In the Absence of Type III Receptor, the Transforming Growth Factor (TGF)-β Type II-B Receptor Requires the Type I Receptor to Bind TGF-β2*

Received for publication, February 6, 2004, and in revised form, February 27, 2004

Published, JBC Papers in Press, March 2, 2004, DOI 10.1074/jbc.M401350200

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Transforming growth factor β (TGF-β) ligands exert their biological effects through type II (TβRII) and type I receptors (TβRI). Unlike TGF-β1 and -β3, TGF-β2 appears to require the co-receptor betaglycan (type III receptor, TβRIII) for high affinity binding and signaling. Recently, the TβRIII null mouse was generated and revealed significant non-overlapping phenotypes with the TGF-β2 null mouse, implying the existence of TβRIII independent mechanisms for TGF-β2 signaling. Because a variant of the type II receptor, the type II receptor (TβRII-B), has been suggested to mediate TGF-β2 signaling in the absence of TβRIII, we directly tested the ability of TβRII-B to bind TGF-β2. Here we show that the soluble extracellular domain of the type II-B receptor (sTβRII-B.Fc) bound TGF-β1 and TGF-β3 with high affinity (Kd values = 31.7 ± 22.8 and 74.6 ± 15.8 pM, respectively), but TGF-β2 binding was undetectable at corresponding doses. Similar results were obtained for the soluble type II receptor (sTβRII.Fc). However, sTβRII.Fc or sTβRII-B.Fc in combination with soluble type I receptor (sTβRI.Fc) formed a high affinity complex that bound TGF-β2, and this complex inhibited TGF-β2 in a biological inhibition assay. These results show that TGF-β2 has the potential to signal in the absence of TβRIII when sufficient TGF-β2, TβRI, and TβRII or TβRII-B are present. Our data also support a cooperative model for receptor-ligand interactions, as has been suggested by crystallization studies of TGF-β receptors and ligands. Our cell-free binding assay system will allow for testing of models of receptor-ligand complexes prior to actual solution of crystal structures.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) E10745.

‡ Supported in part by National Institutes of Health Training Grant T32 DK-07540-17.

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¶ The abbreviations used are: TGF-β, transforming growth factor-β; BMP, bone morphogenetic protein; TβRI, TGF-β type I receptor; TβRII, TGF-β type II receptor; TβRIII, TGF-β type III receptor; TβRII-B, type II-B receptor; ActRII, activin type II receptor; sTβRII.Fc, soluble human TβRII fused to the Fc region of human immunoglobulin; sTβRII-B.Fc, soluble human TβRII-B fused to the Fc region of human immunoglobulin; sActRII-Fc, soluble human activin type II-B receptor fused to the Fc region of human immunoglobulin; Mv1Lu cells, mink lung epithelial cells; ECD, extracellular domain.

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contrast, TGF-β2 has much lower affinity for TβRII than TGF-β1 and -β3. It has been demonstrated that an accessory receptor, TβRIII, is necessary for efficient binding and cross-linking of TGF-β2 and subsequent signaling (28). In this model, TGF-β2 binds to TβRII, which then recruits TβRI and TβRII resulting in phosphorylation of TβRI and downstream signaling (28). Interestingly, the recently published phenotype of the TβRII null mouse (29) is not completely overlapping with the phenotype of the TGF-β2-deficient mouse (8), suggesting the existence of alternative methods for TGF-β2 binding and signaling that do not involve TβRII. Cross-linking studies of cell-surface TGF-β receptors with 125I-TGF-β2 in transfected COS cells have suggested that high affinity TGF-β2 binding and downstream signaling in these cells can occur via complexes of type I and type II receptors (30). Alternatively, Rotzer et al. (16) has proposed that TβRII-B binds TGF-β2 with ensuing signaling in the absence of TβRII. Of note, an earlier report showing the inability of unlabeled TGF-β2 to compete for 125I-TGF-β1 binding to TβRII-B inferred that TβRII-B resembles TβRII in its inability to bind TGF-β2 (14). In general, these studies of TGF-β receptor binding to ligand were performed on receptors expressed at the cell surface where binding can only be measured indirectly via cross-linking to radioligand followed by autoradiography. These studies are therefore limited by an inability to directly quantify binding and thus obtain an accurate measurement of receptor-ligand affinities. In addition, the presence of other extracellular cell-surface-associated proteins that may act as accessory genes to binding cannot be ruled out.

To overcome the limitations of cell surface expression studies and to specifically assess the ability of TβRII-B to bind TGF-β2, we have developed a cell-free system using soluble TGF-β receptors. We demonstrate that in comparison with TGF-β1 and -β3, TGF-β2 bound poorly to soluble TGF-β type II (sTβRII.Fc) or TGF-β type II-B receptors (sTβRII-B.Fc) alone. However, TGF-β2 did bind sTβRII.Fc or sTβRII-B.Fc in complex with the soluble type I receptor (sTβRI.Fc) in solution, and cell-surface TβRII or TβRII-B together with TβRII could mediate TGF-β2 signaling in the absence of TβRII. Our heteroreceptor binding assay system provides supporting evidence for a cooperative model of type II and type I receptor interactions with TGF-β2 ligand, and provides a rapid and straightforward way to measure the binding of receptor and/or ligand mutants that arise from structure-function studies.

EXPERIMENTAL PROCEDURES
cDNA Subcloning—The cDNA encoding the extracellular domain of human TβRII was amplified by PCR using primers: 5′-CCCAAGCTTGGCGCAACCTGGTGTGTTGAGTGAG-3′ (forward) and 3′-TCTGCTGGTATGTTGAGTGAG-5′ (reverse). The cDNA was sequenced on both strands to confirm the fidelity of the construct.

To generate cDNA for the extracellular domain of human TβRII-B, the 26-amino acid insert was generated by an overlapping primer strategy using PCR. The N-terminal half of the insert was generated by PCR using the following primers: 5′-GGGATCCCGCGGATGTCGAGACACGGTCTGCTGGGCTCTACTCTAGCTGACG-3′ (forward) and 3′-GGGATCCCGCGGATGTCGAGACACGGTCTGCTGGGCTCTACTCTAGCTGACG-5′ (reverse). The C-terminal half of the insert and the rest of the extracellular domain was generated by PCR using the following primers: 5′-GGGATCCCGGTGTGATGTCGAGACACGGTCTGCTGGGCTCTACTCTAGCTGACG-3′ (forward) and 3′-GGGATCCCGGTGTGATGTCGAGACACGGTCTGCTGGGCTCTACTCTAGCTGACG-5′ (reverse).

Production and characterization of sTβRII-B.Fc and sTβRII-B.Fc proteins. A schematic representation of sTβRII-B.Fc (top) and sTβRII-B.Fc (bottom), comprised of TβRII and TβRII-B hydrophobic leader sequences (dark gray) and extracellular domains (light gray) fused to human Fc (black). Also shown is the position of Val32 in TβRII, which is replaced by a 26-amino acid insert in TβRII-B. B, purified sTβRII-Fc (RIII) or sTβRII-B.Fc (RIII) proteins were treated with (lanes 2 and 4) or without (lanes 1 and 3) 5000 units of PNGase F at 37 °C for 2 h, and analyzed by reduced SDS-PAGE followed by silver staining. C, and D, purified sTβRII-B.Fc (RIII) and sTβRII-B.Fc (RII) protein (C), or sTβRII-B.Fc (D, RI, R1, and R2) proteins were analyzed by reducing SDS-PAGE followed by Western blot with anti-human Fc antibody (αFc, C, left; and D), anti-TβRII antibody (αRII, C, middle), or anti-TβRII-B insert peptide antibody (α26aa, C, right).
anti-human TβRII antibodies (R&D Systems) or goat anti-human Fc-specific IgG (Jackson ImmunoResearch Laboratories, West Grove, PA), followed by donkey anti-goat IgG conjugated to hors eradish peroxidase (Santa Cruz Biotechnolo gy). The chemiluminescence immunodassay was performed with Renaissance Western blot chemiluminescence reagent (Amersham Biosciences). Polyclonal antibodies directed against a peptide of human TβRII-B (QKEIDEIFCNSRTHAPLRHI, Peptide Core Facility, MGH, MA) were raised in goats (SIGMA-Gemosys, The Woodlands, TX) and employed to specifically detect human recombinant sTβRII-B-Fc compared with human recombinant sTβRII-Fc. Mouse sTβRII-Fc was purchased from R&D Systems.

**Ligand Iodination**—Carrier-free human TGF-β1, -β2, and -β3 were purchased from R&D Systems. Two μg of ligand per reaction was iodinated with [125I] by the modified chloramine-T method as previously described (32).

**Binding Assays on Protein A Plates**—Soluble recombinant human receptors were diluted in TBS/casein blocking buffer (BioFX, Owings Mills, MD) and incubated on Protein A-coated 96-well plates (Pierce) overnight. Plates were then washed with wash buffer (BioFX) and blocked 2 h at room temperature with TBS/casein buffer. For competition binding assays, fixed amounts of radioligands (50,000—100,000 counts) were added to the plates together with increasing amounts (2 pM to 500 nM) of homologous or heterologous non-radioactive ligands.

**Binding Assays in Solution**—Soluble recombinant human receptors were diluted in TBS/casein blocking buffer (BioFX) and incubated overnight in the presence or absence of ligand. For competition binding assays, fixed amounts of radioligands (50,000—100,000 counts) were added to the samples together with increasing amounts (2 pM to 500 nM) of homologous or heterologous non-radioactive ligands. Samples were then plated on Protein A-coated 96-well plates (Pierce) for 90 min, washed 3 times with wash buffer (BioFX), and counted using a γ-counter.

**Luciferase Reporter Assay**—Mink Lung cells (MvLu) were transiently transfected with the (CAGA)12MPL-Luc reporter construct (33) and with a pRL-TK vector (Promega) in a ratio of 10:1 to control for transfection efficiency. Cells were then serum starved for 6 h before treatment with varying amounts of TGF-β ligands in the presence or absence of varying amounts of soluble receptor for 16 h. Experiments were performed in triplicate wells. Cells were lysed and luciferase activity was determined with the Dual Reporter Assay (Promega). Relative light units were calculated as ratios of Firefly (reporter) and Renilla (transfection control) values. Alternatively, MvLu cells were used that had been stably transfected with the (CAGA)12MPL-Luc reporter construct. In this case, relative light units were corrected for total amount of protein in the lysate as determined by a bovine serum albumin protein assay (Pierce). Rat myoblast L6 cells were transfected with the (CAGA)12MPL-Luc reporter construct and with a pRL-TK vector and in addition with empty vector or full-length TGF-β receptors constructs using LipofectAMINE 2000. The same protocol as for the MvLu cells was then followed.

**Data Analysis**—Each experiment was repeated at least three times and different preparations of sTβRII-Fc and sTβRII-B-Fc were tested and used. Data are expressed as mean ± S.E. The Ligand Program from the National Institutes of Health was used to fit binding curves for the binding data (34). The Student’s t test was used with a p value of <0.05 to determine statistical significance.

**RESULTS**

**Production and Characterization of Soluble Type II-B.Fc and Soluble Type II-Fc (sTβRII-Fc) Chimeric Proteins**—cDNA encoding the ECDs of either TβRII or TβRII-B were fused to the Fc portion of human IgG and transfected into HEK 293 cells to generate sTβRII-Fc and sTβRII-B.Fc as described under “Experimental Procedures” and shown schematically in Fig. 1A. The ECD of human TβRII-B contains the 26-amino acid insert that replaces Val125 of TβRII (14–16).

Analysis of soluble receptor proteins purified by one-step Protein A affinity chromatography with SDS-PAGE and silver staining showed that the sTβRII-Fc protein was −50 kDa, whereas the sTβRII-B.Fc protein was −55 kDa (Fig. 1B, lanes 1 and 3), consistent with the presence of the 26-amino acid insert in sTβRII-B.Fc. Under non-reducing conditions, protein bands of −100 kDa for sTβRII-Fc and −110 kDa for sTβRII-B.Fc were visualized, reflecting the disulfide bond formation of the dimeric Fc domain (data not shown).

Both sTβRII-Fc and sTβRII-B.Fc proteins were sensitive to N-glycosidase F treatment (Fig. 1B), indicating that both proteins are N-glycosylated. The molecular mass of the deglycosylated receptors, −40 kDa (sTβRII-Fc, lane 2) and −42 kDa (sTβRII-B.Fc, lane 4), corresponded to the predicted molecular masses of the core protein of each chimeric protein.

Western blot analysis of soluble recombinant receptor chimeric proteins shown in Fig. 1C confirmed that the soluble receptor proteins contained both the human Fc domain (Fig. 1C, left), and the extracellular domain of the type II receptor (Fig. 1C, middle), using an anti-human Fc antibody (αFC) and an anti-type II receptor ECD domain antibody (αRII). As expected, a rabbit polyclonal antibody raised against the peptide encoding the 26-amino acid insertion sequence of TβRII-B recognized only the sTβRII-B.Fc protein with no detectable crossreactivity to sTβRII-Fc protein (α2baa, Fig. 1C, right). The anti-human Fc antibody also recognized sTβRII-Fc (from R&D Systems, Fig. 1D).

sTβRII-Fc and sTβRII-B.Fc Can Bind TGF-β1 and -β2, but Not TGF-β2—Radioligand competition experiments were performed to determine the selectivity and affinity of sTβRII-B.Fc and sTβRII-Fc proteins for different TGF-β isoforms. A non-saturating amount of soluble receptor was incubated overnight with 125I-labeled TGF-β1, -β2, or -β3 with or without serial dilutions of unlabeled TGF-β1, -β2, or -β3 at final concentrations from 2 pM to 500 nM, as indicated. The amount of com-

**TABLE I**

| 125I-Ligand | Cold ligand | sTβRII-B.Fc | sTβRII-Fc |
|---|---|---|---|
| **K_{i}** | **ED_{50} ± S.E.** | **Relative potency** | **ED_{50} ± S.E.** | **Relative potency** |
| β1 | 0.58 ± 0.1 | 1 | 31.7 ± 22.8 | 112 ± 38 |
| β2 | 2.2 ± 0.4 | 0.26 | Not done | 0.38 |
| β3 | 1.1 ± 0.3 | 2.1 | Not done | 1.6 |
| **K_{i}** | **ED_{50} ± S.E.** | **Relative potency** | **K_{i}** | **ED_{50} ± S.E.** | **Relative potency** |
| β1 | 2.3 ± 0.75 | 1 | 74.6 ± 15.8 | 375 ± 185 |

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Fig. 2. Measurement of sTβRII-B.Fc and sTβRII-B.Fc binding affinities. sTβRII-B.Fc and sTβRII.B.Fc were incubated on Protein A-coated plates overnight followed by 100,000 counts of 125I-TGF-β1 or -β3 in the presence of increasing amounts (2 pM to 500 nM) of homologous non-radioactive ligand. Samples were washed and counted using a standard γ-counter. Panels show a representative inhibition curve and Scatchard plot for sTβRII-B.Fc (upper panel) and sTβRII-B.Fc (lower panel) binding to TGF-β1 (closed circles) or -β3 (open circles) from one of three experiments.

Not TGF-β2 Biological Activity—Next we tested whether sTβRII-B.Fc and sTβRII.B.Fc could block TGF-β activity by performing a biological inhibition assay using a TGF-β responsive luciferase reporter assay (Fig. 3). MV1Lu cells transfected with the (CAGA)_n MPL-Luc reporter construct and sTβRII-B.Fc (Fig. 3A) or sTβRII-Luc (Fig. 3B). The relative luciferase activity induced by TGF-β1 (Fig. 3, diamonds) and TGF-β3 (Fig. 3, triangles) was decreased in a dose-dependent manner by either sTβRII-B.Fc or sTβRII.B.Fc. The ED_{50} was 360 pM for sTβRII-B.Fc to both TGF-β1 and TGF-β3. For sTβRII.B.Fc, the ED_{50} was 664 pM for TGF-β1 and 501 pM for TGF-β3. In contrast, there was no inhibition of TGF-β2-induced luciferase activity by either sTβRII-B.Fc or sTβRII.B.Fc (Fig. 3, squares).

Both Full-length TβRII-B and TβRII Expressed in L6 Cells That Lack TβRIII Can Enhance TGF-β2 Signaling Activity—We compared the effect of the three TGF-β ligands on the rat myoblast cell line L6 with or without transfection of full-length TβRII and TβRII-B receptors. L6 cells lack TβRIII (11, 28) and TβRII-B (16), but express TβRII and TβRI and are thus able to transduce TGF-β1 signals (16, 28). L6 cells were transfected with the (CAGA)ₙ MPL-Luc reporter construct and treated with increasing amounts (0–200 pM) of TGF-β2 (Fig. 4A). The dose-response curve obtained with increasing amounts of TGF-β2 suggested that the cells are able to signal through TGF-β2 in the absence of TβRIII and TβRII-B, but require higher doses of TGF-β2 compared with TGF-β1 or -β3.

Competitor that inhibited 50% of 125I-labeled TGF-β2 was defined as the effective dose (ED_{50}). The ED_{50}, relative potency, and calculated dissociation constants (K_d) averaged from at least three separate experiments are summarized in Table I (all slopes were parallel). For sTβRII-B.Fc, the ED_{50} (in ng) obtained when 125I-TGF-β1 was competed with unlabeled TGF-β1 and -β3 was 0.58 ± 0.1 and 2.2 ± 0.4, respectively, and for sTβRII.B.Fc, 0.64 ± 0.16 and 1.7 ± 0.4, respectively. When 125I-TGF-β3 was competed with unlabeled TGF-β1 and -β3, the ED_{50} (in nanograms) for sTβRII-B.Fc was 1.1 ± 0.3 and 2.3 ± 0.8, respectively, and for sTβRII.B.Fc, 2.0 ± 0.36 and 3.2 ± 1.1, respectively. The relative potency was calculated by comparing ED_{50} values and showed that TGF-β1 was more effective than TGF-β3 in all cases. Cold TGF-β2 was unable to compete with 125I-TGF-β1 or 125I-TGF-β3 binding to the soluble receptors, even at a concentration of 1250 ng/ml (500 nM).

Binding affinity values were calculated using Scatchard analysis of the binding data. A Scatchard analysis from one representative experiment is shown in Fig. 2, and averages from at least three separate experiments are summarized in Table I. sTβRII-B.Fc and sTβRII.B.Fc proteins had high affinity for TGF-β1 and -β3, with K_d values in the picomolar range. sTβRII-B.Fc had a 1000-fold higher affinity for TGF-β1 and -β3 than sTβRII.B.Fc, but the difference in calculated affinities was not statistically significant. When 125I-TGF-β2 was employed, no binding could be detected, even when the amount of soluble receptor per well was increased to 100 ng/well.

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Fig. 3. sTβRII-B.Fc or sTβRII-B.Fc can inhibit TGF-β1 and -β3 but not TGF-β2 signaling activity. Mv1Lu cells stably transfected with the TGF-β responsive (CAGA)12MPL-Luc reporter construct were treated for 16 h with 100 pm TGF-β1 (diamonds), -β2 (squares), or -β3 (triangles) and with increasing amounts (30–1300 pm) of purified sTβRII-B.Fc (upper panel) or sTβRII.Fc (lower panel), as indicated. Cell lysates were harvested and luciferase assay was performed. Luciferase values were normalized for total lysate protein by bovine serum albumin protein assay. Relative luciferase activity is presented as the percent decrease in luciferase activity of cells incubated with TGF-β ligand and soluble receptors compared with cells incubated with TGF-β ligand alone. Shown is the mean ± S.E. for three experiments.

Next, we examined the effect on TGF-β2 signaling after transfecting L6 cells with increasing amounts (0–5 μg/6-cm plate) of TβRII-B or TβRII full-length cDNA (Fig. 4, panels B and C, respectively). Western analysis of cell lysates confirmed increased type II receptor protein expression with transfection of increasing amounts of cDNA (data not shown). The luciferase response of L6 cells transfected with reporter vectors did not differ when the cells were treated with 100 pm TGF-β1, -β2, or -β3, confirming that L6 cells respond with similar potency to the three different TGF-β isoforms, in the absence of TβRII or TβRII-B. Transfection of either type II receptor isoform into the L6 cells produced a dose-dependent increase in signaling to TGF-β2 similar to that seen with TGF-β1 and -β3, indicating that both receptors are able to enhance signaling by TGF-β2 with a similar potency to TGF-β1 and -β3.

sTβRII-B.Fc and sTβRII.Fc Require sTβRI.Fc Protein for Binding to TGF-β2—To investigate the possibility that sTβRII.Fc and sTβRIII/II-B.Fc can form high affinity complexes, we mixed the soluble receptors and tested binding to ligands (Fig. 5). Different amounts of sTβRII-B.Fc, sTβRII.Fc (10–50 ng), or sTβRI.Fc (50–100 ng) were incubated with 125I-TGF-β1 or 125I-TGF-β2. As expected, sTβRII-B.Fc or sTβRII.Fc alone significantly bound 125I-TGF-β1 in a dose-dependent manner, whereas there was no significant binding to 125I-TGF-β2. Also, sTβRII.Fc by itself did not bind either 125I-TGF-β1, as has previously been reported (35), or 125I-TGF-β2. Next we mixed sTβRII-B.Fc or sTβRII.Fc at 10 and 50 ng together with 100 ng of sTβRI.Fc in the presence of 125I-TGF-β2. The heterologous receptor complex was now able to bind 125I-TGF-β2 in a dose-dependent fashion with increasing amounts of soluble type II receptors (Fig. 5A). A similar experiment was carried out by mixing increasing amounts of sTβRII.B.Fc receptor (100–500 ng) with a fixed amount of sTβRII-B.Fc or sTβRII.Fc (10 ng), which again lead to binding of 125I-TGF-β2 in a dose-dependent fashion (Fig. 5B).

sTβRII-B.Fc and sTβRII.Fc in Complex with sTβRI.Fc Can Inhibit TGF-β2 Signaling—To determine whether the complex composed of sTβRII-B.Fc or sTβRII.Fc with sTβRI.Fc could effectively inhibit TGF-β2 signaling, we incubated Mv1Lu cells transfected with the (CAGA)12MPL-Luc reporter construct, with 40 pm TGF-β2 in the presence or absence of 5 μg/ml sTβRII-B.Fc, sTβRII.Fc, or sTβRI.Fc alone, or the mixture composed of 5 μg/ml each of sTβRII-B.Fc plus sTβRII.Fc or sTβRII.Fc plus sTβRI.Fc. As shown in Fig. 6, when sTβRII-B.Fc or sTβRII.Fc were incubated with sTβRII.Fc a functional heterocomplex could be reconstituted that significantly inhibited TGF-β2-induced luciferase activity compared with each of the soluble receptors alone.
**Figure 5.** sTβRII-B.Fc and sTβRII.Fc require sTβRII.Fc for binding to TGF-β2. A, sTβRII-B.Fc (RIIB, 10 and 50 ng), sTβRII.Fc (RII, 10 and 50 ng), or sTβR.LFce (RI, 50 and/or 100 ng) were incubated overnight with 100,000 counts of 125I-TGF-β1 (black bars) or 125I-TGF-β2 (middle gray bars) as indicated. Combinations of increasing doses of sTβRII-B.Fc (RIIB, 10 and 50 ng) or sTβRII.Fc (RII, 10 and 50 ng) were mixed with a fixed dose of sTβRII.Fc (RI, 100 ng) and 100,000 counts of 125I-TGF-β2 (right gray bars). Samples were placed on Protein A-coated plates, washed, and counted using a standard γ-counter. B, sTβRII-B.Fc (RIIB, 10 ng), sTβRII.Fc (RII, 10 ng), or sTβR.LFce (RI, 50 and/or 100 ng) were incubated overnight with 100,000 counts of 125I-TGF-β1 (black bars) or 125I-TGF-β2 (middle gray bars) as indicated. Combinations of fixed doses of sTβRII-B.Fc (RIIB, 10 ng) or sTβRII.Fc (RII, 10 ng) were mixed with increasing doses of sTβRII.Fc (RI, 100 and 500 ng) and 100,000 counts of 125I-TGF-β2 (right gray bars). Samples were placed on Protein A-coated plates, washed, and counted using a standard γ-counter.

**Figure 6.** sTβRII-B.Fc and sTβRII.Fc in solution can form a complex with sTβRII.Fc to block TGF-β2 signaling. Mv1Lu cells were transiently transfected with the (CAGA)6-MPL-Luciferase reporter mixture. 48 h after transfection, cells were incubated for 16 h with (lanes 2–7) or without (lane 1) 40 μg TGF-β2 in the absence (lanes 1 and 2) or presence of 5 μg/ml each of sTβRII-B.Fc, sTβRII.Fc, or sTβR.LFce alone (lanes 3–5), or of the mixture composed of 5 μg/ml each of sTβRII-B.Fc or sTβRII.Fc plus sTβR.LFce (lanes 6 and 7) as indicated. Cells were lysed and lysates were assayed for luciferase activity. Luciferase values were normalized for transfection efficiency relative to Renilla activity, and data are presented as -fold increase in luciferase activity of cells treated with TGF-β ligand relative to untreated cells. Shown are results from one of three representative experiments.

**DISCUSSION**

There are several advantages to using cell-free systems for analyzing binding properties of receptor complexes. First, the ability to quantitate specific binding is straightforward compared with indirect cell surface binding assays and thus allows an accurate measurement of binding affinity and specificity. This is especially helpful in cases such as the TGF-β family members in which the ligands often have multiple binding proteins of various affinities on or near the cell surface that lead to a high background of nonspecific binding. Second, the absence of any confounding co-expressed accessory proteins that might be present at the cell surface allows the determination of the binding properties of single types of receptors by themselves in isolation. Of note, the x-ray crystal structures of proteins are determined in a cell-free manner and the validity and utility of their data are well recognized and accepted.

The results presented here using a cell-free radioligand binding assay provide evidence that sTβRII.Fc and sTβRII-B.Fc by themselves can bind with high affinity to TGF-β1 and -β2 but do not bind to TGF-β2. Corroborating data from a biological inhibition assay of TGF-β activity in cells were consistent with the selectivity and specificity of sTβRII.Fc and sTβRII-B.Fc seen in the binding data. Our measured binding affinities (30 to 500 pM) are consistent with previously published affinities for the ECD of TβRII (31, 36, 37), and compare well with indirect estimates for membrane-bound complexes (1).

The precise role and effect on ligand binding of the 26-amino acid insert in TβRII-B is unknown. It has been suggested that iodinated TGF-β2 could be cross-linked to TβRII-B but not TβRII, thus indicating that the 26-amino acid insert might confer the ability to bind and signal via TGF-β2 to the type II-B receptor (16). Indeed, the 26-amino acid insert is at the N-
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terminus of the type II-B receptor, and would be in the unresolved region of the crystal structure of the ECD of the type II receptor (25). This unresolved region is in close proximity to residues in the type II receptor that interact with TGF-β3 (25), and could conceivably participate in altering ligand binding properties of the receptor. However, these prior studies were performed in COS cells, which express TβRII (11, 12), and thus could not demonstrate the ability of TβRII or TβRII-B to bind TGF-β2 in the absence of TβRII. In our cell-free system, we found no discernable difference in affinity or specificity between the TGF-β type II and type II-B receptors. Importantly, neither receptor by itself could bind TGF-β2 with measurable affinity. Although there was a trend toward a higher affinity for TGF-β1 and -β3 for the sTβRII-B-Fc than the sTβRII-Fc protein, as well as a severalfold increase in the ED₅₀ in the biological inhibition assay, this increase was not statistically significant.

Interestingly, both full-length TβRII and TβRII-B, when expressed in L6 myoblasts that lack TβRIII and native TβRII-B, could increase TGF-β2 signaling in a dose-dependent manner (Fig. 5), a finding that appeared paradoxical to the binding data. However, these results are consistent with a prior study showing responsiveness of L6 cells to increasing doses of TGF-β2 and signaling augmentation by transfection with TβRII-B (16). Although this prior study did not find augmentation of signal by transfection with TβRII, a dose-response curve was not performed and so adequate doses of receptor may not have been used (16). Of note, another study demonstrated that type I and type II receptors could be cross-linked to 125I-TGF-β2 when they were overexpressed in COS cells, suggesting the possibility that TGF-β2 can signal via complexes of type I and type II receptors (30). However, interpretation of this study is also limited by the presence of TβRIII in COS cells as a potential confounding factor.

To understand the apparent discrepancy between our binding data showing high affinity binding of soluble type II receptors for TGF-β1 and -β3 but not β2, and the ability of the full-length receptor to mediate TGF-β2 binding when expressed at the cell surface of L6 cells, we tested the ability of sTβRII-Fc or sTβRII-B-Fc and sTβRI-B-Fc to form a functional complex with TGF-β2 in vitro in the absence of TβRII. We found that the mixture of sTβRI-Fc and sTβRII-Fc or sTβRII-B-Fc was sufficient to reconstitute binding to TGF-β2. In addition, we demonstrate that the heterocomplex of sTβRI-Fc and sTβRII-Fc or sTβRII-B-Fc was a functional inhibitor of TGF-β2 in a biological inhibition assay compared with sTβRI-Fc, sTβRII-Fc, or sTβRII-B-Fc alone.

These results imply that the complexed TGF-β type I and type II receptors are sufficient to bind TGF-β2 in the absence of type III receptors when there are sufficient quantities of type II receptors, type I receptors, and TGF-β2 ligand present. This could result in TGF-β2 binding and subsequent signaling in the absence of type III receptors, and could explain in part the difference between phenotypes of the type III receptor null mouse and the TGF-β2 null mouse. For example, bone defects seen in the TGF-β2 knockout phenotype were not evident in the TβRIII null mutants, suggesting that bone cells are either exposed to sufficient TGF-β2 ligand or express enough type II and type I receptors to allow TGF-β2 signaling in the absence of the type III receptor.

Thus, we propose two pathways for TGF-β2 signaling, one TβRIII-dependent and one TβRIII-independent (Fig. 7). As has previously been described, TGF-β2 can bind to TβRIII, which then recruits TβRII and TβRI, leading to phosphorylation of TβRI and downstream signaling (Fig. 7, left; Ref. 28). Alternatively, in the absence of TβRIII, TGF-β2 can still bind and signal through the heterocomplex of TβRII and TβRI (Fig. 7, right). Further studies will be needed to determine the relative affinities of TGF-β2 for TβRIII compared with the heterocomplex of TβRII and TβRI, and the relevance of these two pathways in vivo.

Our data also have important implications for the structure of the active TGF-β2 signaling complex. The high resolution crystal structures of TGF-β2 (21, 22), TGF-β3 (23), and more recently, the soluble ECD of the type II receptor (24), and the complex of the ECD-TβRII and TGF-β3 (25) have been solved, in addition to those of the BMPRIA-BMP2 (26) and ActRII-BMP7 complexes (27). In aggregate, these studies suggest that TGF-β receptor and ligand interactions may involve a cooperative model of binding with direct protein-protein contact between the type II and type I receptors as part of the assembly process, whereas the BMP/ActRII and BMP ligands may utilize an allosteric model of binding in which the type II and type I receptors do not necessarily make contact with each other. Our data strongly supports a cooperative model of receptor-ligand interaction for TGF-β2 and predicts that a stable binding complex in the absence of TβRIII will require the presence of the type II receptor, the type I receptor, and the TGF-β2 ligand.

Many unanswered questions remain regarding the structure of the active TGF-β2 signaling complex. Our model does not predict whether type II receptors, type I receptors, or both directly bind to TGF-β2, only that the presence of both receptors is required for binding. One possibility is that both receptors bind directly to TGF-β2. Another possibility is that the presence of one receptor causes a conformational change of the second, which then allows the latter to bind TGF-β2. Perhaps most intriguing is the question of how the type III receptor is able to enhance TGF-β2 binding to the type II and type I receptors in a multimeric ternary signaling complex. We have generated preliminary data using a soluble type III receptor-Fc construct suggesting that the interaction of the type III recep-
tor and type II receptor are also cooperative.\textsuperscript{2}

Our assay system provides a rapid, potentially high-throughput and straightforward system to measure the binding of receptors or ligands to test hypotheses regarding receptor-ligand interactions. For example, it is unclear exactly why T\textsubscript{ßRII} and T\textsubscript{ßRII-B} cannot bind TGF-\beta 2 alone, because the amino acid residues that constitute the contact region of TGF-\beta 2 and TGF-\beta 2 are highly conserved with only conservative changes in the corresponding amino acid residues that constitute the contact region of the type II and type II-B receptors to attempt to "restore" TGF-\beta 2 binding. Finally, mixing and matching experiments using different type I and type II receptors could allow the creation of new soluble complexes with novel specificities tailored to particular TGF-\beta 2 to serve as antagonists.

Acknowledgments—We thank Dr. Dennis Brown, Dr. Dennis A. Ausiello, and Dr. William F. Crowley, Jr. for their continued support.

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