Syndecan-2 Regulates Transforming Growth Factor-β Signaling*

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Transforming growth factor-β (TGF-β) has multiple functions including increasing extracellular matrix deposition in fibrosis. It functions through a complex family of cell surface receptors that mediate downstream signaling. We report here that a transmembrane heparan sulfate proteoglycan, syndecan-2 (S2), can regulate TGF-β signaling. S2 protein increased in the renal interstitium in diabetes and regulated TGF-β-mediated increased matrix deposition in vitro. Transfection of renal papillary fibroblasts with S2 or a S2 construct that has a truncated cytoplasmic domain (S2ΔS) promoted TGF-β binding and S2 core protein ectodomain directly bound TGF-β. Transfection with S2 increased the amounts of type I and type II TGF-β receptors (TβRI and TβRII), whereas S2ΔS was much less effective. In contrast, S2ΔS dramatically increased the level of type III TGF-β receptor (TβRIII), betaglycan, whereas S2 resulted in a decrease. Syndecan-2 specifically co-immunoprecipitated with betaglycan but not with TβRI or TβRII. This is a novel mechanism of control of TGF-β action that may be important in fibrosis.

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§ The abbreviations used are: TGF-β, transforming growth factor-β; TβRI, TβRII, and TβRIII, type I, II, and III TGF-β receptors; V region, variable region; S2, syndecan-2; S4, syndecan-4; S2ΔS, S2 truncated in the V region; GAG, glycosaminoglycan; HS, heparan sulfate; PDZ, post-synaptic density-95, disks large, zona occludens-1 domain binding motif; PDZ-Domain containing protein GIPC (GAP-interacting protein, C terminus) leads to increased betaglycan expression (9).

Syndecan-2 (S2) is one of four mammalian members of a transmembrane proteoglycan family (14). Syndecans act as coreceptors for growth factor binding, cell-matrix interactions, and cell-cell interactions (15–18). They have divergent ectodomains and highly homologous transmembrane domains, and their cytoplasmic domains have two regions of homology (C1 and C2) flanking V regions unique to each syndecan. S2 interacts with matrix proteins such as laminin (19) and fibronectin (20), and its cytoplasmic V region (Fig. 1) controls matrix assembly at the cell surface (21). This may be due to oligomerization-dependent V region phosphorylation (22). Cells expressing an S2 construct that is truncated in the V region (S2ΔS) lack matrix deposition (21, 23). The V region of S2 also controls left-right asymmetry during Xenopus development through the actions of TGF-β family members (23), with parallel phosphorylation requirements (24). Interestingly, S2 has similar motifs to betaglycan (Fig. 1) including the GAG attachment sites, a possible serine phosphorylation site (Ser-ser-ala-ala; SSAA) and a C-terminal PDZ-domain binding motif that binds GIPC. Since both fibrosis and determination of left-right asymmetry involve TGF-β family members (5, 23), we investigated whether S2 and TGF-β act in concert in fibrosis.

EXPERIMENTAL PROCEDURES

Materials—General chemicals, Tween, and coverslips and cultureware were from Sigma, Bio-Rad, and Fisher, respectively. TGF-β1 and biotinylated TGF-β1 were from R & D Systems (Minneapolis, MN). RNAzol™ B was from Leedo Medical Laboratories (Houston, TX). pcDNA3 vector was obtained from Invitrogen. Antibodies against S2 (T-17), TβRII (V-22), TβRII (L-21), TβRII (C-20), and donkey anti-goat IgG-horseradish peroxidase (HRP) were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal anti-S2 (R1891), monoclonal anti-S4 (150.9), and anti-fibropectin have been described previously (14, 25, 26). Protein-G-Sepharose, blocking agent for detection of biotinylated proteins, and streptavidin-HRP were obtained from Amersham Biosciences. Fluorescein isothiocyanate (FITC)-conjugated Fab(′)2, fragments of goat anti-rabbit or anti-mouse IgG were purchased from Cappel (Durham, NC). Heparinase I (EC 4.2.2.8) and chondroitinase ABC (EC 4.2.2.4) were from Seikagaku America (Fallmouth, MA). UltraLink immobilized streptavidin gel and NHS-Sulfo-Biotin were from Pierce.

Immunohistochemical Analysis of S2 Expression in Renal Sections—Frozen sections from control and diabetic type II patients were incu-

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FIG. 1. Schematic structures of syndecan-2, S2AS, and betaglycan. Cytoplasmic domain residues are shown. C1, V, and C2 regions of S2 are indicated. PDZ-domain binding residues are capitalized. s represents putative phosphorylation sites.

FIG. 2. Syndecan-2 mediates TGF-β function. A, S2 and S4 labeling in normal and diabetic kidney sections. Note a lack of interstitial labeling (stars) in normal kidney but labeling of a nerve (arrowhead) and blood vessel (arrow). No significant differences were seen with S4. Bar in left panel = 50 μm. Bars in right three panels = 100 μm. B, S2 levels ± TGF-β treatment were analyzed by Northern analysis and densitometry. Relative levels of S2 to GAPDH mRNA are shown. C, anti-fibronectin labeling in vector- (left), S2AS- (middle) and S2- (right) panels transfected RPF cells in the presence (bottom) or absence (top) of TGF-β treatment. Arrowheads represent cells lacking fibrils; arrows represent fibrils. Bar = 10 μm.

centrifugation (10,000 rpm, 10 min, 4 °C), the supernatant was incubated (1 h, 4 °C) with protein-G-Sepharose beads preblocked with 5% fetal bovine serum. Beads were removed by centrifugation (400 × g, 10 min, 4 °C) and the supernatant incubated with fresh preblocked beads and 5 μg/ml antibody (2 h, 4 °C). Beads were washed three times with lysis buffer, three times with PBS, and bound proteins were eluted by boiling with SDS sample buffer (5 min), subjected to SDS-PAGE, and blotted with streptavidin-HRP and ECL. Membranes were stripped and blotted with antibodies against S2.

RESULTS

When renal sections from age-matched controls (Fig. 2A, left panel) were compared with those from type II diabetic patients (Fig. 2A, second panel), S2 was increased in the tubulointerstitium. In contrast, despite some increase in the glomerulus, syndecan-4 (right two panels) was not dramatically increased in the tubulointerstitium. Similar results were obtained with streptozotocin-induced diabetic rats (data not shown). TGF-β increases S2 expression in several cell types (28, 29). We, therefore, tested whether the increase in S2 was due to TGF-β, a known mediator of fibrosis (5). However, S2 mRNA or protein levels were not increased (Fig. 2B and data not shown) in RPF cells treated with TGF-β at concentrations sufficient to increase fibronectin matrix in these (Fig. 2C) and other cells (30–32).
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We then tested whether S2 can mediate TGF-β-induced increased matrix deposition. Immunofluorescent labeling indicated that overexpression of S2 in RPF increased fibronectin matrix deposition with more fibrils/cell and larger fibrils (arrows) than in cells transfected with vector only (Fig. 2C). This difference was not statistically significant in a semiquantitative assay using exposure time. Construct expression levels vary among cells, although immunoblotting (data not shown) indicated transfection with S2 or S2ΔS increased S2 levels 2.5-fold overall. In contrast, expression of S2ΔS decreased basal fibronectin deposition (Fig. 2C), compared with cells transfected with vector only (102 ± 11 versus 76 ± 6.0, p < 0.001), and several cells (arrowheads) lacked fibrils. This suggests that S2 cytoplasmic domain regulates matrix deposition in RPF cells, as it does in CHO-K1 cells (21). Differences in matrix deposition were exaggerated after TGF-β treatment. The percentage decrease of exposure time after TGF-β treatment parallels increased matrix deposition. Treatment of vector-only transfected cells with TGF-β (Fig. 2C) increased matrix deposition (18% ± 9.7% decrease in exposure time). Increased matrix deposition in S2 cells was exaggerated (21% ± 7.0%), but S2ΔS cells showed (Fig. 2C) less decrease (14% ± 9.1%).

These results suggest that S2 regulates TGF-β-mediated matrix increase. Transfection of cells with either S2 or S2ΔS increased surface binding of TGF-β over that to cells transfected with empty vector (Fig. 3, A and B). Cleavage of HS chains reduced TGF-β binding (Fig. 3B), suggesting that some binding is through HS chains. Surprisingly, recombinant S2 ectodomain bound biotinylated TGF-β1, and TGF-β1 could capture S2 from RPF cell lysates (Fig. 3, C and D). Thus, the core protein of both S2 and S2ΔS binds TGF-β, and binding is independent of the cytoplasmic domain.

Immunoblotting of total cell lysates demonstrated that S2ΔS slightly, and S2 dramatically, increased protein levels of TβRI and TβRII (Fig. 4, A, B, D, and E). The expression of TβRIII, betaglycan, was increased in S2ΔS cells but decreased in S2 cells (Fig. 4, C and F). When cell surface receptor levels were monitored by biotinylation followed by immunoprecipitation (Fig. 5, A–C), these were consistent with the total levels. In Fig. 5C, the lowest species is coprecipitated S2, as confirmed in Fig. 5D.

The regulation of the expression of TGF-β receptors by S2 may be through formation of a complex between S2 and the receptor(s). Syndecan-2 was not detected in TβRI or TβRII immunoprecipitates (data not shown) but was detected in TβRIII immunoprecipitates (Fig. 5D). Although S2ΔS cells expressed the highest levels of betaglycan (Fig. 4C), less S2 co-precipitated with betaglycan from S2ΔS cells than from vector-only transfected cells, and that detected may be endogenous full-length S2. Thus, the cytoplasmic domain of S2 may be
needed for association with betaglycan. Low amounts of coprecipitated S2 were detected from S2 cells, probably due to the decreased level of betaglycan in S2 cells (Fig. 4C). To confirm differential association of betaglycan with S2ΔS and S2, further coprecipitation experiments were performed with increased loading on SDS-PAGE and longer time exposure on the film (Fig. 5E).

**DISCUSSION**

Syndecans can act as co-receptors for growth factors (16–18) by delivery of ligands to their signaling receptors and the formation of a ternary complex (18, 33). Most growth factor receptor down-regulation (36) involves proteosome degradation (9). If S2 competes for GIPC binding and betaglycan level in S2ΔS may differentially regulate cell surface degradation of TGF-β receptors; TβRII can be cleaved at the cell surface (39), although cleavage of TβRI and TβRII was not detected (35). Finally, S2 and S2ΔS may differentially regulate endocytosis-dependent degradation and recycling of TGF-β receptors. The distinct effects of S2 and S2ΔS on betaglycan levels are intriguing. Effects on betaglycan (increased by S2ΔS but not by S2) may be explained by similarities between S2 and betaglycan cytoplasmic domains. Both contain a PDZ-domain binding motif (Fig. 1) that binds GIPC (9, 40). GIPC binding up-regulates betaglycan levels on the cell surface by preventing proteosome degradation (9). If S2 competes for GIPC binding, less GIPC will be available for betaglycan in S2 cells but more in S2ΔS cells. All three receptors may be required for efficient receptor down-regulation (36). Decreased cell surface betaglycan in S2 cells may then reduce down-regulation of TβRI and TβRII. Whatever the mechanism, the finding that S2 can regulate the cell surface levels of TGF-β receptors adds a new level of complexity to TGF-β action.

Despite increased TGF-β binding and betaglycan level in S2ΔS cells, TGF-β did not increase matrix deposition in S2ΔS cells, suggestive of defective downstream signaling. Both betaglycan and S2 cytoplasmic domain contain a SSAE sequence (Fig. 1). Phosphorylation of betaglycan cytoplasmic domain is needed for downstream signaling (8), and, although the site has not been determined, this can be by TβRII. The SSAE sequence in S2 can be phosphorylated if not oligomerized (22), and phosphorylation is needed for both matrix assembly and the regulation of left-right asymmetry in *Xenopus* embryos (23, 24). Whether S2 and betaglycan compete for the same kinase(s) is not known, but overexpression of S2 may promote oligomerization, as it does with syndecan-4 (41), and thus reduce its phosphorylation potential. Finally, we found that betaglycan coprecipitates with S2, and the cytoplasmic domain of S2 is needed for this association. Whether this is a direct interaction is now being determined. There may be two distinct signaling mechan-

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