PBD fusion protein and purified using glutathione-agarose beads. The glutathione-agarose bead-bound GST-PBD was washed with lysis buffer three times and mixed with transfected HCT116 cell lysates of equal volume and concentration for 2 h at 4 °C. The beads were washed four times with lysis buffer, and bound rac1 proteins were detected by Western blotting using a polyclonal antibody against rac1.

**RNA Extraction and RT-PCR**—Total RNA extracted from transfected cells was used as a template for the reverse transcriptase reaction. Aliquots of cDNA were amplified using the following primers: rat SDC2, 5'-ATGCGGGTACGAGCC-ACGTC-3' (forward) and 5'-CGGGAGCAGCACTAGTAGG-3' (reverse); rat SDC4, 5'-ATGGCGCCTGTCTGCCT-GTT-3' (forward) and 5'-GCTGCCCTGGGAAGTGC-TGG-3' (reverse); and  $\beta$ -actin, 5'-TGGAATCCTGTGGCAT-CCATGAAA-3' (forward) and 5'-TAAAACGCAGCTCAGT-AACAGTCCG-3' (reverse). After an initial denaturation at 94 °C for 5 min, we performed 25 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 60 s, and extension at 72 °C for 60 s. The reaction products were analyzed in 1% agarose gels.

**Statistical Analysis**—Data are presented as the means from at least three independent experiments. Statistical analysis was performed using unpaired Student's *t* test. *p* < 0.05, 0.01, or 0.001 was considered statistically significant.

## Results

**The Conserved Phenylalanine Regulates SDS-resistant Heterodimer Formation of Syndecan-2**—We have reported previously that syndecan-2 and -4 formed SDS-resistant heterodimers through the GXXXG motif in their TMDs (19) and that the unique phenylalanine (Phe-167) on the TMD of syndecan-2 strengthened its homodimerization (27). In addition to Phe-167, the syndecan-2 TMD contains additional phenylalanines near the GXXXG motif. In particular, Phe-169 is conserved in all syndecan family members, as Phe-269 in syndecan-1, Phe-399 in syndecan-3, and Phe-164 in syndecan-4 (Fig. 1A). To assess the functional role of this residue, we analyzed its involvement in the regulation of SDS-resistant dimerization, a characteristic of syndecan family members. When Phe-167 was replaced by tyrosine (S2(F167Y); Fig. 1B, bottom panel), SDS-resistant homodimer syndecan-2 formation was reduced markedly. However, replacement of Phe-169 (S2(F169Y)) or Phe-172 (S2(F172Y)) by tyrosine did not affect SDS-resistant formation of syndecan-2 dimers (Fig. 1B, bottom panel), suggesting that Phe-169 is not involved in regulating the homodimeric interaction of the syndecan-2 TMD. Because this TMD also mediates heteromeric interactions of syndecans, we investigated whether Phe-169 could regulate SDS-resistant heterodimer formation. Consistent with a previous report (19), we found that wild-type syndecan-2 (SDC2) and a syndecan-4 mutant (4eTC) formed SDS-resistant heterodimers, whereas the S2(F169Y) mutant showed reduced SDS-resistant heterodimer formation with 4eTC compared with the S2(F172Y)



**FIGURE 1. The conserved phenylalanine in the syndecan transmembrane domain enhances the SDS-resistant formation of syndecan heterodimers.** *A*, amino acid sequences of the rat syndecan-1, -2, -3, and -4 transmembrane domains. The conserved GXXXG motif is *underlined*, and the phenylalanines are shown in *boldface*. *B*, *top panel*, schematic of syndecan-2 and -4 single point mutants. The extracellular domain (E), transmembrane domain (T), cytoplasmic domain (C), and the four amino acid residues in the membrane-flanking region (ERTE, KRTE) are shown. *Bottom panel*, recombinant His-tagged thioredoxin (Trx), wild-type syndecans (SDC2 and SDC4), syndecan single point mutants (S2(F167Y), S2(F169Y), S2(F172Y), and S4(F164Y)), and syndecan mutants (2eTC and 4eTC) were purified using Ni-NTA columns, separated by 10% SDS-PAGE, and stained with Coomassie Blue. ●● and ○○, syndecan-2 or -4 homodimer, respectively; ● and ○, syndecan-2 or -4 monomer, respectively. *C* and *D*, the indicated syndecan wild type and mutants were purified and mixed for 10 min on ice, separated by 10% SDS-PAGE, and stained with Coomassie Blue. ●● and ○○, syndecan-2 or -4 homodimer, respectively; ● and ○, syndecan-2 or -4 monomer, respectively; ●○, syndecan-2 and -4 heterodimer. *E*, 15 stained heterodimer/monomer syndecan bands were analyzed quantitatively using Image Studio Lite software. The average levels are indicated by *horizontal bars*. The results are represented as mean  $\pm$  S.D. \*\*\*,  $p < 0.001$ .

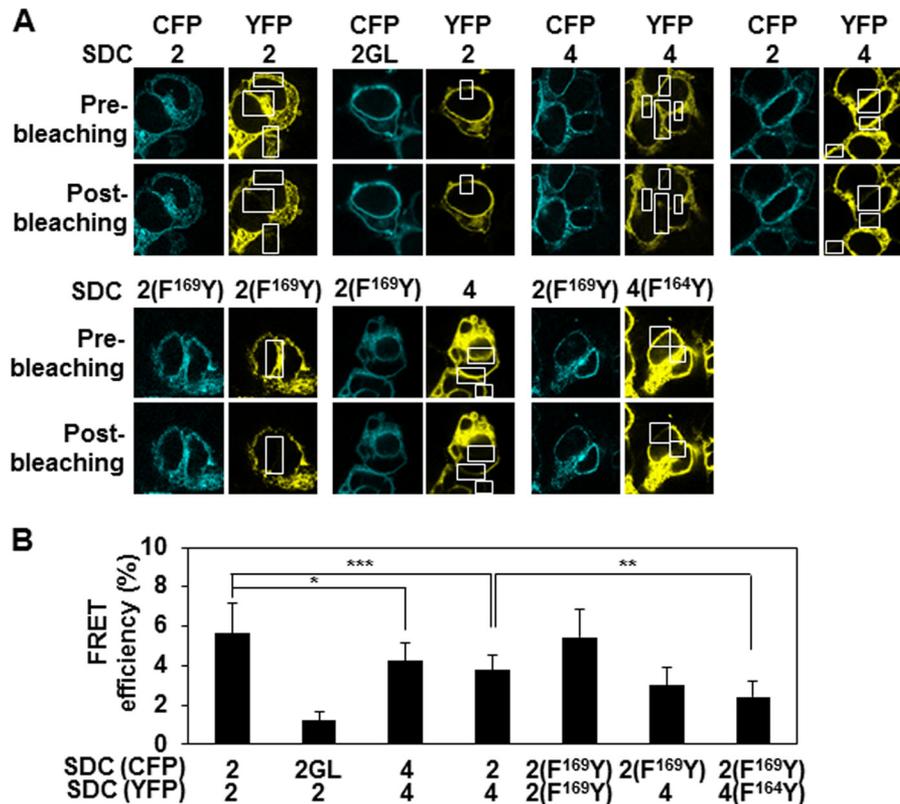
mutant or SDC2 wild type (Fig. 1C). Because S2(F172Y) did not show this effect, these findings suggest a specific role for Phe-169 in TMD-mediated heteromeric interactions between syndecan TMDs. Syndecan-4 substitution mutants (S4(F164Y)) in which the conserved phenylalanine was replaced by tyrosine consistently showed reduced SDS-resistant heterodimer formation with syndecan-2 (2eTC) (Fig. 1D). Quantitative results showed that heterodimer formation by the syndecan-2 (S2(F169Y)) and syndecan-4 (S4(F164Y)) substitution mutants was reduced compared with wild-type syndecan-2 and -4 (Fig. 1E). Taken together, these results indicate that Phe-169 is involved in regulating syndecan-2-mediated intermolecular interactions with other syndecans and that the conserved phenylalanine on syndecan TMDs is critical for the formation of SDS-resistant syndecan heterodimers.

*The Conserved Phe-169 in the Syndecan-2 TMD Mediates Intermolecular Interactions with Syndecan-4*—To assess the involvement of Phe-169 of the syndecan-2 TMD in heteromolecular interactions with the syndecan-4 TMD, either wild-type syndecan-2 or the S2(F169Y) mutant were mixed with different amounts of 4eTC, analyzed by SDS-PAGE following Coomassie Blue staining, and compared with SDS-resistant heterodimer formation abilities. Compared with syndecan-2, the S2(F169Y) mutant showed reduced SDS-resistant heterodimer formation with 4eTC (Fig. 2A). To further investigate whether the con-

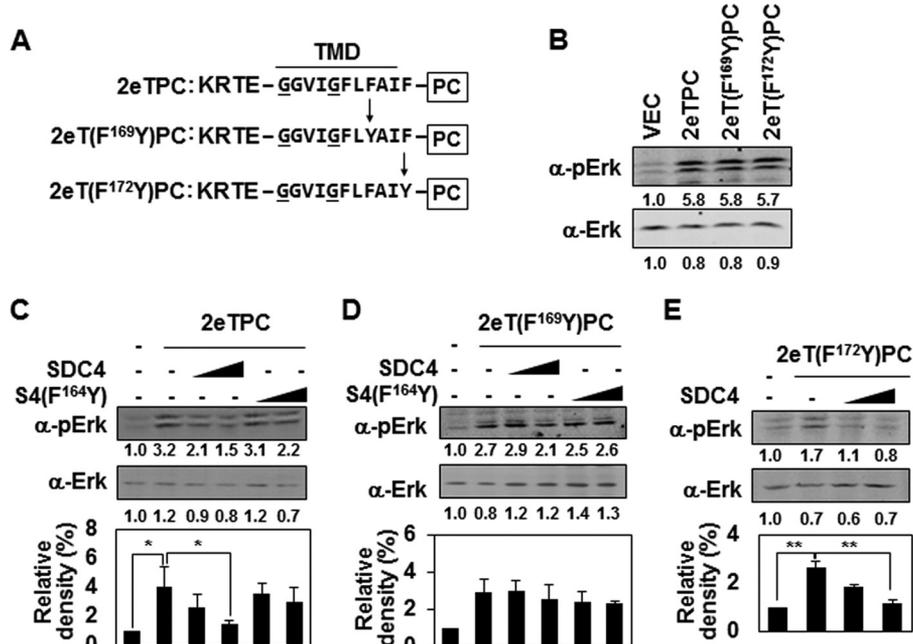
served Phe-169 in the syndecan-2 TMD mediated the intermolecular interaction with syndecan-4, purified His-tagged syndecan-2 was subjected to SDS-PAGE and transferred to PVDF membranes. These membranes were incubated with GST-tagged 4eTC proteins, and then heteromolecular interactions were analyzed by Western blotting. Compared with wild-type syndecan-2, the syndecan-2 mutant (S2(F169Y)), but not (S2(F172Y)), showed a significantly reduced interaction with GST-tagged 4eTC proteins (Fig. 2B), suggesting that the replacement of Phe-169 reduced the intermolecular interaction between syndecan-2 and -4. Interestingly, the heteromolecular interactions of syndecan-4 with syndecan-2 were reduced significantly for dimeric syndecan-2 but not for monomeric syndecan-2 (Fig. 2B). In addition, when GST-4eTC was immobilized on glutathione beads and its interaction with His-tagged syndecan-2 was analyzed, the His-tagged syndecan-2 mutant (S2(F169Y)) showed a significantly reduced interaction with GST-4eTC (Fig. 2C). Taken together, all of these findings indicate that the conserved Phe-169 in syndecan-2 regulates heteromolecular interactions with syndecan-4.

*The Conserved Phenylalanine on the Syndecan TMD Regulates the Intermolecular Interactions of Syndecan-2 and -4 in Living Cells*—FRET was used to investigate whether the conserved phenylalanine of TMDs was involved in regulating heterodimeric interactions between syndecans. To directly inves-



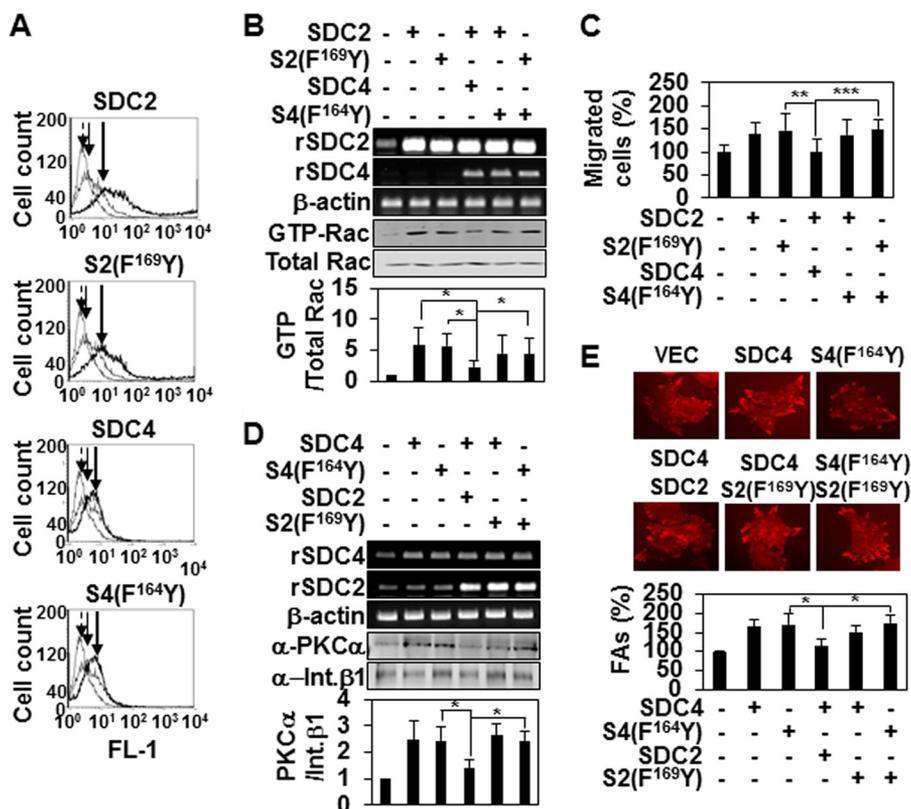


**FIGURE 3. The conserved phenylalanine in the syndecan transmembrane domain regulates the intermolecular interactions between syndecan-2 and -4 in living cells.** *A*, HEK293T cells were co-transfected with plasmids encoding the syndecan fusion proteins linked to CFP and YFP. The CFP and YFP images were obtained before and after photobleaching with high-intensity argon laser light. Ten regions of interest were analyzed for each image, and at least five images were quantified. *B*, FRET efficiencies calculated with Leica LAS AF software during YFP photobleaching. The results are represented as mean  $\pm$  S.D. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ .



**FIGURE 4. The conserved phenylalanine affects transmembrane domain-mediated heterodimer formation and chimera-induced MAPK activation.** *A*, schematics of the 2eTPC, 2eT(F<sup>169</sup>Y)PC, and 2eT(F<sup>172</sup>Y)PC chimeras in which four amino acid residues of the extracellular domains of rat syndecan-2 (KRTE) and their corresponding TMDs were linked to the cytoplasmic domain of the human PDGF receptor. PC, cytoplasmic domain of the PDGF receptor. *B–E*, HEK 293T cells co-expressing 2eTPC or mutant and SDC4 or mutant were lysed with radioimmune precipitation assay buffer. VEC, pcDNA 3.1 (empty vector). Equal amounts of cell lysates were analyzed by Western blotting using antibodies against phospho-Erk and Erk. The data shown are representative of three independent experiments. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ .

## Role of the Conserved Phenylalanine in Syndecan Interaction



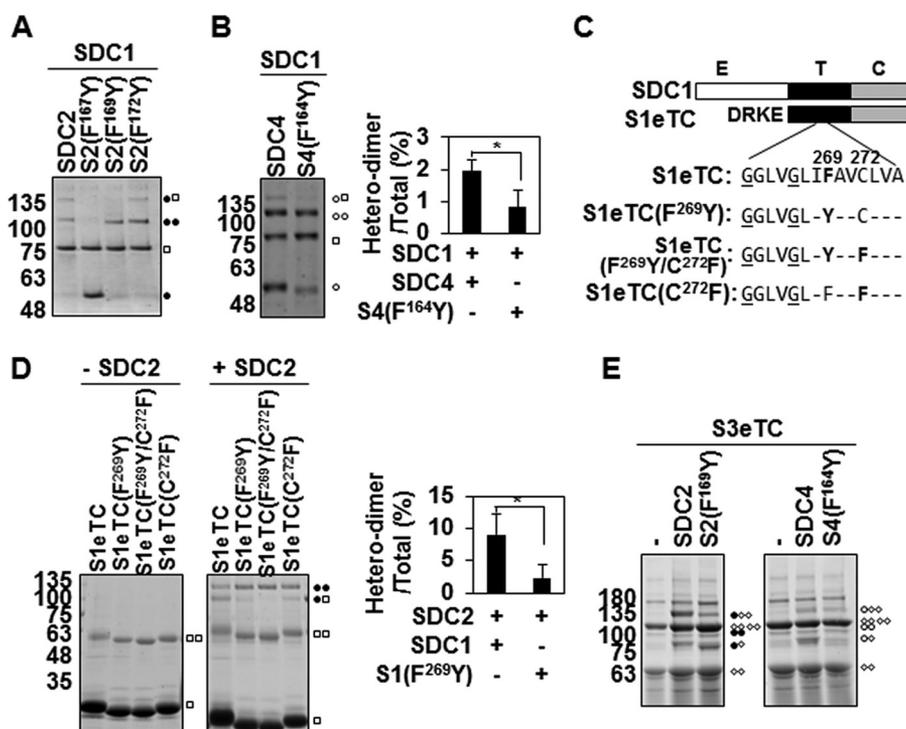
**FIGURE 5. The conserved phenylalanine is critical for heterodimerization-mediated inhibition of syndecan-2 and -4 functions.** *A*, transfected cells were analyzed by flow cytometry as described under "Experimental Procedures." Dotted arrows, IgG; solid arrows, vector; thick arrows, syndecan-2, -4, or mutant constructs. *B*, Rac activity of HCT116 cells co-transfected with the indicated syndecan-2 and -4 wild type or mutants. Total cell lysates were used as a positive control, and the transfection efficiency was analyzed by RT-PCR. The data shown are representative of three independent experiments. \*,  $p < 0.05$ . *C*, HCT116 cells were transfected with the indicated cDNAs, and Transwell migration assays were performed as described under "Experimental Procedures." The data shown are representative of three independent experiments. \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ . *D*, REF cells were co-transfected with the indicated syndecan-4 and -2 wild type or mutants, and the amount of PKC $\alpha$  in the membrane fraction was determined by immunoblotting with anti-PKC $\alpha$  antibody. An antibody against integrin  $\beta 1$  was used as a loading control. The data shown are representative of three independent experiments. \*,  $p < 0.05$ . *E*, REF cells co-transfected with the indicated cDNAs were incubated on fibronectin-coated plates for 2 h and stained with anti-paxillin antibody (top panel). The results are expressed as the average percentage of cells showing focal adhesion from three independent experiments (bottom panel). \*,  $p < 0.05$ .

the chimeras showing a significant increase in Erk phosphorylation (Fig. 4B). Interestingly, 2eTPC-mediated Erk phosphorylation was reduced by co-expression with syndecan-4, confirming that the homodimeric interactions of syndecan-2 were disrupted by the heterodimeric interaction of the latter with syndecan-4. In contrast, 2eTPC-mediated Erk phosphorylation was relatively unaffected by expression of S4(F164Y) (Fig. 4C), and 2eT(F169Y)PC-mediated Erk phosphorylation was not affected by expression of syndecan-4 (Fig. 4D), whereas 2eT(F172Y)PC-mediated Erk phosphorylation was reduced significantly by syndecan-4 (Fig. 4E). Collectively, these results indicate that the conserved phenylalanine on TMDs of syndecans is crucial in regulating the intermolecular interactions between syndecan-2 and -4.

**The Conserved Phenylalanine Regulates Heterodimerization-mediated Syndecan-2 and -4 Functions**—Because our research group has shown that increased heterodimerization, which disrupts homo-oligomerization, inhibits individual functions of syndecans (19), we expected that a change in the ability to heterodimerize may alter the function of heterodimers. To investigate this possibility, we examined whether altering heterodimer formation affected syndecan-2-mediated tumorigenic signal transduction. Transfection into HCT116 cells of plasmid constructs encoding wild-type or mutant syndecan-2

(S2(F169Y)) or wild-type or mutant syndecan-4 (S4(F164Y)) resulted in expression of the respective proteins and their localization on the cell surface (Fig. 5A).

Expectedly, expression of syndecan-2 or S2(F169Y) in HCT116 colon cancer cells enhanced Rac activity, a crucial regulator of syndecan-2-mediated cell migration (34, 35). In addition, co-expression of syndecan-2 and -4 reduced syndecan-2-mediated Rac activation compared with cells transfected with syndecan-2 (Fig. 5B). In contrast, co-expression of either wild-type syndecan-2 and S4(F164Y) or S2(F169Y) and syndecan-4 had no effect on the inhibition of syndecan-2-mediated Rac activation (Fig. 5B). As expected, syndecan-4 induction of hetero-oligomerization reduced syndecan-2-mediated cell migration but did not inhibit S2(F169Y)-mediated cell migration activity (Fig. 5C). Consistent with this, co-expression of syndecan-4 and -2 reduced syndecan-4-mediated PKC $\alpha$  membrane localization in REF cells whereas co-expression of wild-type syndecan-4 and S2(F169Y) did not (Fig. 5D). As expected, S2(F169Y) did not reduce syndecan-4-mediated focal adhesion formation (Fig. 5E). Taken together, these results suggest that the conserved phenylalanine plays a role in regulating heteromeric interactions of syndecans and heterodimer-mediated syndecan functions.



**FIGURE 6. The conserved phenylalanine has a critical role in syndecan-1 heterodimer formation.** *A, B, and E*, the indicated syndecan wild type and mutants were mixed for 10 min on ice, separated by 8% SDS-PAGE, and stained with Coomassie Blue. The data shown are representative of three independent experiments. \*,  $p < 0.05$ . ●● or ○○, syndecan-2 or -4 homodimer, respectively; ● or ○, syndecan-2 or -4 monomer, respectively; ●□ and ○□, syndecan-2 or syndecan-1 or syndecan-4 or syndecan-1 heterodimer, respectively; □, syndecan-1 monomer; ◇◇ or ◇◇◇◇, syndecan-3 homodimer or homo-oligomer; ●◇◇ or ○◇◇, syndecan-3 and -2 or -4 hetero-oligomer. *C*, the TMD amino acid sequences of rat syndecan-1 wild-type and all point mutants. The GXXXG motif is underlined, and phenylalanine and the substituted amino acids are shown in **boldface**. *E*, extracellular domain; *T*, transmembrane domain; *C*, cytoplasmic domain. *D*, recombinant His-tagged thioredoxin (*Trx*) wild-type syndecan-1, syndecan-1 single point mutants (S1eTC(F269Y) and S1eTC(C272F)), and the syndecan-1 double point mutant (S1eTC(F269Y,C272F)) were each purified on Ni-NTA columns, separated by 12% SDS-PAGE, and stained with Coomassie Blue (*left panel*). The indicated syndecan wild type and mutants were incubated for 10 min on ice, separated by 12% SDS-PAGE, and stained with Coomassie Blue (*right panel*). The data shown are representative of three independent experiments. \*,  $p < 0.05$ .

*The Conserved Phenylalanine in the TMD Regulates Heterodimeric Interactions between Syndecans*—To confirm the importance of the conserved Phe in TMD-mediated heterodimerization, heterotypic interactions with syndecan-1 and other syndecans were investigated. Similar to syndecan-2 and -4, syndecan-1 formed SDS-resistant dimers with syndecan-2 (Fig. 6A) and -4 (Fig. 6B). However, mutants such as S2(F169Y) or S4(F164Y) showed reduced heterodimer formation with syndecan-1 (Fig. 6, A and B). We also constructed several His-tagged syndecan-1 wild types and substituted mutants (Fig. 6C). The ability of SDS-resistant homodimer formation in syndecan-1 mutants to form SDS-resistant heterodimers was assayed by SDS-PAGE (Fig. 6D). After showing that homodimer formation by the mutants (S1eTC(F269Y), S1eTC(F269Y, C272F), and S1eTC(C272F)) was similar to that of the wild type (Fig. 6D, *left panel*), purified His-tagged syndecan-1 proteins were mixed, and their SDS-resistant heterodimer patterns were analyzed (Fig. 6D, *right panel*). Syndecan-1 that contained the conserved Phe (S1eTC and S1eTC(C272F)) could form TMD-mediated heterodimers with syndecan-2, whereas mutants such as S1eTC(F269Y) and S1eTC(F269Y, C272F), in which the conserved Phe was replaced by tyrosine, could not form heterodimers even when an extra Phe residue (Phe-272) was added to their TMD sequences (Fig. 6D). Syndecan-3 could form TMD-mediated SDS-resistant heterodimers with syndecan-2 and -4 and showed decreased SDS-resistant heterodimer for-

mation with heterodimer-defective mutants (S2(F169Y) and S4(F164Y)) (Fig. 6E). Collectively, these results show that the conserved phenylalanine plays a critical role in heterodimer formation between syndecan family members.

## Discussion

Syndecans have been found to self-associate through the GXXXG motif in their highly conserved TMDs (17, 18), and our group has shown previously that this motif is critical for the heterodimerization of syndecan-2 and -4 and that this binding inhibits the functions of each of these syndecans (19). In addition, we showed that the unique phenylalanine residue in the syndecan-2 TMD strengthened the homodimeric interactions of syndecan-2 (27), suggesting a critical role of Phe in the TMD. Interestingly, the numbers of phenylalanine residues differ in the TMD of each syndecan family member. For example, syndecan-2 has three phenylalanine residues, but syndecan-1 has only one. Nevertheless, one Phe residue, located at positions 269, 169, 399, and 164 in rat syndecan-1 through -4, respectively, is conserved in all mammalian syndecans. Because aromatic amino acids are involved in  $\pi$ - $\pi$  and cation- $\pi$  interactions (26, 37, 38), the conserved phenylalanine may be involved in regulating the intermolecular interactions of syndecan TMDs. Indeed, our results showed that the conserved phenylalanine in rat syndecan-2 and -4 enhanced SDS-resistant heterodimer formation and intermolecular interactions of syndecan

## Role of the Conserved Phenylalanine in Syndecan Interaction

can-2 and -4 at the molecular and cellular levels and regulated syndecan-2 TMD-mediated cytoplasmic domain function and heterodimerization with the TMDs of other syndecans, like syndecan-1. Taken together, these data strongly indicate that the conserved phenylalanine regulates heterodimeric but not homodimeric interactions of syndecan TMDs.

We have reported previously that the heterodimer formation between syndecan-2 and -4 inhibited the homodimeric interaction-dependent functions of each of these proteins (19), suggesting that the conserved Phe in the TMD could affect heterodimeric interaction-dependent functions. As expected, the inhibition of homodimer related-functions by heterodimers was not observed when cells were co-transfected with heterodimer-defective mutants. Indeed, we observed that Rac activity was not reduced in cells co-transfected with heterodimer-defective mutants and that syndecan-4 homodimer-related PKC $\alpha$  membrane localization and focal adhesion formation were not inhibited by the S2(F169Y) mutant. These results provide further evidence that the conserved phenylalanine on syndecan TMDs contributes to the higher affinity of heterodimer compared with homodimer formation.

Our findings that the unique Phe near the GXXXG motif in the syndecan-2 TMD contributes to homodimer formation (27), whereas the conserved Phe, located at a certain distance from the GXXXG motif in the TMDs of syndecan core proteins, contributes to heterodimeric interactions, provide insights into the molecular mechanisms underlying the syndecan-2 TMD-mediated associations. First, the GXXXG motif of the syndecan TMD helix induces non-covalent dimerization through van der Waals interactions. Second, the unique Phe (Phe-167 of rat syndecan-2) located near the GXXXG motif cooperates with the GXXXG motif to strengthen homodimer/oligomer formation through  $\pi$ - $\pi$  interactions. Third, the conserved Phe in the C-terminal regions of the syndecan TMDs, at positions 269, 169, 399, and 164 in rat syndecan-1 through -4, respectively, participates in the regulation of heterodimerization. All of these TMD motifs provide insights into the mechanisms regulating the biological functions of syndecans.

Syndecan expression has been reported to be altered during tumorigenesis and disease progression (30, 36, 39), enhancing interest in syndecan family heterodimerization. Analyzing the role of the conserved phenylalanine in heterodimerization may be useful in assessing the mechanism underlying these interactions and determining strategies to inhibit syndecan heterodimers. The numbers of phenylalanine residues in syndecan TMDs differ, and these differences may be associated with the strengths of intermolecular interactions and the ability to form oligomers. The tendency of TMDs to form oligomers may regulate the oligomeric status of syndecan extracellular and/or cytoplasmic domains and of the interactions of signaling molecules. Each syndecan may have unique biological functions, with these activities regulated by the intermolecular interactions of the syndecan TMDs, including interactions mediated by conserved Phe residues.

In summary, the results of this study suggest that the TMDs of syndecans may regulate the intermolecular interactions of these proteins through a conserved Phe. This may increase the

dimeric diversity of syndecan receptors, enabling them to convey distinct signaling functions as cell surface receptors.

**Author Contributions**—M. J. K., J. P., and E. S. O. conceived and coordinated the study and wrote the paper. M. J. K., J. P., S. J., C. Y. E., and E. S. O. performed the experiments and analyzed the data. All authors reviewed the results and approved the final version of the manuscript.

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## **The Conserved Phenylalanine in the Transmembrane Domain Enhances Heteromeric Interactions of Syndecans**

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