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The type III TGF-β receptor betaglycan transmembrane–cytoplasmic domain fragment is stable after ectodomain cleavage and is a substrate of the intramembrane protease γ-secretase

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ABSTRACT

The Type III TGF-β receptor, betaglycan, is a widely expressed proteoglycan co-receptor for TGF-β superfamily ligands. The full-length protein undergoes ectodomain cleavage with release of a soluble ectodomain fragment. The fate of the resulting transmembrane–cytoplasmic fragment, however, has never been explored. We demonstrate here that the transmembrane–cytoplasmic fragment is stable in transfected cells and in cell lines expressing endogenous betaglycan. Production of this fragment is inhibited by the ectodomain shedding inhibitor TAPI-2. Treatment of cells with inhibitors of the intramembrane protease γ-secretase stabilizes this fragment, suggesting that it is a substrate of γ-secretase. Expression of the transmembrane–cytoplasmic fragment as well as γ-secretase inhibitor stabilization are independent of TGF-β1 or -β2 and are unaffected by mutation of the cytoplasmic domain serines that undergo phosphorylation. γ-Secretase inhibition or the expression of a transmembrane–cytoplasmic fragment in HepG2 cells blunted TGF-β2 signaling. Our findings thus suggest that the transmembrane–cytoplasmic fragment remaining after betaglycan ectodomain cleavage is stable and a substrate of γ-secretase, which may have significant implications for the TGF-β signaling response.

1. Introduction

The transforming growth factor-β (TGF-β) superfamily is a widely expressed family of peptide growth factors with broad functions including the control of cell growth, differentiation, and adhesion. TGF-β superfamily members, including the TGF-βs, activin/inhibin, the bone morphogenetic proteins (BMPs), and Müllerian inhibiting substance, signal through pairs of structurally similar but functionally distinct cell surface receptors, termed type I and type II receptors (TGFRI and TGFRII), which have serine/threonine kinase cytoplasmic domains. An additional TGF-β binding protein, known as the type III TGF-β receptor or betaglycan, is a broadly distributed heparin and chondroitin sulfate proteoglycan that acts as a signaling modifier and co-receptor [1–6]. Betaglycan is an important regulator of development and differentiation [7–12] and tumor growth and metastasis [13–18] and can modulate MAPK signaling associated with TGF-β activation [19,20]. Betaglycan binds all three TGF-β isoforms with high affinity through two distinct binding sites on the core ectodomain [21]. Mechanistically, betaglycan enhances TGF-β dependent signaling by increasing the affinity of the ligand for TGFRII [4,5,22]. This is particularly important for signaling by the isoform TGF-β2, which, unlike the TGF-β1 and -β3 isoforms, has low affinity for TGFRII alone and appears to require the presence of preformed TGFRI/TGFRII complexes in order to signal in the absence of betaglycan [22–25].

Recent studies suggest that betaglycan has additional functions in addition to enhancing TGF-β2 signaling. Superfamily members inhibin and BMP-2 bind to the betaglycan ectodomain core, and FGF-2 binds via the glycosaminoglycan (GAG) chains [1,26–30]. Betaglycan binds to inhibin, in some contexts mediating formation of a complex with the type II activin receptor and thereby inhibiting binding and signaling by activin [29–31]. Betaglycan serves similar inhibitory functions in BMP-2 signaling by binding BMP-2 and, in certain settings, has an inhibitory function in TGF-β signaling as well [28,32,33]. Additionally, through the action of as yet undetermined matrix metalloproteinases, betaglycan undergoes ligand-independent ectodomain shedding [34–36] with release of a stable soluble

Abbreviations: TGFRI, Type I TGF-β receptor; TGFRII, Type II TGF-β receptor; GAG, glycosaminoglycan; TM/cytoplasmic, transmembrane–cytoplasmic; GSI, γ-secretase inhibitor

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fragment that sequesters TGF-β; this fragment may have therapeutic value in certain malignancies and fibrotic diseases as an inhibitor of TGF-β signaling [37–44]. In contrast to the ectodomain, the short (44 amino acid) betaglycan cytoplasmic domain, which lacks kinase activity or identifiable signaling motifs, has not been well studied. Some, although not all, studies suggest that deletion of the entire cytoplasmic domain reduces TGF-β2 sensitivity [32,45]. Interactions between the betaglycan cytoplasmic domain and other proteins, including β-arrestin and the PDZ domain protein GIPC, can modulate TGF-β signaling by various mechanisms, including activation of alternative pathways [46,47] and endocytosis of other TGF-β receptors [48–51]. The cytoplasmic domain of betaglycan also serves as a modulator of TGF-β dependent and independent activation of p38/MAPK through an undetermined mechanism [19,20,52]. The full-length betaglycan forms a complex with syndecan-2, an interaction that may be important in regulating TGF-β signaling in fibrosis [53]; the syndecan-2 cytoplasmic domain appears to be required for this interaction, although the role of the cytoplasmic domain of betaglycan has not been determined.

Our goal was to investigate the fate of the betaglycan fragment remaining after ectodomain cleavage. Many other transmembrane receptors, including Notch, ErBb4, the insulin-related growth factor receptor, the Met receptor, the p75 neurotrophin receptor, and syndecan-3 undergo ectodomain shedding followed by γ-secretase-mediated intramembrane proteolytic cleavage. Many of the resulting free cytoplasmic domains have important functions independent of the full-length receptors [54–61]. Ectodomain shedding is a prerequisite step for cleavage of the receptor intracellular domain since the γ-secretase complex preferentially cleaves single transmembrane proteins with short ectodomain regions [60,62,63].

We hypothesized that betaglycan, by virtue of undergoing ectodomain cleavage, is also a substrate for γ-secretase. We provide evidence here that the betaglycan transmembrane–cytoplasmic (TM/cytoplasmic) fragment is stable, undergoes cleavage by γ-secretase, and may have important effects on TGF-β signaling.

2. Materials and methods

2.1. cDNA constructs and cell lines

cDNA constructs used are shown in Supplemental Fig. 1. HA-tagged full-length rat betaglycan (wtBG), a GAG-minus mutant (ΔGAG), and a cytoplasmic domain minus mutant (ΔCyto) were constructed as previously described [2,32]. Cytoplasmic domain residues $Ser^{833,834}$ were mutated to $Ala^{833,834}$ using the QuikChange II PCR mutagenesis kit (Stratagene, San Diego, CA) to generate the ΔSer construct. An HA-tagged ectodomain-minus receptor (ΔEcto) was constructed by first inserting *Cla*I restriction sites by site-directed mutagenesis at positions 87 and 2346 (numbering as per [6]) of the HA-tagged rat betaglycan cDNA. The resulting cDNA was then digested with *Cla*I, the smaller fragment (encoding the bulk of the ectodomain) removed, and the larger fragment ligated closed. The resulting construct encodes a protein with an ectodomain composed of the betaglycan signal sequence, the two amino acids distal to the signal sequence, the nine amino acid HA epitope tag, and the seven amino acids GluProSerAsnArgLeuAsp, plus the complete betaglycan transmembrane and cytoplasmic domains. Cell lines were obtained from the ATCC (COS-7, CR-1651; HepG2, HB-8065; NIH-3T3, CRL-1658; CHO, CCL-61). cDNAs were introduced into COS-7 cells using the Effectene reagent (Qiagen, Valencia, CA) and into HepG2 cells using FuGENE 6 (Roche, Indianapolis, IN). Transfection efficiency for HepG2 cells was approximately 15%–20%.

2.2. Inhibitor treatments and Western blotting

COS-7 cells 48 hours after transfection were incubated in serum free media overnight with 200 pM TGF-β1 or TGF-β2 (R&D Systems, Minneapolis, MN), the γ-secretase inhibitors (GSI) X or XVII (100 nM for COS-7, 1 μM for other cell lines; Calbiochem, La Jolla, CA), or cycloheximide (10 μM; Sigma, St. Louis, MO). Some cells were treated similarly for 14 hours with TAPI-2 (25 μM; Calbiochem). Cell lysates were separated by SDS–PAGE followed by immunoblotting with antibodies against the cytoplasmic domain of betaglycan (α-BG/cyto; 0.8 μg/mL; Santa Cruz Biotechnology, Santa Cruz, CA), the ectodomain of betaglycan (α-BG/ecto; 0.5 μg/mL; R&D Systems), the HA epitope tag (0.3 μg/mL; Sigma), or cyclophilin B (0.5 μg/mL; Affinity Bioreagents, Rockford, IL). In addition to HRP conjugated secondary antibodies, infrared conjugated secondary antibodies were used in conjunction with the LI-COR Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE). Images of blots acquired with the Odyssey system were adjusted to a gamma setting of 1.0 for use in figures. Conditioned media from transfected COS-7 cells treated with TAPI-2 were concentrated and adjusted for protein levels based on the protein concentration of corresponding cell lysates. Concentration-adjusted media were then separated by SDS–PAGE for analysis of soluble betaglycan ectodomain levels.

2.3. Dual luciferase assay

HepG2 cells were transfected with empty vector or ΔEcto construct as well as the 3TP-lux [64] and Renilla luciferase (Promega, Madison, WI) constructs. After 12–16 hours, cells were trypsinized, re-seeded on 48 well plates, and incubated for an additional 12–16 hours before being serum starved for 6 hours. They were then treated with 30 pM TGF-β1 or -β2 and 1 μM GSI-X or XVII in serum free media for 12–16 hours. Luciferase activity was assayed using the Promega Dual Luciferase assay kit (Promega) and was corrected for Renilla levels.

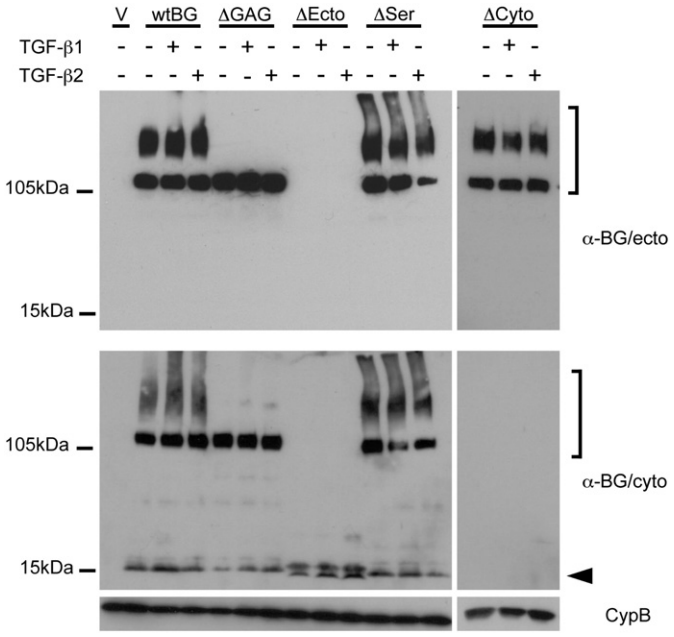

Fig. 1. Betaglycan ectodomain cleavage results in the production of a stable transmembrane–cytoplasmic domain fragment. A) COS-7 cells were transiently transfected with the indicated betaglycan constructs or empty vector (V). Cells were treated for 12–14 hours with 200 pM TGF-β1 or TGF-β2 in serum free medium. Immunoblotting was carried out with α-BG/ecto and α-BG/cyto antibodies, as indicated. Full-length HA-tagged betaglycan species (bracket) migrate as a combination of a discrete band of 120 kDa, reflecting the N-glycosylated core protein, and as a smear from ~140 kDa to above 200 kDa, reflecting the heterogeneously GAG-modified forms. The TM/cytoplasmic domain fragment (arrowhead) migrates at 17 kDa. The doublet seen for this construct likely represents variable cleavage of the short, HA-tagged ectodomain and is not seen consistently. The blot was stripped and probed with an antibody against cyclophilin B (CypB) as a loading control. The blot shown is representative of three independent experiments.