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Production, isolation, and structural analysis of ligands and receptors of the TGF- β superfamily

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Abstract

The ability to understand the molecular mechanisms by which secreted signaling proteins of the TGF- β superfamily assemble their cell surface receptors into complexes to initiate downstream signaling is dependent upon the ability to determine atomic-resolution structures of the signaling proteins, the ectodomains of the receptors, and the complexes that they form. The structures determined to date have revealed major differences in the overall architecture of the signaling complexes formed by the TGF- β s and BMPs, which has provided insights as to how they have evolved to fulfill their distinct functions. Such studies, have however, only been applied to a few members of the TGF- β superfamily, which is largely due to the difficulty of obtaining milligram-scale quantities of highly homogenous preparations of the disulfide-rich signaling proteins and receptor ectodomains of the superfamily. Here we describe methods used to produce signaling proteins and receptor ectodomains of the TGF- β superfamily using bacterial and mammalian expression systems and procedures to purify them to homogeneity.

Keywords

TGF- β ; ligand and receptor; protein expression; protein purification; structural analysis

1. Introduction

The transforming growth factor-beta (TGF- β) superfamily is comprised of a diverse family of signaling proteins, with three known family members in *C. elegans*, seven in *D. melanogaster*, and more than thirty in humans and other vertebrates [1]. The proteins of the superfamily arose as developmental factors responsible for embryonic patterning and morphogenesis in invertebrates, but have further evolved to fulfill many extraembryonic roles as organisms have diversified.

The proteins of the superfamily are produced as pre-pro proteins and are secreted either as mature disulfide-linked dimers, or as mature disulfide-linked dimers non-covalently bound to their pro-domain [2,3]. The mature homodimers signal by binding and bringing together two transmembrane receptors, known as receptor types I and II, to form heterotetrameric complexes with two type I and two type II receptors [4,5]. The proteins of the superfamily

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can be divided into two phylogenetic clades based on the type I receptors they bind and Smad proteins they activate [6]. The more recently evolved members of the superfamily, including the TGF- β s, activins, nodal, and some of the GDFs and BMPs (GDF-9, -11, and -15 and BMP-15), bind and signal through type I receptors that couple to and activate R-Smads 2, 3, while the more distantly related GDFs (GDF-1, -3, -5, -7, and -10) and BMPs (BMP-2, -3, -4, -5, -6, -7, -8, -9, and -10) bind and signal through type I receptors that couple to and activate R-Smads 1, 5, and 8 [1]. The two subclasses of R-Smads, upon association with the co-mediator Smad, Smad4, assemble distinct transcriptional complexes and thus activate (or repress) distinct subsets of genes [7].

The recent structures of TGF- β s (TGF- β 1 and - β 3) and BMPs (BMP-2 and BMP-7) bound to the ectodomains of their receptors (T β RI and T β RII and BMPRIa/BMPRIb, ActRII/ActRIIb, respectively) show that although the TGF- β s and BMPs and their receptors share the same overall folds, they nevertheless bind and assemble their receptors in a distinct manner (Fig. 1) [8–13]. The differences are especially pronounced for the type II receptors: BMP type II receptors use the concave surface of their β -sheet to complement the convex surface of the knuckle epitope of the BMP, while the TGF- β type II receptor inserts one of its edge β -strands into the cleft between the fingertips of the TGF- β . Though the differences are less pronounced, the type I receptors also bind differently: BMP type I receptors bind to the wrist and have extensive contact with both BMP monomers, while the TGF- β type I receptor is shifted away from the wrist toward the fingertips where it contacts T β RII, the TGF- β monomer to which T β RII is bound, and to a limited extent, the adjacent monomer. These structural differences are significant as the distinct interfaces expand the range of specificity and segregate the actions of TGF- β s, which signal through receptors that activate Smads 2 and 3, away from the many BMPs and GDFs, which signal through receptors that activate Smads 1, 5, and 8 [14–17].

The determination of the structures of the TGF- β s and BMPs bound to the ectodomains of their type I and type II receptors has been highly dependent on the ability to produce and isolate highly homogenous forms of each these proteins in quantities large enough for structural studies. The objective of this review is to provide an overview of the different strategies that have been used to obtain these proteins and to characterize their binding properties and structures *in vitro*. The high disulfide content of both the signaling proteins and receptor ectodomains of the superfamily necessitates that procedures specifically adapted for these types of proteins be used. The primary methods that have been used for these purposes include overexpression as secreted proteins in cultured eukaryotic cells, and overexpression in bacteria, followed by renaturation into native signaling proteins or receptors. The methods commonly used to obtain signaling proteins of the superfamily will be described first, followed by a summary of the methods used to obtain the receptor ectodomains of the superfamily.

2. Materials

2.1 Reagents and buffers for *E. coli* expression

1. *E. coli* host strain: BL21(DE3) (Stratagene)

2. Expression constructs: The coding sequences of mature human TGF- β 2 and extracellular domain of T β RII were synthesized by Genscript (Piscataway, NJ) to optimize the codon usage for *E.coli* and minimize formation of secondary structure by the mRNA. The coding sequences were inserted between the NdeI and HindIII sites in plasmid pET32a (Novagen, Madison WI).
3. Standard LB medium: Dissolve 10 g tryptone, 5 g yeast extract, and 10 g NaCl in 1 L H₂O and adjust to pH 7.4 by addition of 10 M NaOH, then sterilize by autoclaving. Add ampicillin (150 μ g/mL) before use.
4. Ampicillin: Dissolve 1.5 g ampicillin in a final volume of 10 mL H₂O to yield 150 mg/mL stock solution, sterilize by filtration through a 0.22 μ m filter, and store in the dark at -20°C .
5. Isopropyl- β -D-thiogalactoside (IPTG): Dissolve 2.38 g IPTG in a final volume of 10 mL H₂O to yield 1 M stock solution, sterilize by passage through a 0.22 μ m filter, and store at -20°C .

2.2 Reagents and buffers for mammalian expression

1. Cell line for transfection: CHO-lec3.2.8.1 (see ^{note 1} in section 4)
2. CMV-based expression construct: Construct is that initially reported by Zou, et. al [18] in which the human TGF- β 1 open reading frame is subcloned into pcDNA3.1(+) modified to include a glutamine synthetase gene for gene amplification. The leader peptide of TGF- β 1 was replaced with that of rat serum albumin, and an eight-histidine tag was inserted immediately after the leader sequence to facilitate protein purification. In addition, Cys 33 of TGF- β 1, which forms a disulfide bond with latent TGF- β 1-binding protein (LTBP) was replaced by a serine residue to increase secretion.
3. Cell culture medium: DMEM/F12 medium, 10% fetal bovine serum (FBS), antibiotics (penicillin-streptomycin), pre-warmed to 37°C .
4. Minimal Essential Medium (MEM): Opti-MEM medium (Invitrogen)
5. Lipid transfection reagent: Lipofectamine 2000 (Invitrogen)
6. Purified plasmid DNA: Maxi Prep (Qiagen)
7. Cell culture flasks and plates
8. Phosphate-buffered saline (PBS)
9. Glutamine-free Glasgow's Minimal Essential Medium (GMEM) (SAFC biosciences)
10. Glutamine synthase (GS) supplement (50X) (SAFC Biosciences)
11. L-methionine sulfoximine (MSX) (Sigma)

¹CHO-lec3.2.8.1 cells have multiple glycosylation defects for production of glycoproteins with minimal carbohydrate heterogeneity [46].

12. Serum-free medium for protein expression: CHO-S-SFM II (Gibco) or SFM4CHO-A (HyClone)
13. Dimethyl sulfoxide (DMSO)

2.3 Reagents and buffers for protein purification

1. Tris(hydroxymethyl)aminomethane (Tris)
2. 2-(N-morpholino)ethanesulfonic acid (MES)
3. Phenylmethanesulfonyl fluoride (PMSF)
4. Dithiothreitol (DTT)
5. 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS)
6. Disruption buffer: 100 mM Tris, 10 mM EDTA, 1 mM PMSF, pH 8.0
7. TGF- β solubilizing buffer: 8 M urea, 20 mM Tris, 1% DTT (w/v), pH 8.0
8. SP denaturing buffer: 8 M urea, 20 mM sodium acetate, 0.1% DTT (w/v), pH 4.2
9. TGF- β folding buffer: 100 mM Tris, 30 mM CHAPS, 1 M NaCl, 5 mM reduced glutathione, pH 9.5
10. Source 15S buffer: 20 mM sodium acetate, 30 % isopropyl alcohol, pH 4.0
11. Ni buffer: 50 mM Tris, 150 mM NaCl and 10 mM imidazole at pH 8.0.
12. RII solubilizing buffer: 8M urea, 20 mM Tris, pH 7.0
13. RII folding buffer: 200 mM Tris, 2 mM reduced glutathione (GSH), 0.5 mM oxidized-glutathione (GSSG), pH 8.0
14. Source 15Q buffer: 20 mM MES, 1 mM PMSF, 1 mM EDTA, pH 6.0

2.4 Equipment and materials

1. Temperature-controlled orbital shaker
2. Water-jacketed CO₂ incubator
3. Refrigerated high-speed centrifuge
4. Handheld glass/teflon homogenizer
5. UV spectrophotometer
6. Chromatography columns
7. Dialysis tubing
8. Centrifugal concentrators
9. Amicon stirred cell concentrator
10. Apparatus for SDS-PAGE

11. Äkta Fast Protein Liquid Chromatography (FPLC) system (GE Healthcare) or equivalent.

3. Methods

3.1 Overview of signaling protein production and isolation

TGF- β s are disulfide-linked dimers of identical 112-residue monomers. The monomers have nine cysteines, one of which participates in the formation of the inter-chain disulfide and eight of which form four internal disulfides [19–22]. Three of the four internal disulfide bonds form a conserved structure known as a cysteine knot [23]. BMPs, GDFs, activins, and most other signaling proteins of the TGF- β superfamily share a similar structure, though the number of internal disulfide bonds varies, with some, such as activins/inhibins possessing four disulfides, but others, such as BMPs and GDFs possessing only the three that form the cystine knot (Fig. 1B).

TGF- β s and other proteins of the superfamily are produced as pre-pro proteins [2]. The pro-domains promote the maturation, including the formation of the inter-chain disulfide bond that stabilizes their dimeric structure [24,25]. TGF- β 's pro-domain, also known as latency associated peptide, or LAP, is almost three times the length of the mature signaling protein (251 – 280 vs. 112 residues, respectively) and contains three cysteines, two of which (C223 and C225 in TGF- β 1) pair cross-wise with the same cysteines in another molecule of the pro-domain to form the pro-domain homodimer [3]. The pro-domains for proteins of the superfamily vary widely in size and sequence: all are cleaved from the mature N-terminal signaling protein prior to secretion, and while the pro-domains of some superfamily proteins, such as TGF- β , associate tightly with the mature signaling dimer and are secreted in complex with them, some, such as those for activins, do not [2]. The approaches used to produce the proteins of the superfamily include a) overexpression of the full-length pre-pro proteins in cultured eukaryotic cells, and b) overexpression of the mature monomers in bacteria, followed by renaturation of the overexpressed polypeptide into native dimers. The procedures commonly used to obtain proteins of the superfamily by expression in eukaryotic cells will first be described, followed by a summary of procedures used to renature bacterially expressed protein into native dimers.

3.1.1 Expression of signaling proteins in eukaryotic cells—The majority of superfamily proteins produced in eukaryotic cells have been produced in mammalian cells, such as CHO or HEK-293 cells [18,26–41]. The preference for mammalian cells is largely for practical reasons, as most investigators are interested in producing mammalian signaling proteins and mammalian cells have the appropriate molecular machinery in the endoplasmic reticulum (ER) for folding and proteolytic processing. Though mammalian hosts are widely used, it has been observed that the proteolytic processing of the pro domain in the ER is often incomplete. This can sometimes complicate the purification as it then becomes necessary to not only separate the mature signaling dimer from its pro-domain, but also the pro-domain-mature precursor. There are several reports of the overexpression of mammalian signaling proteins in non-mammalian cells, including human BMP-2, bovine activin A, and bovine inhibin A in insect cells [42,43] and human activin A in yeast [44]. These expression

hosts have some advantages over mammalian cells, including the absence of native binding partners that can interfere with maturation [18] and potentially higher yields [44,45]. The higher yields have only been obtained when the protease cleavage site between the pro-domain and mature signaling dimer have been altered to more closely match the endogenous proteases in the expression host [44,45]. Thus, successful heterologous expression has required some alterations to account for differences in molecular machinery relative to mammalian hosts, but so far these have been relegated to the enzymes responsible for proteolytic processing, not enzymes involved in catalyzing disulfide exchange or folding.

The signaling proteins are usually expressed along with their native pro-domains, although the signal peptides are often substituted to improve secretion [18,33]. The overexpressed proteins are purified from the conditioned medium using either conventional means (ion-exchange, gel filtration, etc.) or purification tags, such as hexahistidine tags. The N-terminus of the mature signaling dimer is accessible and flexible and thus provides a suitable site for tagging, while the C-terminus is structurally ordered and buried in the interior of the structure, and thus does not provide an appropriate site for tagging. If N-terminal tags are used, they should be removed after the signaling protein has been purified as the N-terminus lies near the binding site for the type I receptor and tags attached here have been found to block receptor binding and signaling [44]. The most straightforward means of removing the tag is to engineer a protease cleavage site between the purification tag and the N-terminus and then treat with the corresponding protease after the mature signaling protein has been purified. The alternative is to place the tag downstream of the predicted signal peptide cleavage site, but before the beginning of the pro-domain. Tagging at this position has the advantage that the tag is removed along with the pro-domain after the pro-domain has been separated away from the mature signaling dimer. The disadvantage of tagging at this position is that some pro-domains, such as those for activin A, associate rather weakly with their mature domain, which may lead to significant loss of the mature signaling protein during the wash steps of the purification.

The complexes between the pro-domains and the mature signaling dimer are generally soluble and can be isolated under non-denaturing conditions. The TGF- β 1:TGF- β 1 pro-domain complex, for example, is isolated in 50 mM Tris, 150 mM NaCl, pH 8.0 [18,33]. The mature signaling dimers, on the other hand, are generally poorly soluble in standard buffers at neutral pH after the pro-domain has been removed. The most common procedure is to acidify the pro-domain:signaling dimer complex after it has been purified from the medium. The acidification serves to release the mature signaling dimer from its pro-domain and to increase its charge, which generally improves solubility. Though acidification irreversibly unfolds many proteins, this is not generally observed with the disulfide-rich proteins of the TGF- β superfamily, and thus it is common for the final purification to be performed under acidic conditions. The procedures most commonly used include cation-exchange chromatography in acidic buffers (such as acetate at pH 4.0) or reverse phase chromatography with 0.05 –0.1% trifluoroacetic acid (and either acetonitrile or methanol for the gradient elution). The concentrated stocks of most superfamily proteins are also commonly stored under acidic conditions, such as 1 mM HCl or 100 mM acetic acid. Though it varies, most superfamily proteins are soluble when diluted 1:100 or 1:1000 from 1 mg/mL stocks in acidic conditions to phosphate buffered saline or other similar solutions

near neutral pH. The procedure used to express and purify human TGF- β 1 from CHO cells as initially described by Zou, et. al [18] is described below.

3.1.2 Protocol for expression and purification of human TGF- β 1 using CHO cells

Establishment of a stable TGF- β 1-expressing cell line

1. Seed CHO-lec3.2.8.1 cells at a density of 3×10^6 cells in 5 mL DMEM/F12 medium containing 10% FBS in T-25 flask and culture them at 37°C with 5% CO₂.
2. After 24 h, replace the medium with 4 mL fresh DMEM/F12 medium containing 10% FBS.
3. Dilute 10 μ g of CMV-based expression plasmid (see section 2.2 above) in 500 μ L of Opti-MEM (mix A) and dilute 30 μ L of lipofectamine 2000 in 500 μ L Opti-MEM (mix B).
4. Incubate at room temperature for 20 min.
5. Add mix A to mix B with gentle pipetting up and down.
6. Incubate for 20 min at room temperature.
7. Add the mix A + B to cells.
8. Replace the medium (DMEM/F12 with 10% FBS) after 24 h. (see ^{note 2}).
9. After two days the cells were trypsinized, seeded into ten 96-well plates, and cultured with glutamine-free GMEM-S medium supplemented with GS supplement, 10% FBS, and 30 μ M MSX. (see ^{note 3})
10. After about three weeks, take the supernatant from growing wells (with visible colony) and assayed for TGF- β 1 expression by ELISA. (see ^{note 4})
11. Pick the ten highest expression clones and do the second round selection with 500 μ M MSX. For each clone, seed one 96-well plate with 100–1000 cells per well. Wait for another three weeks and repeat the step above.
12. After two rounds of selection and amplification, the clone with the highest expression of TGF- β 1 was chosen for large-scale recombinant protein production. Make stocks of the chosen clone and store them in liquid nitrogen.

Expression of TGF- β 1 from CHO cells and purification

1. Expand the highest expression clone of TGF- β 1 into T-225 flasks or T-500 triple layer flasks and culture the cells in glutamine-free GMEM-S medium supplemented with GS supplement, 10% FBS, and 500 μ M MSX.

²It is normal from some of the cells to detach and die after transfection.

³In each well there should be 100–1000 cells and the transfection rate is approximately 0.1%, thus 0–1 colonies are expected in each well.

⁴Colonies that do form will grow at different rates and thus the medium should be removed only when the color of the medium turns yellow. Protein expression levels detected by ELISA do not generally correlate with growth rates.

2. When the cultured cells reach confluence, wash the cells twice with PBS and change the medium to CHO-S-SFM II serum-free medium.
3. After 3 days, harvest the medium and replace with fresh CHO-S-SFM II serum-free medium, repeat 5–6 times or until desired volume. (see ^{note 5})
4. Freeze harvested medium at -20°C for further purification.
5. Thaw 500 mL harvested medium add imidazole to final concentration of 10 mM, filter with 0.22 μM membrane. (see ^{note 6})
6. Equilibrate 20 mL Ni-NTA column with Ni buffer containing 50 mM Tris, 150 mM NaCl and 10 mM imidazole at pH 8.0.
7. Load 500 mL filtered medium onto Ni column and wash the column with Ni buffer for two column volumes.
8. Elute the protein by a linear concentration gradient of imidazole (10–500 mM) in Ni buffer.
9. Pool fractions that contain protein and adjust the pH to below 3.0 by adding 6 M HCl (see ^{note 7}); dialyze the solution against 100 mM acetic acid (minimum 20-fold dilution, three times).
10. Purify TGF- β 1 on Source 15S column following the same protocol as TGF- β 2 (steps 4–8 in 3.1.4 Isolation of native dimer) except using the linear gradient of 0–600 mM NaCl. (see ^{note 8})

3.1.3 Expression of signaling protein monomers in bacteria and renaturation into native dimers—

The other major avenue by which signaling proteins of the superfamily have been obtained is by expression of the mature monomers in bacteria, followed by renaturation of these into disulfide-linked dimers. The majority of family members are expressed using pET vectors, which are based on the T7 promoter [47]. To optimize expression, synthetic genes in which the codons have been optimized for bacteria may be used. Though N-terminal tags are sometimes added, these are not generally required, as the proteins invariably misfold and form highly refractile inclusion bodies. The formation of inclusion bodies aids in the purification since after disrupting the cells in non-denaturing buffer, most of the contaminating soluble proteins can be removed by repeatedly washing the inclusion bodies with non-denaturing buffers containing either salt (e.g. 0.5 – 1.0 M NaCl) or non-ionic detergents (e.g. 0.5 – 2.0% Triton X-100). The isolated inclusion bodies are almost always reconstituted in high concentrations of denaturant, such as 6 M GdmCl or 8 M urea. Typically, the denaturant is buffered at pH 8.0 – 9.0 and includes a reductant, such as dithiothreitol, to convert any disulfides into free cysteines. Tris is also often included, especially if urea is used as the denaturant, as its primary amine group will react with

⁵Replace the medium when the color changes from red to yellow; can be repeated until the cells are no longer viable (as many as 10 times). Protein secretion levels generally decrease over time.

⁶Add 10 mM imidazole in the sample to prevent non-specific binding to the Ni-NTA column

⁷Acidification of the solution will dissociate the mature TGF- β 1 from LAP; however, this step will not separate the mature TGF- β 1 from incompletely processed pro-domain-mature precursor (usually 5 – 10%).

⁸Only dimeric TGF- β will be observed (but not monomeric TGF- β as in the purification of refolded bacterial TGF- β)

isocyanic acid which is formed from the breakdown of urea. Though typically already quite pure at this stage, the proteins are nevertheless often further purified in denaturant using ion-exchange, gel filtration, or metal affinity chromatography to remove residual contaminants that might interfere with the subsequent folding.

The native signaling dimers are most often formed by diluting the reduced denatured monomers into a much larger volume of folding buffer. The folding buffers used for most of the proteins of the superfamily are based on the buffers reported by Cerletti and co-workers for refolding the TGF- β s [48]. These include, among other components, low (mM) concentrations of redox agents, such as cysteine/cystine, reduced glutathione/oxidized glutathione, or dithiothreitol/*trans*-4,5-dihydroxy-1,2-dithiane (oxidized dithiothreitol) and relatively high concentrations (20 – 30 mM) of the non-denaturing detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, also known as CHAPS. The particular choice of redox agents is highly protein-dependent: sometimes only reductants are used, while in other cases a combination of reductants and oxidants are used. The majority of proteins folded have included CHAPS in the folding buffer, though notably, a recent study by Ejima and co-workers found that the CHAPS derivative sodium taurodeoxycholate (TDCA) was about 10 – 20 times more effective than CHAPS for folding human activin A [49]. The inclusion of CHAPS, or derivatives such as TDCA, is the most expensive reagent used in the folding since to be effective they must be used at concentrations comparable or higher than their critical micelle concentrations (which for CHAPS is near 10 mM). The folding mixture sometimes also include a co-solvent: TGF- β s, for example, have been folded in buffers containing 10 – 20 % DMSO, while activin A has been folded without any co-solvent. The requirement for co-solvents, such as DMSO, seems to be related to the solubility of the mature signaling dimer, as activins are reasonably soluble at milligram per milliliter concentrations at pH 8.0, while TGF- β s are not. The pH of the folding buffer is almost always 8.0 or higher, which is essential as cysteine thiols have a pK_a of approximately 8.5 and are not capable of forming disulfides unless their sidechain thiols are deprotonated.

The concentration of protein in the folding buffer is important: concentrations higher than about 0.4 mg/mL are generally undesirable as this tends to lead to significant precipitate and lower yields of native dimers. Though less precipitate and greater yields of native dimers can generally be obtained with lower protein concentrations, there is a limit since the formation of disulfide-linked dimers is concentration-dependent and is favored at higher protein concentrations. Thus, the protein concentration must be adjusted to achieve a balance between minimizing loss due to increased formation of aggregates at higher concentration, and a slow rate of dimer formation at low protein concentrations. Though not yet in widespread use, it has been proposed that the folding be performed in two phases, one in which native monomers are formed, and another in which the native monomers are oxidized into disulfide-linked dimers [50]. To demonstrate feasibility, BMP-2 was initially folded under relatively dilute conditions (0.2 mg/mL) in the presence of redox buffers that promote disulfide exchange (0.1 mM reduced glutathione, 0.1 mM oxidized glutathione), and after a significant fraction of protein had folded into native monomers, the protein was concentrated five-fold (1 mg/mL) and transferred into buffer that only included oxidized glutathione (25 mM). The authors reasoned that this should lead mainly to the formation of native disulfide-

linked dimers since all of the other cysteine residues had already formed their native disulfide-pairings (and were therefore not accessible for misfolding). This procedure was quite successful for BMP-2, with yields roughly 2-fold higher than that of single phase folding at 0.2 mg/mL for a period three times longer. The two-step folding method described above is predicated on the assumption that the monomers form the cystine knot and adopt their native folds independent of the other monomer. This may not be so for all family members, but it does appear to be for some, as native monomers of both GDF-5 and TGF- β 3 have been isolated [10,51–53]. Thus, this procedure may lead to refolding of additional family members that have not been previously folded, but whether this procedure will be applicable to all family members has not yet been established.

The folding of most superfamily proteins can be monitored by running samples of the folding mixture on non-reducing SDS-gels; over a period of several days, one observes an increase in dimeric forms and a concomitant decrease in monomeric forms as the cysteines are oxidized and the proteins fold into native dimers. The primary species in the folding buffer after several days of folding include, in addition to native dimers, non-dimerized monomers and high molecular weight disulfide-linked aggregates. Ion-exchange chromatography is well-suited for isolation of the native dimers from folding mixtures owing to charge differences among the different species present. In practice, the folding mixtures are usually acidified, concentrated, and loaded onto cation-exchange columns under acidic conditions and eluted with a salt gradient. In some cases, the native dimers are directly isolated from the folding mixture using reverse-phase chromatography under acidic conditions as described above; more commonly, reverse phase chromatography is used as a polishing step following initial isolation of the native dimers using ion-exchange chromatography.

The yields of mature signaling dimer obtained using the procedures described here vary greatly. The procedure shown below typically yields 8 – 10 milligrams of TGF- β 2 from each liter of bacteria, while similar procedures afford 3 – 4 milligrams of TGF- β 3, and almost no TGF- β 1. The differences in yield are due to differences in the efficiency of the folding – all three isoforms are produced at comparable levels and are present in equal amounts (ca. 50 – 60 milligrams per liter of culture medium) prior to folding. This emphasizes the point that the folding is highly dependent on the amino acid sequence and folding conditions. The most reasonable strategy that can be pursued if the folding does not lead to native dimer would be to adhere to the procedures described above for isolating the purified denatured monomers, and then screen for formation of native dimers under different folding conditions using an ELISA-based approach. The types of screens that have been previously described for folding proteins of the superfamily can be tried [48,49] as well as other screens that have been reported for folding disulfide rich proteins more generally [54]. Though the development of a folding protocol that affords native dimers with high efficiency (defined as the amount of native dimer isolated relative to the amount of purified denatured monomer) can be time-consuming, this investment can pay off as folding efficiencies as high as 40% have been reported, affording large quantities (20 – 30 mg) of highly purified dimer from just a few liters of cultured bacteria. The procedure used to bacterially express, refold, and purify human TGF- β 2 is described below.

3.1.4 Protocol for *E. coli* expression, refolding, and purification of human TGF- β 2

Expression

1. Transform *E. coli* strain BL21(DE3) with the plasmid containing TGF- β 2 sequence. Spread onto a LB-agar plate supplemented with ampicillin (150 μ g/mL) and incubate overnight at 37°C.
2. Inoculate 1 L LB medium, supplemented with ampicillin (150 μ g/mL), with colonies from the plate and incubate at 37°C with orbital shaking at 250 rpm in a 2.8 L flask. Add 0.8 mM IPTG at mid-log phase (0.6 OD₆₀₀) to induce protein expression and incubate at 37°C for 3 h.
3. Harvest the cells by centrifuge at $6,000 \times g$ for 15 min and freeze the pellet.

Recover insoluble, monomeric TGF- β 2

1. Cell pellets from 1 L of culture were resuspended in 30 mL of disruption buffer (100 mM Tris, 10 mM EDTA, 1 mM PMSF, pH 8.0) and sonicated.
2. Centrifuge at $20,000 \times g$ for 20 min and discard the supernatant (see note 1).
3. Resuspend pellet in 100 mL disruption buffer containing 1M NaCl.
4. Centrifuge at $20,000 \times g$ for 20 min and discard the supernatant.
5. Resuspend pellet in 100 mL disruption buffer containing 1% Triton X-100 (v/v).
6. Centrifuge at $20,000 \times g$ for 20 min and discard the supernatant.
7. Resuspend pellet in 100 mL TGF- β solubilizing buffer (8 M urea, 20 mM Tris, 1% DTT (w/v), pH 8.0), stir overnight at room temperature.
8. Add solid sodium acetate to a final concentration of 20 mM and adjust the pH to 4.0 using glacial acetic acid.
9. Centrifuge at $20,000 \times g$ for 20 min and collect supernatant.

Initial purification

1. Equilibrate 20 mL SP-sepharose cation exchange column with SP denaturing buffer (8 M urea, 20 mM sodium acetate, 0.1% DTT (w/v), pH 4.2) for 2 column volumes.
2. Load the supernatant onto column and wash the column with SP denaturing buffer for 2 column volumes.
3. Elute with a linear 0–300 mM NaCl gradient in SP denaturing buffer.
4. Identify fractions containing purified TGF- β 2 using SDS-PAGE; pool purified denatured TGF- β 2 and dialyze against 100 mM acetic acid at 4°C (minimum 20-fold dilution, 3 times).

Folding

1. Dialyzed protein is slowly diluted into TGF- β folding buffer containing 100 mM Tris, 30 mM CHAPS, 1 M NaCl, 5 mM reduced-glutathione, pH 9.5 to a final concentration of 0.2 mg/mL at 4° C (see ^{note 2}). Stir gently for 3 – 4 days at 4 °C (see ^{note 3}).

Isolation of native dimer

1. Adjust the pH of folding mix to 3.5 using acetic acid and concentrate the mixture to roughly 50 mL using an Amicon stirred cell concentrator with a YM-10 membrane (Millipore).
2. Dialyze the concentrated refolding mix against 100 mM acetic acid (minimum 20-fold dilution, three times) (see ^{note 4}).
3. Centrifuge at 20,000 $\times$ g for 20 min, collect supernatant and filter through 0.22 μ m membrane (see ^{note 5}).
4. Equilibrate a Source 15S HR 10/10 column with 20 mM sodium acetate, 30 % isopropyl alcohol, pH 4.0 (Source 15S buffer) at a flow rate of 2.0 mL/min.
5. Load sample onto Source 15S column and wash column with two column volumes Source 15S buffer.
6. Elute with a linear gradient 0 – 400 mM NaCl over 10 column volumes in Source 15S buffer.
7. Collect peak fractions corresponding to the dimer peak (see Fig. 2) and then dialyze against 100 mM acetic acid (minimum 20-fold dilution, 3 times).
8. Measure the concentration of purified TGF- β 2 by OD₂₈₀ absorbance, aliquot, and lyophilize.

3.2 Overview of receptor ectodomain production and isolation

The structurally characterized receptor ectodomains of the TGF- β superfamily, which includes the type I receptors Alk1 [55,56], Alk3 [11,57], Alk5 [10,12,58], and Alk6 [59] and the type II receptors ActRII [8,60], ActRIIb [13,61], BMPRII [62], and T β RII [63–65], have each been shown to adopt a three finger toxin fold, consisting of three pairs of antiparallel β -strands stabilized by a characteristic pattern of four disulfides (Fig. 1C). The seven type I receptors, and all but one of the type II receptors of the superfamily, include one additional disulfide, while the TGF- β type II receptor, includes two. The procedures that have been used to produce the ectodomains of the superfamily are similar to those used to produce the signaling proteins and are described below.

3.2.1 Expression of receptor ectodomains in eukaryotic cells—The majority of receptor ectodomains that have been produced and studied are from mammalian species, such as humans and rodents, yet an almost equal number have been produced in mammalian and non-mammalian hosts. The mammalian hosts used include CHO, HEK-293, and mouse myeloma cells [56,66–70], while the non-mammalian hosts include insect cells and the

methylotrophic yeast, *Pichia pastoris* [61,70–73]. The majority of receptors have been produced as secreted proteins, either alone as monomers, or as dimers by fusing them with the Fc region of an antibody. The Fc-dimerized receptors typically bind their cognate signaling proteins with affinities that are 1 – 2 orders of magnitude greater than their non Fc-dimerized counterparts [55,66,74], which is expected for a bivalent interaction between a covalently dimerized receptor and a covalently dimerized signaling protein. The Fc-dimerized receptor ectodomains are useful for increasing potency for use in binding studies [66] or as inhibitors in cells or animals [75–77].

The N- and C- termini are accessible and flexible in all of the type I and type II receptors of the superfamily, and thus affinity tags, such as hexahistidine tags, can be readily appended to the N- or C-termini without interfering with folding. To date, most of the ectodomains have been produced with a N-terminal tag followed by a protease cleavage site though a few have been produced with cleavable C-terminal tag. The receptors can be directly purified from the conditioned medium if they have an affinity tag. This generally yields protein free of most contaminants, though in many cases, some high molecular weight material may also be present. This high molecular weight material often disappears if the receptors are reduced prior to loading on the gel, indicating that while most of the overexpressed protein folds and adopts native disulfide pairings, some misfolds and forms disulfide-linked multimers. Thus, in many cases, receptors that have been purified with an affinity tag are further purified to eliminate multimers as well as other contaminating proteins. The multimers can be relatively easily eliminated using either size exclusion chromatography, which separates based on size, or ion-exchange chromatography, which separates based on charge (monomers have lower overall charge compared to multimers, and thus elute at lower salt concentrations than the multimers). The purification tag is often removed by treatment with appropriate protease prior to the second step of the purification, as such tags can potentially interfere crystallization. If an affinity tag was not included in the construct, the medium is usually desalted, and the dialyzed medium is then applied to anion or cation exchange column equilibrated at a pH where the protein is expected to bind (usually determined by calculating the net charge as a function of pH using a tool such as the Scripps Protein Calculator [78]). In many cases, this affords protein that is free of most other contaminating proteins, as well as disulfide-linked multimers, though if not, an additional purification step, such as size exclusion or hydrophobic interaction chromatography, can be used. Importantly, non-reducing SDS-PAGE must be used to analyze the purity of the final preparation since reducing agents, such as β -mercaptoethanol or dithiothreitol (DTT), will convert disulfide-linked oligomers or multimers into monomers. It is important to be aware of the presence of disulfide-linked oligomers or multimers and to remove them if they are present since these will interfere in subsequent binding and structural studies.

3.2.2 Protocol for expression of human T β RII-Fc using CHO cells

Establishment of a stable T β RII-Fc expressing cell line

1. The human T β RII ectodomain is inserted in the polylinker of plasmid pFUSE-hIgG4-Fc (Invivogen) to yield an expression construct in which T β RII is in frame with a C-terminal human IgG4 Fc domain. The T β RII-Fc expression cassette is then PCR amplified and inserted downstream of the rat serum albumin signal

peptide in the modified form of pcDNA3.1(+) with a glutamine synthase gene described in Section 2.2.

2. The CMV-based T β RII-Fc expression plasmid is then transfected into CHO-lec3.2.8.1 cells and a stable expressing clone is selected using the same procedure described in Section 3.1.2 above.

Expression of T β RII-Fc from CHO cells and purification

1. Stably transfected cells are cultured in serum free medium in T-225 flasks and the conditioned medium is collected as described in Section 3.1.2 above.
2. Conditioned medium is diluted three-fold into 0.1 M Tris, pH 8.2 and passed over a HiTrap protein A column (GE Healthcare) equilibrated with 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.2
3. Column is washed with ten column volumes of 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.2 and the protein is then eluted by passing over five column volumes of 0.1 M citrate, pH 3.0.
4. Eluted protein is adjusted to pH 7.0 by addition of 0.1 M Tris base, followed by concentration to roughly 10 mg/mL using a Amicon ultrafiltration stirred cell (Millipore).
5. One milliliter aliquots of the T β RII-Fc fusion is passed over a 1.6 $\times$ 60 cm Superdex 200 gel filtration column (GE Healthcare) equilibrated in 0.1 M Tris, pH 7.0. Peak fractions are pooled, concentrated to 10 mg/mL using an Amicon ultrafiltration stirred cell. Protein purity is checked by non-reducing SDS-PAGE and pooled stocks are stored at -20 °C until ready for use.

3.2.3 Expression of receptor ectodomains in bacteria—The other major avenue by which the receptor ectodomains have been obtained is by expression in bacteria. The ectodomains, if expressed alone, form highly refractile inclusion bodies and must be renatured [10,52,55,58,64,73,79]. The overall procedure parallels that used for the signaling proteins, including initial isolation of the inclusion bodies, subsequent purification of the reduced protein in denaturant, and folding by dilution into non-denaturing buffers that include redox-active species that promote disulfide exchange. The procedure used to fold and purify the human T β RII ectodomain is described in Section 3.2.4 below.

The receptor ectodomains, like the signaling proteins, do not require a purification tag as the overexpressed protein forms inclusion bodies, and are therefore largely pure, even before the initial purification in denaturant. Tags are however sometimes included as they aid in the isolation of the protein from the folding buffer. The buffers used to fold the receptors are generally simpler than those used to fold the signaling proteins: most include redox-active species, such as reduced and oxidized glutathione, but other additives such as non-denaturing detergents and co-solvents are not generally required. The ectodomains generally have favorable solubility properties and can be concentrated to high concentration (10 mg/mL, or even higher) in standard buffers (Tris, Hepes, phosphate, etc.) at neutral pH. Thus, the receptors are usually concentrated following folding, and then dialyzed into a

buffer suitable for subsequent ion-exchange or gel filtration chromatography. These two methods are both effective for purifying the receptors following folding as both eliminate disulfide-linked oligomers, which are the major 'contaminant' present in the folding. These two purification methods do not, however, have sufficient resolution to separate native monomers from non-native monomers, and thus in cases where the monomer pool is heterogeneous, it is recommended that the receptor be further purified using C8 or C18 reverse phase chromatography. This method of purification, which separates based on hydrophobicity, has outstanding resolution and has been found to be effective for separating native monomers away from the non-native monomers. This type of purification denatures the receptors (since it is commonly performed in 0.1 % trifluoroacetic acid with either acetonitrile or methanol elution), but is not problematic since the disulfides remain intact and the receptors readily refold after the trifluoroacetic acid and acetonitrile/methanol have been removed by either lyophilization or dialysis. The final purity of the receptor ectodomains, like the signaling proteins, should be assessed by non-reducing SDS-PAGE, since as before reductant will mask any misfolded disulfide-linked dimers and higher order oligomers.

Several receptors of the superfamily, including BMPRIa, BMPRIb, BMPRII, and T β RII have been previously produced as soluble proteins in *E. coli* by expressing them as fusion proteins with thioredoxin [62,80–84]. The advantage of producing the receptors in this manner is that the proteins remain soluble and can be extracted from the bacteria in their native form. These types of fusion proteins are usually constructed with an intervening hexahistidine tag and protease cleavage site between thioredoxin and the receptor. To increase the proportion of protein that remains soluble, the growth temperature can be lowered from 37 °C to 20 °C prior to the induction of protein expression. The protein is extracted from the cells by disrupting them in non-denaturing buffer with protease inhibitors. The proteins are usually purified by metal affinity chromatography, followed by a subsequent polishing step using gel filtration. The latter is important as the proteins produced in this manner often include some disulfide-linked dimer and higher order oligomers.

3.2.4 Protocol for expression of the human T β RII ectodomain using *E. coli*

Expression

1. Transform *E. coli* strain BL21(DE3) with the plasmid coding for the human T β RII ectodomain. Spread onto an LB-agar plate supplemented with ampicillin (150 μ g/mL) and incubate overnight at 37°C.
2. Inoculate 1 L LB medium, supplemented with ampicillin (150 μ g/mL), with colonies from the plate and incubate at 37°C with orbital shaking at 250 rpm in a 2.8 L flask. Add 0.8 mM IPTG at mid-log phase (0.6 OD₆₀₀) to induce protein expression and incubate at 37°C for 3 h.
3. Harvest the cells by centrifugation at 6,000 $\times$ g for 15 min and freeze the pellet.

Recover non-soluble T β RII

1. Cell pellet from 1 L of culture is resuspended in 30 mL of disruption buffer (100 mM Tris, 10 mM EDTA, 1 mM PMSF, pH 8.0) and sonicated
2. Centrifuge at $20,000 \times g$ for 20 min and discard the supernatant (see ^{note 1})
3. Resuspend pellet in 100 mL disruption buffer containing 1 M NaCl
4. Centrifuge at $20,000 \times g$ for 20 min and discard the supernatant
5. Resuspend pellet in 100 mL disruption buffer containing 1% Triton X-100 (v/v)
6. Centrifuge at $20,000 \times g$ for 20 min and discard the supernatant
7. Resuspend pellet in 100 mL RII solubilizing buffer (8M urea, 20 mM Tris, pH 7.0), stir overnight at room temperature
8. Centrifuge at $20,000 \times g$ for 20 min and collect supernatant

Initial purification

1. Equilibrate 20 mL DEAE-sepharose anion exchange column with RII solubilizing buffer (8M urea, 20 mM Tris, pH 7.0) for two column volumes.
2. Load the supernatant onto column and wash the column with RII solubilizing buffer for two column volumes.
3. Elute with a linear 0 – 300 mM NaCl gradient in RII solubilizing buffer.
4. Identify fractions containing purified T β RII using reducing SDS-PAGE; pool purified denatured T β RII; add solid DTT to final concentration of 25 mM, stir for 30 min and dialyze against 100 mM acetic acid at 4°C (minimum 20-fold dilution, 3 times).

Folding

1. Dialyzed protein is slowly diluted into RII folding buffer containing 200 mM Tris, 2 mM reduced-glutathione, 0.5 mM oxidized-glutathione, pH 8.0 to a final concentration of 0.5 mg/mL at 4°C. (see ^{note 2}). Stir gently for 18 – 24 h at 4 °C in an open vessel.

Isolation of native T β RII

1. Concentrate the folding solution to roughly 50 mL using an Amicon stirred cell concentrator with a YM-3 membrane (Millipore).
2. Dialyze the concentrated folding solution against 20 mM MES, pH 6.0 at 4°C (minimum 20-fold dilution, 3 times).
3. Centrifuge at $20,000 \times g$ for 20 min, collect supernatant and filter through 0.22 μ M membrane.
4. Equilibrate a Source 15Q HR 10/10 column with 20 mM MES, 1 mM PMSF, 1 mM EDTA, pH 6.0 (Source 15Q buffer) at a flow rate of 2.0 mL/min.

5. Load sample onto Source 15Q column and wash column with 2 column volumes Source 15Q buffer.
6. Elute with a linear gradient 0 – 300 mM NaCl in Source 15Q buffer over 10 column volumes.
7. Pool the fractions corresponding to purified native receptor (see Fig. 3) and dialyze against Source 15Q buffer to remove salt (minimum 20-fold dilution, 3 times).
8. Measure the concentration of purified T β R II by OD₂₈₀ absorbance, concentrate to 5 – 10 mg/mL, aliquot and store at –20°C.

3.3 Methods to assess ligand-receptor binding

The binding properties of signaling proteins and receptors of the superfamily have been predominantly studied using surface plasmon resonance (SPR) [8–10,12,13,55,56,58,59,66,67,77,85–102]. The feature of this method that has led to its widespread use is that the signaling proteins can be readily coupled to SPR sensor surfaces at low pH where the proteins are soluble, and then the conditions can be changed to study receptor binding near neutral pH. The other advantage of performing SPR experiments with immobilized signaling proteins is that the amount of protein required is minimal (0.2 – 2 micrograms). These quantities can usually be purchased at a reasonable cost, though if they are, it is important to purchase ‘carrier-free’ protein so that the coupling of the signaling protein to the SPR sensor chip is not blocked by the carrier.

The signaling proteins are most commonly coupled to sensor chips by a carbodiimide coupling reaction in which amino groups of lysine residues on the signaling protein are coupled to carboxylates on the surface of the sensor chip. Though reliable and easy to perform, this has the disadvantage that it results in a random coupling of the signaling protein to the sensor chip. This can result in a heterogeneous surface in which some of the protein is coupled such that receptor binding is unimpaired, but another portion in which receptor binding is partially or fully impaired. The heterogeneous surfaces are generally undesirable since this complicates the pattern of receptor binding. One way to minimize heterogeneity is to form the complex in solution between the signaling protein to be studied and its receptors and in turn biotinylate the complex using an amine-reactive biotin derivative. Once modified, the biotinylated signaling protein can be separated from the receptor(s) by purifying it under denaturing conditions (such as C8 or C18 reverse phase chromatography with either acetonitrile or methanol gradient elution). This separation step also removes free biotin from signaling protein. After it has been dialyzed to remove the acetonitrile or methanol, it can be captured onto a sensor chip coated with a high density of streptavidin or neutravidin.

The binding measurements are performed by using the microfluidic system of the SPR instrument to inject the receptor ectodomain for a given period of time, followed by a disassociation period in which the instrument is switched back to the running buffer. Two types of experiments are commonly performed, kinetic and equilibrium experiments. Kinetic experiments are performed by injecting at a high flow rate for a period long enough to determine the rate of binding, but not so long that the response reaches equilibrium, while

equilibrium experiments are performed by injecting at a low flow rate for a period long enough so that equilibrium is reached. Kinetic experiments are analyzed by fitting the time-dependence of the association and disassociation to the simplest mathematical model describing the binding, while equilibrium experiments are analyzed by fitting the equilibrium response (R_{eq}) as a function of concentration of the injected receptor to a standard binding equation. The kinetic experiments therefore provide the association and disassociation rate constants (k_a and k_d , respectively, as well as the K_d from k_d/k_a) as well as the maximal response (R_{max}), while the equilibrium experiments provide only the K_d and R_{max} . Kinetic experiments therefore provide more information and are therefore generally desirable, though they have the disadvantage that this comes at the cost of using larger amounts of receptor (owing to the higher flow rates). The amount of receptor used in a typical kinetic SPR experiment in which the receptor concentration is varied from 0 – 4 μ M is approximately 0.2 mg.

SPR experiments, in addition to providing rates and affinities for binding of a single receptor, also affords the opportunity to study binding of more than one receptor. Such measurements can provide insight into whether the receptors compete or cooperate with one another and have been instrumental in delineating the very different modes of receptor complex assembly for the TGF- β s and BMPs [9,10,12]. Such experiments can be performed in one of two ways: the first and simplest is to perform a co-injection experiment in which the first half of the injection loop is filled with a near-saturating concentration of the first receptor (e.g. type II receptor), while the second half is filled with a variable concentration of the second receptor (e.g. type I receptor) on top of the same concentration of the first receptor in the first half. Such experiments can therefore be used to determine whether the first receptor (e.g. type II receptor) alters the binding of the second receptor (e.g. type I receptor), by comparison to a control experiment in which the binding of the second receptor (e.g. type I receptor) is studied in the absence of the first receptor (e.g. type II receptor).

Several alternatives to SPR have been proposed in recent years, including bio-layer interferometry [103] and microscale thermophoresis [104]. Studies using these methods for studying interactions between signaling proteins and receptors of the TGF- β superfamily have not been reported in the literature, but it is expected that these will be increasingly used in the future, owing to their lower cost and ease of use. Binding studies using titration calorimetry have not been reported, presumably because most superfamily signaling proteins are poorly soluble and must therefore be stored under conditions (low pH) where they bind their cognate receptors poorly, or not at all. Such solubility properties require that the signaling protein and receptor be contained in different buffers, which has the disadvantage that the heat evolved from mixing solvents is large compared to the heat evolved by binding.

3.4 Structural analyses of ligand-receptor complexes

The relatively small size of the signaling proteins (ca. 25 kDa) and receptor ectodomains (11 – 16 kDa) has enabled studies of both using NMR [20,55,57,58,64,79,105–107] and X-ray crystallography [19,21,22,96,98,102,108–110]. The strategies used to obtain the NMR solution structures and X-ray crystal structures of the signaling proteins and receptor ectodomains more or less follow the standard methodologies. The larger sizes of the

complexes, on the other hand, have relegated these to being studied mostly by X-ray crystallography [8–13,61,99,101]. The strategies used to obtain the structures of the signaling protein:receptor ectodomain complexes have mostly been performed by first adding an excess of receptors relative to signaling protein, followed by isolation of the complex by gel filtration. The rationale for this strategy is that it allows for the isolation of stoichiometric complexes, which due to their homogeneity, are more likely to form diffracting crystals. This strategy clearly has limits in light of weaker affinities and thus is likely to be successful for studying many of the signaling protein:receptor ectodomain complexes of the superfamily, but not all. The alternative strategy, in light of weaker affinities, is to combine the proteins in the appropriate stoichiometric ratio (two equivalents of type I or type II receptor ectodomain per equivalent of signaling homodimer), concentrate, and setup the crystallization trials at the highest protein concentration feasible (this has to be empirically determined based on the inherent solubility of the complex). The strategy is based on the presumption that the high protein concentration (typically on the order of 5 – 10 mg/mL) will drive the equilibrium toward complex formation given that the concentration of the complex (0.05 – 0.1 mM, depending on the size and concentration of the complex) is typically several orders of magnitude greater than the disassociation constant (typically 10 μ M or lower). The alternative approach, in light of moderate to lower affinities, is to isotopically label either the signaling protein or receptor ectodomain with NMR-active stable isotopes (15 N or 13 C) and monitor the signals of the labeled component as increasing concentrations of the unlabeled partner are added [53]. The NMR signals of residues that lie directly in the binding site will be most strongly perturbed, thus providing a “map” of the contact surface. This procedure can be repeated with the labeling pattern reversed to obtain the complementary contact surface of the partner. These maps, together with the known structures of the two proteins, can in turn be used to construct a model of the complex using a data-driven docking algorithm, such as HADDOCK [111,112]. This strategy has not yet been used to determine the structures of any signaling protein:receptor complexes of the TGF- β superfamily, but has been widely used to study other systems [113], and may therefore be used to study signaling protein:receptor complexes of the superfamily, especially receptors, such as some of the BMP type II receptors, that bind their cognate signaling proteins weakly.

4. Notes

¹CHO-lec3.2.8.1 cells have multiple glycosylation defects for production of glycoproteins with minimal carbohydrate heterogeneity [46].

²It is normal from some of the cells to detach and die after transfection.

³In each well there should be 100–1000 cells and the transfection rate is approximately 0.1%, thus 0 – 1 colonies are expected in each well.

⁴Colonies that do form will grow at different rates and thus the medium should be removed only when the color of the medium turns yellow. Protein expression levels detected by ELISA do not generally correlate with growth rates.

⁵Replace the medium when the color changes from red to yellow; can be repeated until the cells are no longer viable (as many as 10 times). Protein secretion levels generally decrease over time.

- ⁶Add 10 mM imidazole in the sample to prevent non-specific binding to the Ni-NTA column
- ⁷Acidification of the solution will dissociate the mature TGF- β 1 from LAP; however, this step will not separate the mature TGF- β 1 from incompletely processed pro-domain-mature precursor (usually 5 – 10%).
- ⁸Only dimeric TGF- β will be observed (but not monomeric TGF- β as in the purification of refolded bacterial TGF- β)
- ⁹The protein expressed in inclusion bodies will remain insoluble until dissolved in 8 M urea.
- ¹⁰Small aliquots of 1 M NaOH are added during the addition to maintain the pH at 9.5.
- ¹¹The folding mixture will appear slightly cloudy and the folding can be monitored by running small aliquots of the folding mixture on a non-reducing SDS-PAGE gel.
- ¹²Leave extra room in the dialysis tubing, because the volume will increase about 50% after dialysis.
- ¹³The precipitate is mostly comprised of misfolded disulfide-linked multimers; the protein that remains soluble is mostly natively folded dimers and monomers.
- ¹⁴The protein expressed in inclusion bodies will remain insoluble until dissolved in 8 M urea.
- ¹⁵Small aliquots of 1 M NaOH are added to maintain the pH 8.0 during the addition.

5. Summary and future prospects

Structural studies of signaling proteins and signaling receptors of the TGF- β superfamily have shown that the TGF- β s, evolutionary latecomers to the superfamily, diverged from the BMPs and GDFs, the ancestral signaling proteins of the superfamily, to bind and assemble their type I and type II receptors in a distinct manner [14,16,17]. The receptors appear to have evolved these new modes of receptor binding in a stepwise manner, first by evolution of a new mode of type I receptor binding and later by evolution of a new mode of type II receptor binding. This idea is based on the observation that evolutionary intermediates of the superfamily, such as activins and nodal, bind and signal through type I receptors that are shared with the TGF- β s, evolutionary latecomers to the superfamily, and type II receptors that are shared with many of the distantly related BMPs and GDFs. There is at present only one structure of one of these intermediates bound to a receptor (activin A bound to its type II receptor, ActRIIb, or its close homolog, ActRII) [61,99], thus there is an urgent need to determine the structures of more of the evolutionary intermediates, such as activin, nodal, and myostatin, to their cognate type I and type II receptors. There are also several receptors yet to be structurally characterized, including the type I receptors Alk2, Alk4, and Alk7, and the type II receptor MISRII. The hope is that the principles and procedures described here for producing and studying the binding properties and structures of the signaling proteins and receptor ectodomains of the superfamily will lead to a better understanding of the different possible signaling complexes that the proteins of the superfamily form, and in turn, this will inform the underlying biology.

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Figure 1.

Different modes of receptor complex assembly by TGF- β s and BMPs. (A) TGF- β (left) and BMP (right) type I receptor, type II receptor ternary complex structures. TGF- β type I and type II receptors are shaded yellow and green, respectively, and extensively contact one another. BMP type I and type II receptors are shaded cyan and magenta respectively, and do not contact one another. (B) Representative dimeric ligand structures, with the two monomers of TGF- β depicted in blue and red, and those of BMP-2 in orange and brown. (C) Receptor extracellular domains of the TGF- β superfamily adopt the same three finger toxin fold, as shown by an overlay of the BMP and TGF- β type I receptors on the left (cyan and yellow, respectively), the BMP and TGF- β type II receptors in the middle (magenta and green, respectively), and the TGF- β type I and type II receptors on the right (yellow and green, respectively). F1, F2, and F3 designate the three fingers of the receptor three-finger toxin fold.

Figure 2.

Isolation of the human TGF- β 2 dimer. Cation-exchange (Source 15S, GE Healthcare) elution profile of the TGF- β 2 folding mixture. Peaks corresponding to monomeric and dimeric forms are labeled. Column was equilibrated in 20 mM sodium acetate, 30% isopropanol, pH 4.0 and eluted with a linear NaCl gradient in the same buffer (as shown by the grey line and accompanying axis on the right).

Figure 3.

Isolation of the human TβRII ectodomain. Anion-exchange (Source 15Q, GE Healthcare) elution profile of the TβRII folding mixture. Peaks corresponding to native monomer and non-native disulfide-linked oligomeric forms are labeled. Column was equilibrated in 20 mM MES, pH 6.0 and eluted with a linear NaCl gradient in the same buffer (as shown by the grey line and accompanying axis on the right).