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Structure-guided engineering of TGF-βs for the development of novel inhibitors and probing mechanism

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Abstract

The increasing availability of detailed structural information on many biological systems provides an avenue for manipulation of these structures, either for probing mechanism or for developing novel therapeutic agents for treating disease. This has been accompanied by the advent of several powerful new methods, such as the ability to incorporate non-natural amino acids or perform fragment screening, increasing the capacity to leverage this new structural information to aid in these pursuits. The abundance of structural information also provides new opportunities for protein engineering, which may become more and more relevant as treatment of diseases using gene therapy approaches become increasingly common. This is illustrated by example with the TGF-β family of proteins, for which there is ample structural information, yet no approved inhibitors for treating diseases, such as cancer and fibrosis that are promoted by excessive TGF-β signaling. The results presented demonstrate that through several relatively simple modifications, primarily involving the removal of an α-helix and replacement of it with a flexible loop, it is possible to alter TGF-βs from being potent signaling proteins into inhibitors of TGF-β signaling. The engineered TGF-βs have improved specificity relative to kinase inhibitors and a much smaller size compared to monoclonal antibodies, and thus may prove successful as either as an injected therapeutic or as a gene therapy-based therapeutic, where other classes of inhibitors have failed.

Keywords

TGF-beta; protein engineering; cell signaling; inhibitor

1. Introduction

There has been an exponential increase in the amount of detailed structural information about all classes of proteins, including membrane proteins, that has occurred over the past 20 years [1]. This increase has largely been driven by improvements in the capacities for structure determination using X-ray crystallography [2, 3], and while there were some indications this trend was beginning to ebb, it is likely only to be momentary, as recent advances in single particle cryo-electron microscopy [4] and cryo-electron tomography [5] are likely to push boundaries to enable the determination of larger and even more complex macromolecular structures at atomic resolution.

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The increasing availability of detailed structural information on many biological systems provides an avenue for manipulation of these structures, either for probing mechanism or for developing novel therapeutic agents for treating disease. Traditionally, to probe mechanism, prominent residues in the active site or regulatory regions are substituted and evaluated in functional assays. To develop novel therapeutic agents, compounds are identified by screening chemical libraries and in turn the activity of the hit compounds are optimized by establishing structure activity relationships (SAR) and by altering the physicochemical properties for drug-likeness. Though these still represent important avenues, innovative new approaches, such as incorporation of non-natural amino acids for probing mechanism [6, 7], or fragment screening for discovery of novel therapeutic agents [8], have emerged and are now becoming increasingly used as alternatives to conventional approaches.

The objective of this article is to present, by way of example with the TGF-β family of secreted signaling proteins, how protein engineering approaches can be used for probing mechanism or for developing novel therapeutic agents. The idea of protein engineering is by no means new, and indeed some of the most important therapeutic agents used today, such as recombinant insulin for treating diabetes [9] or vaccines protecting us from deadly viruses [10], are a product of protein engineering. There is nonetheless a renewed interest in protein engineering for development of novel therapeutic agents, based on the recent success in reprogramming patient-derived T-cells with engineered chimeric antigen receptors [11] for treatment of hematologic malignancies, such as relapsed acute lymphoblastic leukemia [12] and refractory large B-cell lymphoma [13]. This opens the door to an entirely new era in medicine, in which drugs are not manufactured and administered, but instead patient's own cells are genetically modified with coding sequences for engineered proteins that confer a therapeutic benefit [14]. To illustrate how protein engineering has been used with the TGF-β family of growth factors, which are studied in my lab, I will first present an overview of the TGF-β pathway and how TGF-βs bind their receptors to initiate a signaling response. I will then describe the engineered TGF-β monomer my lab has developed, which inhibits TGF-β signaling and has potential use as a therapeutic agent for treating cancer or fibrosis, or for probing mechanism.

2. TGF-β family signaling

Transforming growth factor beta isoforms, TGF-β1, −β2, and −β3, are 25 kDa secreted homodimeric signaling proteins. They are present only in vertebrates and perform functions essential for the long-term survival of humans and other higher vertebrates, including regulation of the adaptive immune system [15] and coordination of wound healing [16]. The TGF-βs belong to a highly diversified family of signaling proteins, with 33 members in humans, known as the TGF-β family [17]. The other members of the family include the bone morphogenetic proteins (BMPs) which regulate embryonic patterning, the closely related growth and differentiation factors (GDFs) which regulate cartilage and skeletal development, the activins (Acts) and inhibins (Inhs) which regulate the release of pituitary hormones, and others, such as Müllerian inhibiting substance (MIS) that regulate sex determination during embryonic development.

The TGF-βs, and other proteins of the family, are synthesized are pre-proproteins, and after maturation and secretion, are found as pro-complexes in which the mature growth factor homodimer is non-covalently bound by a homodimer of its pro-domain (Fig. 1A) [18-21]. The mature growth factor homodimers, which consist of two cystine-knotted monomers held together in most, but not all cases by a single inter-chain disulfide bond (Fig. 1B) [22-34], signal by binding and bringing together two serine-threonine kinase receptors, known as receptor types I and II (Fig. 1C) [35]. The assembly of receptor types I and II into heterotetrameric receptor complexes [36] leads to the activation of the type I receptor kinase, which in turn activates cytoplasmic effectors, known as receptor-regulated Smads or R-Smads [37] (Fig. 1C). R-Smads, together with the co-mediator Smad, Smad4, are dependent on other co-activators and co-repressors to effect transcriptional responses [38]. This dependence on co-activators and co-repressors, coupled with the variation of such factors from cell to cell, is thought to underlie the pronounced cell- and context-dependent activities characteristic of GFs of the family [39].

There are seven type I and five type II receptors in mammals [17]. Thus, there are many more GFs than receptors, and by necessity, there is significant growth factor-receptor promiscuity. The type I receptors of the family additionally couple to and activate only two classes of R-Smads: the more recently evolved members of the family, such as TGF-βs, activins, nodal and some of the GDFs and BMPs, such as GDF-9, −11, and −15 and BMP-15, bind and signal through type I receptors that activate R-Smads 2, 3, while the more distantly-related GDFs and BMPs, such as GDF-1, −3, −5, −7 and BMP-2, −3, −4, −5, −6, and −7, bind and signal through type I receptors that couple to and activate R-Smads 1, 5, and 8 [17]. The functional diversity that can be attained through intrinsic differences in signaling is therefore limited as there only two types of intracellular Smad signals induced by the 33 GFs of the family. The interactions of the GFs with the signaling receptors therefore represents only a portion of the ‘molecular recognition’ that results in the distinctive activities of the proteins of the family. The multitude of accessory binding proteins, which regulate access of the GFs to the signaling receptors and thereby determine which cells and groups of cells are targeted by TGF-β family GFs, instead provide much of the molecular recognition that results in the distinctive activities of GFs of the family.

3. TGF-βs bind and assemble their receptors in a distinct manner

TGF-β1, −β2, and −β3 are unique among the proteins of the family in that they are the only known GFs that bind and signal through the TGF-β type II receptor, TβRII[17, 40, 41]. Through purified component binding studies, it also known that TGF-β1 and TGF-β3, the two TGF-βs that have high intrinsic affinity for TβRII, bind and assemble TβRII, and their cognate type I receptor TβRI, in an ordered manner, first by binding TβRII and then by recruiting TβRI [42, 43]. The BMPs and GDFs, which exhibit promiscuous binding to both type I and type II receptors, have in contrast been shown through purified component binding studies to bind their type I and type II receptors in manner that is largely independent of one another [25, 44].

These findings, together with differences in the promiscuity of receptor binding, hinted that the TGF-βs/activins and BMPs/GDFs might differ in the manner by which they bind and assemble their receptors into signaling complexes.

These differences in receptor binding have been borne out by the structures of the TGF-β and BMP type I type II receptor ternary complexes determined over the past several years. The structures of the ternary complexes show that although the signaling proteins and receptors of the BMP and TGF-β subfamilies share the same overall fold (Fig. 2 and Fig. 3, respectively), they nevertheless bind their receptors in a distinct manner (Fig. 4A-B) [25, 44-50]. The BMP type I and type II receptors bind to the underside of "fingers" and "knuckle", respectively, and do not contact one another (Fig. 4b), while the TGF-β type I and type II receptors bind to the underside of the "fingers" and to the "fingertips", respectively, and have extensive contact (Fig. 4A). The direct contact between the type I and type II receptors in the TGF-β complex, but not the BMP, has been shown to be responsible for the pronounced stepwise manner by which TGF-βs bind and assemble their type I and type II receptors into a signaling complex [46, 51], thus distinguishing them both structurally and functionally from all other proteins of the family.

4. TGF-βs in human disease and the need for novel inhibitors

TGF-β1, −β2-, and −β3 play essential roles in tumor suppression [52], maintain the balance between the tolergenic and immunogenic arms of the adaptive immune system [15], maintain the extracellular matrix [53], and orchestrate wound healing [16]. The importance of the TGF-βs in these processes is demonstrated through inherited human diseases, such as hereditary nonpolyposis colorectal cancer (HNPCC) which is caused by mutations in TβRI [54] and the connective tissue disorders, Marfan's and Loey's-Dietz syndromes, which are caused by mutations in the proTGF-β binding matrix protein fibrillin-1, or TβRI, that lead to too much or too little TGF-β signaling, respectively [55, 56]. TGF-β signaling also plays a significant role in soft tissue cancers and fibrotic disorders [52, 53, 57], where the TGF-β pathway remains intact, but excessive TGF-β signaling drives disease progression. Though a role for excessive TGF-β signaling in promoting cancer progression may seem at odds with its tumor suppressive growth inhibitory activity, many cancer cells dysregulate their cell cycle, which antagonizes TGF-β's ability to inhibit cell growth [58]. The many tumor promoting activities of the TGF-β's, including their ability to potently suppress the immune system, induce EMT, promote cell migration and invasion, and increase the population of cancer stem cells, nonetheless remain intact [59]. This, coupled with overexpression of TGF-βs at levels 10- to 100-fold higher than in non-transformed cells, is thought to underlie TGF-β's role in the growth and invasiveness of many cancers, including those of the breast, brain, bone, prostate, pancreas, colon, lung, and bladder [60-66]. The fibrotic disorders caused by dysregulated TGF-β signaling, which include idiopathic pulmonary fibrosis (IPF), renal fibrosis, cardiac fibrosis, and coronary restenosis, are a result of hyperactive TGF-β signaling following tissue injury or disease progression that leads to the accumulation of extracellular matrix proteins [53].

The disease promoting activities of the TGF-βs have prompted vigorous TGF-β inhibitor development and testing in clinical trials [67, 68]. TGF-β inhibitors include small molecule

TGF-β receptor kinase inhibitors, antisense oligonucleotides, and synthetic peptides, or protein-based biologics, such as TGF-β or TGF-β receptor neutralizing antibodies, TGF-β receptor traps, or antibodies that interfere with TGF-β maturation. Though pre-clinical and clinical studies have shown that TGF-β inhibitors offer significant promise for the treatment of cancer and fibrosis [59, 69-81], to date none have been approved for use in humans [67]. Small molecule TGF-β receptor kinase inhibitors have progressed slowly in clinical settings, primarily because they lack specificity for TβRI [82], resulting in a narrow therapeutic window [83]. Biologics offer promise as alternatives to SMRKIs due to their inherent high specificity, but they also have the limitation that they tend to occupy the vascular space and penetrate poorly into dense tissues such as tumors [84, 85].

5. TGF-βs high specificity for TβRII provides an avenue for inhibitor design

To address the need for novel TGF-β inhibitors, it occurred to us that it might be possible to specifically inhibit TGF-β signaling by developing a modified form of TGF-β1 or −β3 that retained its high inherent affinity for TβRII, but was fully disrupted in terms of its ability to bind and recruit TβRI. This approach, if successful, would have several advantages over existing inhibitors, for example it would be expected to have high target selectivity, like antibodies and unlike small molecule TGF-β receptor kinase inhibitors, but at the same time to have a greater propensity to penetrate dense tissues, like small molecule TGF-β receptor kinase inhibitors, but unlike antibodies.

Though one might consider designing such an inhibitor by introducing residue substitutions that selectively disrupt TβRI binding, this approach was considered unlikely to be viable since our previous experience showed us that the folding of the TGF-βs to form the intricate cystine knot within each monomer and inter-chain disulfide was very sensitive to residue substitutions [86], with single substitutions sometimes leading to the formation of little to no native disulfide-liked dimer. The other reason is that TGF-β homodimers are notoriously insoluble, thus posing a significant challenge for delivery, even if variants unable to bind TβRI could be generated.

To overcome these challenges, we considered covalently monomeric TGF-β1 or TGF-β3, formed by substituting the cysteine residue that normally forms the inter-chain disulfide bond, Cys77, with serine. TGF-β1 C77S and TGF-β3 C77S had been previously reported [51, 87] and importantly these monomers had at least one of the essential characteristics for the development of a dominant negative inhibitor, specifically the retention of high affinity TβRII binding. This characteristic was predicted from the structure of the TGF-β receptor complex, which showed that TβRII binds to residues from a single TGF-β monomer [41, 46, 49]. Though possessing this one very desirable characteristic, they nonetheless possessed one very undesirable characteristic, namely sinaling activity that was only slightly (ca. 10-fold) diminished relative to the corresponding disulfide-linked homodimer [51, 87]. This was not predicted from the structure of the TGF-β receptor complex since TβRI was shown to bind to a composite interface formed by both monomers of TGF-β, as well as TβRII.

To reconcile this, we hypothesized that that the signaling capacity of TGF-β1 C77S or TGF-β3 C77S arise from their ability to non-covalently dimerize and in turn assemble the same heterotetrameric receptor complex as native disulfide-linked TGF-β homodimers [51]. To prevent the formation of such complexes, we reasoned this might be possible by replacing the heel helix, which connect β-strands 4 and 5 and forms a substantial component of both the hydrophobic dimer interface and TβRI binding site, with a flexible loop. The elimination of α-helix 3 should interfere with self-association of the monomers, and binding and recruitment of TβRI, but should not impair TαRII binding. Though uncertain, it was hypothesized that removal of the heel helix, which includes a large number of hydrophobic residues that interdigitate and pack against hydrophobic residues in the palm of the opposing monomer and replacement of this with a flexible loop bearing several charged residues, might decrease the hydrophobic character enough so that solubility of the engineered monomer is improved relative to that of either TGF-β homodimers or full TGF-β monomers (TGF-β1 or −β3 C77S), which are also poorly soluble.

6. TGF-β engineered monomer

The simple design described above, in which Cys77 was substituted with serine and the heel helix of the TGF-βs was removed and replaced with a short flexible loop to form a so-called TGF-β mini monomer (Fig. 5A), was investigated and remarkably had all the desired design characteristics, including retention of the same growth factor fold and manner of TβRII binding relative to that of TGF-β1 and −β3 dimers (Fig. 5C-D), the same high affinity TβRII binding as TGF-β1 and TGF-β3 dimers (Fig. 6A-B), an inability to bind TβRI (Fig. 6C) and signal (Fig. 6D), and inhibitory activity against TGF-β1, −β2, and −β3, with potencies (IC_{50s}) in the range of 20 – 60 nM (Fig. 6E) [88] Though only a minor component of the design, an additional property was greatly improved solubility relative to TGF-β2 dimers (Fig. 5B) [88].

The combination of properties that make the TGF-β mini monomer a favorable inhibitor may seem like a stroke of luck, but in fact several of these, including the ability to fold and bind TβRII with high affinity, were anticipated based on previous studies. The ability to fold was anticipated based on the previous structure of an “open” TGF-β3 homodimer previously determined in our laboratory [41], in which the core of monomer, including the TβRII binding fingertips, retained its structure in the absence of any contacts with the other monomer. To increase the likelihood of isolating natively folded protein, the TGF-β mini monomer was based on the backbone of TGF-β2 [88], which from our previous studies was known to fold the most efficiently of all three TGF-βs [86], but at the same time to bind TβRII with low affinity [42]. To engender the TGF-β2 mini monomer with high affinity TβRII binding, Lys25 and Lys94, along with five other residues, were substituted with the corresponding residues from TGF-β1 and TGF-β3, which we knew from our earlier studies could confer TGF-β2 with ability to bind TβRII with high affinity [40, 42].

The properties of design of which we were less certain were the inability to bind and recruit TβRI and the improved solubility. These properties were anticipated based on the structure, but we had no *a priori* information to indicate this would be successful or not. To investigate TβRI binding, we developed a highly sensitive time-resolved fluorescence energy resonance

energy transfer (TR-FRET) assay to monitor assembly of TβRI and TβRII into a complex by TGF-βs and showed that the TGF-β3 homodimer, but as well the full TGF-β3 monomer, TGF-β3 C77S, led to a TR-FRET signal 38 - 42-fold above background (Fig. 6C) [88]. The engineered monomer, which we designated as the TGF-β2 mini monomer with 7 substitutions, or mmTGF-β2-7M, in contrast, led to a TR-FRET signal indistinguishable from background (Fig. 6B) [88]. The fact that TGF-β3 C77S could assemble a complex under these assay conditions, but mmTGF-β2-7M could not, indicated that mmTGF-β2-7M was likely devoid of any capacity to bind and recruit TβRI. To investigate solubility, we diluted TGF-β dimers, as well as the full TGF-β monomer and mmTGF-β2-7M from acidic stocks where they were highly soluble into phosphate buffer at neutral pH and quantitated how much protein precipitated versus remained in solution (Fig. 5B) [88] Though an improvement in solubility was expected, based on the removal of the heel helix, which includes a large number of hydrophobic residues, the degree of this improvement was nonetheless striking. The reason for this has is not known, but may be that elimination of the heel helix reduced the longest possible stretch of hydrophobic amino acids to below a critical value, a property well known to be important increasing protein solubility [89].

Though we describe this protein as an engineered monomer, it is in fact only covalently monomeric, not monomeric in solution. This was shown by sedimentation velocity analytical ultracentrifugation experiments in which the sedimentation profiles could only be fit assuming mmTGF-β2-7M was undergoing a monomer/dimer equilibrium in solution [88]. The fact that mmTGF-β2-7M has some propensity to non-covalently dimerize is not surprising given that some remnants (i.e. hydrophobic residues) of the dimer interface remain. The fact that mmTGF-β2-7M has some propensity to dimerize, does however, show that perhaps the most important element of its design is elimination of a large portion of the TβRI binding site – in other words, the design of mmTGF-β2-7M does not require that the engineered protein behave as a perfect monomer in solution, but instead that enough of its TβRI binding site be removed so that if the molecule does dimerize, it is still unable to bind and recruit TβRI.

7. Perspective

The results presented here have demonstrated how a simple a modification, including substitution of a single cysteine residue with serine and replacement of a helix with a flexible loop, can change a potent signaling protein into a potent specific inhibitor of the same signaling protein. Though further studies are required, this relatively small and highly specific TGF-β inhibitor has several possible uses, such as an injected therapeutic, possibility conjugated with the F_c domain of an antibody or with albumin to diminish renal filtration, or as a secreted protein, either alone or conjugated to an F_c domain or albumin, for gene therapy applications. Though not demonstrated or described, this engineered form of TGF-β also has several potential uses for further mechanistic studies β for example, this form of TGF-β could be used to probe and possibly localize the binding site for other proteins known to bind and alter the functions of TGF-βs, such as the non-signaling type III receptor [90, 91], or soluble TGF-β binding proteins, such as decorin or biglycan [92, 93].

Though the changes required to completely alter the function of this protein are simple, it in fact required significant knowledge of the structure, and accompanying binding properties of the receptors, to enable the design. This type of detailed structural and mechanistic knowledge is being increasingly employed to develop other types of engineered growth factors, such as vascular endothelial growth factor [94-97], fibroblast growth factor [98-100], and Wnts [101, 102], with altered functions for use as therapeutic agents. Through advances in capabilities for structure determination by NMR, X-ray, and EM continue, it is expected that the type of detailed structural and biophysical knowledge that enabled the design of the engineered TGF-β monomer, as well as other growth factors, will become more commonplace, thus expanding a potentially important avenue for the altering the function of many different classes of growth factors for therapeutic gain.

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Abbreviations:

TGF-β	transforming growth factor-beta
GDF	growth and differentiation factor
BMP	bone morphogenetic protein
SPR	surface plasmon resonance
EM	electron microscopy
NMR	nuclear magnetic resonance
EMT	epithelial-to-mesenchymal transition
TβRI	TGF-β type I receptor
TβRII	TGF-β type II receptor
SA	streptavidin

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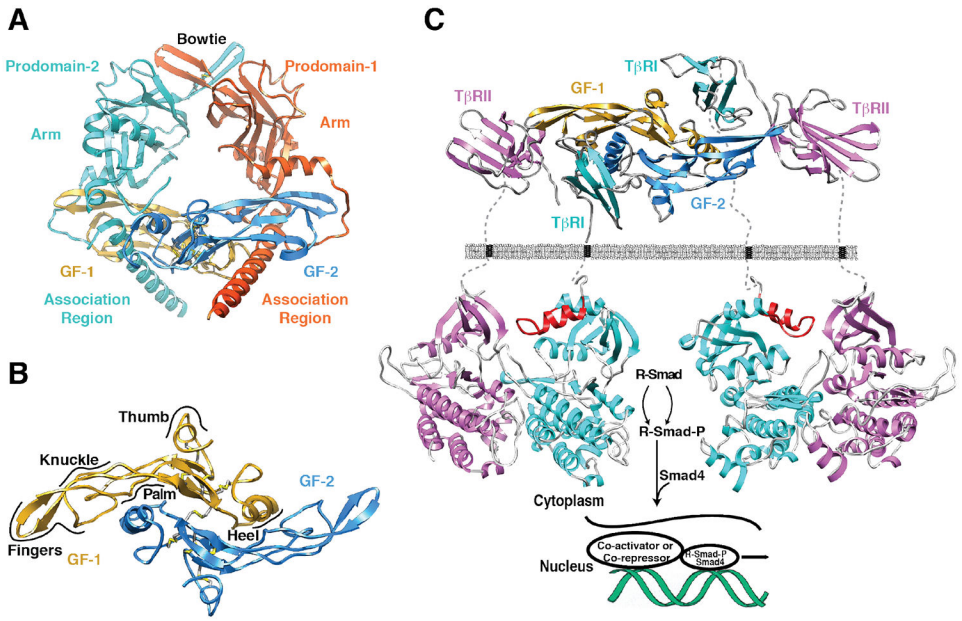

Figure 1.

Structure of a representative TGF-β family member, its type I and type II receptors, and a schematic of it signaling mechanism. A. Structure of proTGF-β1 (PDB 3RJR) [20], in which the disulfide-linked growth factor homodimer, is surrounded and non-covalently bound by a homodimer of its prodomain. Growth factor (GF) monomers (GF-1 and GF-2) are depicted in dark blue and gold, while the prodomain monomers are depicted in cyan and orange. B. Structure of the disulfide-linked TGF-β2 homodimer (PDB 2TGI) [24]. GF-1 and GF-2 are shaded as in panel A and the four intramolecular disulfides, and one inter-molecular disulfide, are depicted in yellow. GF monomers are described as a curled left hand, in which the heel of one hand packs into the palm of the other. Other features of the of GF fold, including the fingers, the knuckles, and the thumb, are labeled. C. Structure of the heterotetrameric signaling complex formed by a TGF-β3 homodimer, and two molecules each of its cognate type I and type II receptor, TβRI and TβRII. Structure of the TGF-β3 homodimer bound to the TβRI and TβRII ectodomains was determined experimentally. Structure of the TβRI kinase domain was determined experimentally (PDB 1IAS), while that of TβRII or the TβRI:TβRII kinase domain complex has not been reported (structure shown for the TβRII kinase domain is that of ActRIIB, PDB 2QLU). GF-1 and GF-2 are shaded as in panel A and TβRI and TβRII are depicted in cyan and magenta, respectively. GS domain of TβRI, which regulates its activity, is depicted in red.

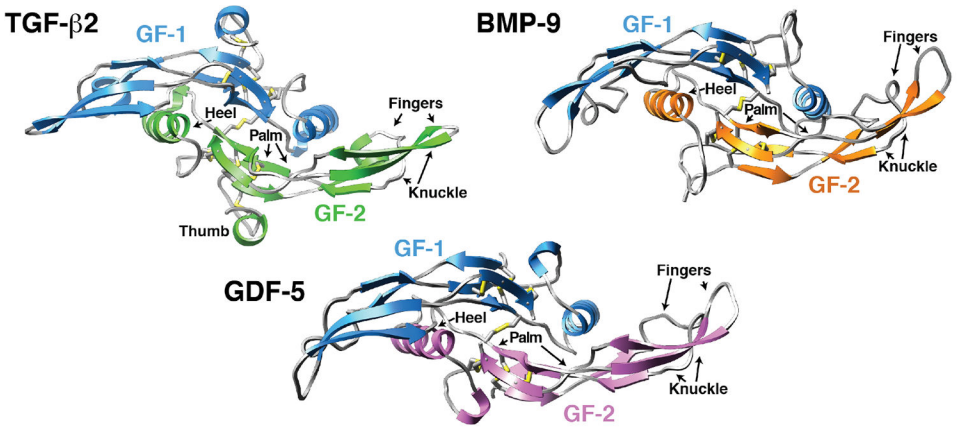

Figure 2.

Structures of three representative TGF-β family GFs. Structures are disulfide-bonded homodimers, with a single inter-chain disulfide connecting each of the monomers. Structures are color coded, with one of the monomers in blue and the other monomer in another shade. Sulfur atoms of the intramolecular and intermolecular disulfides are depicted in yellow. Structures shown correspond to the following PDB entries: TGF-β2 – 2TGI [24], BMP-9 – IZKZ [23], and GDF-5 – 1WAQ [31].

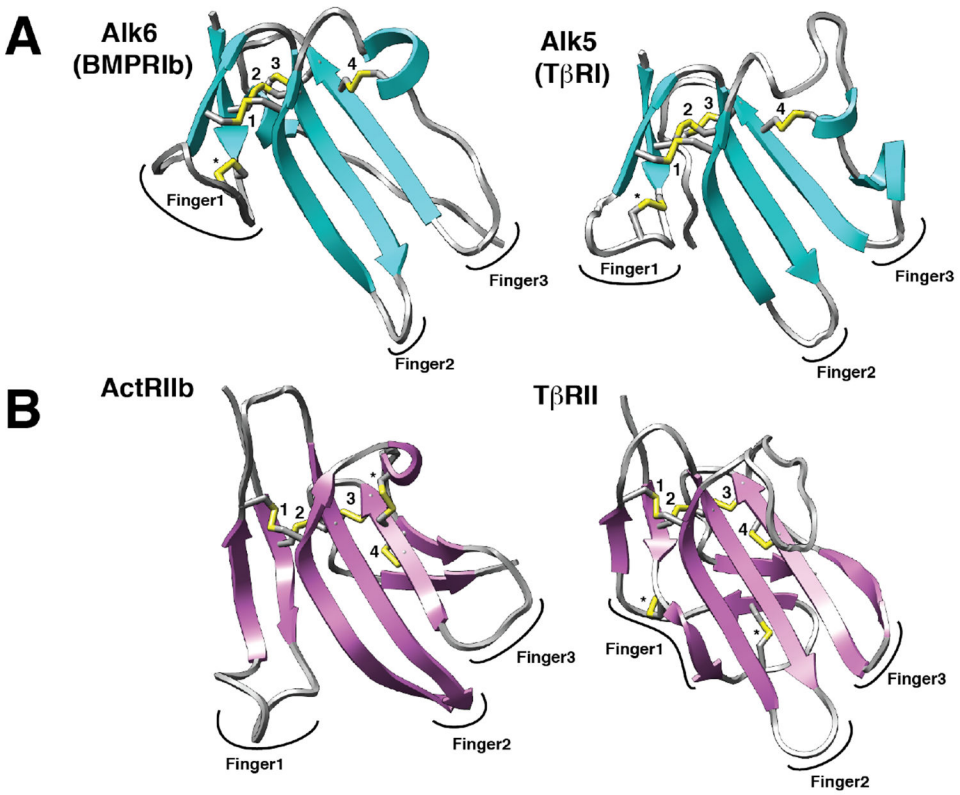

Figure 3.

Structures of representative type I and type II receptors of the TGF-β family. A, B. Structures of type I and type II receptors (A and B, respectively) shown as ribbon diagrams. Disulfide bonds are depicted as sticks with yellow sulfur atoms. Four disulfide bonds that are positionally conserved in all type I and type II receptors of the TGF-β family are labeled 1 – 4. Disulfide bonds that are unique are labeled with an asterisk (*). The three fingers of the type I and type II receptors that define them as having a three-finger toxin fold are indicated. Structures shown correspond to the following PDB entries: Alk6 – 3EVS [103], Alk5 – 2L5S [104], ActRIIb – 1NYU [105], and TβRII – 1M9Z [106].

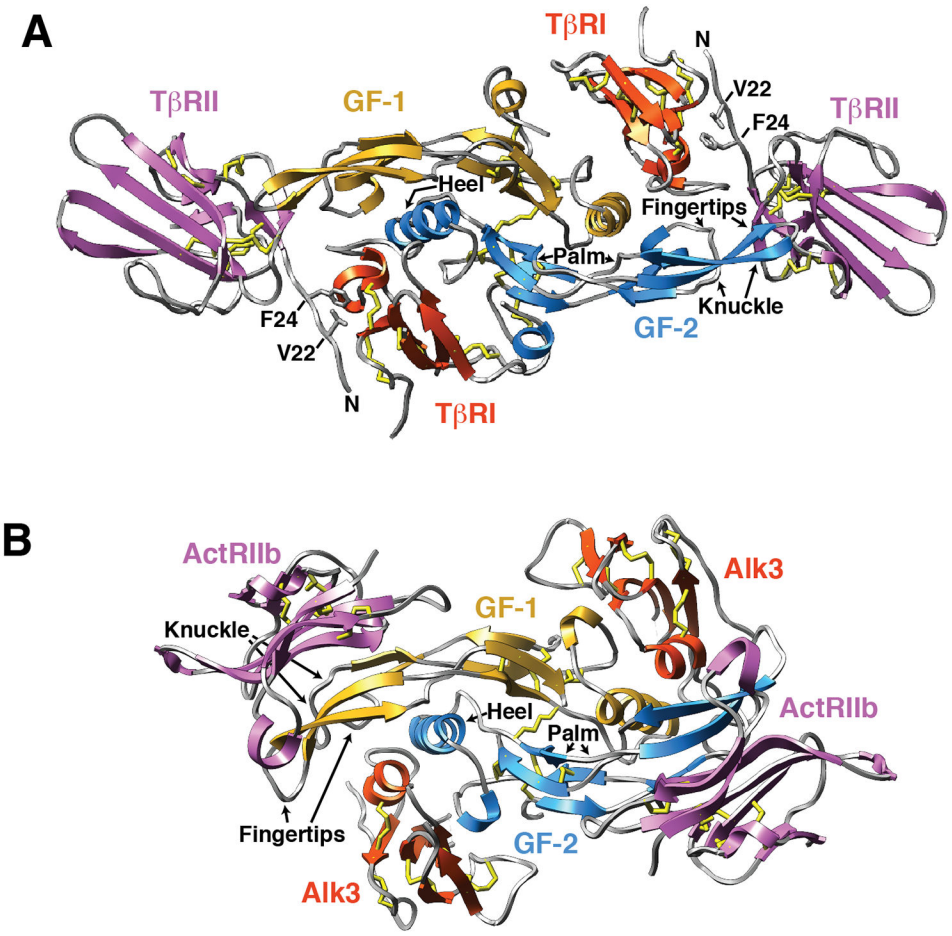

Figure 4.

Structures of two representative GF:type I receptor:type II receptor complexes. A. Structure of the TGF-β receptor complex shown as a ribbon diagram with the two growth factor monomers shown in blue and gold, the two bound TβRII ectodomains in magenta, and the two bound TβRI ectodomains in orange. Disulfides are shown as sticks, with the sulfur atoms depicted in yellow. Structure shown is from PDB 2PJY [46]. Structure of the BMP receptor complex shown as a ribbon diagram with the same shading scheme as that used in panel A. Structure shown is from PDB 2H62 [44].

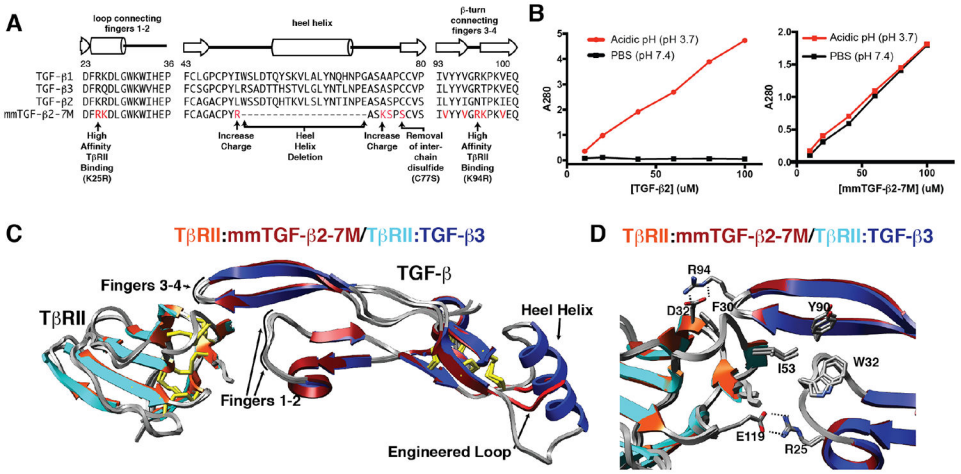

Figure 5.

Engineered TGF-β monomer and characterization of its structure and solubility. A. Design of the engineered TGF-β monomer, known as mmTGF-β2-7M, which is based on the backbone of TGF-β2. mmTGF-β2-7M, in addition to lacking the heel helix and bearing a substitution of Cys77 with Ser, also has two substitutions to increase the charge in the loop that serves to replace the heel helix and seven substitutions in the loops connecting fingers 1-2 and 3-4 that contact TβRII. B. Solubility of TGF-β2 dimers (left panel) and mmTGF-β2-7M (right panel). Solubility was assessed by measuring the absorbance at 280 nm of the supernatant after TGF-β2 or mmTGF-β2-7M are diluted from an acidic stock where they are highly soluble into either acidic solution (pH 3.7) or phosphate buffered saline (PBS) at neutral pH (pH 7.4) and centrifuged. C. Overlay of the 1.8 Å crystal structure of mmTGF-β2-7M:TβRII complex (dark red and orange ribbons, respectively) with one of the TGF-β3 monomers and its bound TβRII from the 3.0 Å crystal structure of the TGF-β3:TβRII:TβRI complex (PDB 2PJY, TGF-β3 monomer and TβRII shown in dark blue and cyan ribbon, respectively; TβRI not shown for clarity). Newly created loop in mmTGF-β2 (red) which takes the place of the heel (α3) helix in TGF-β2 is depicted in red. D. Overlay as in panel *C*, but expanded to show the near identity of critical hydrophobic and hydrogen-bonding/ electrostatic interactions shown previously to be essential for high affinity TGF-β3:TβRII binding [40, 42]. Figure is adapted and reproduced with permission from Kim, et. al, *J. Biol. Chem.*, 292, 7173-7188 (2017).

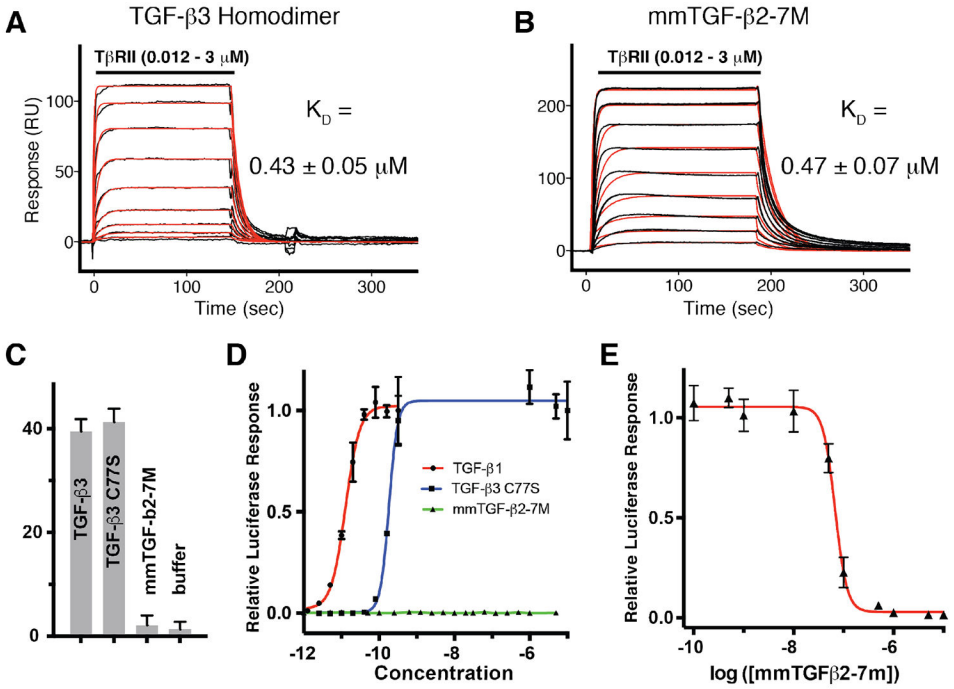

Figure 6.

Receptor binding properties and cell-based inhibitory activity of the engineered monomer. A-B. SPR sensorgrams for injection of a two-fold dilution series from 3 – 0.012 μM of TβRII ectodomain monomer over immobilized avi-TGF-β3 or avi-mmTGF-β2-7M. Sensorgrams were fitted to a 1:1 binding model – raw data is shown in black and the fitted curve is shown in red. C. Time resolved FRET assay for ligand-mediated assembly of TβRI:TβRII complexes. Preassembled TGF-β3:TβRII-His (1:2), TGF-β3 C77S:TβRII-His (1:1), and mmTGF-β2-7M:TβRII-His (1:1) complexes at a concentration of 250 nM were incubated with 50 nM biotinylated TβRI-Avi and 2 nM Tb-anti-His and 30nM SA-665 for 2 hours at room temperature as previously described. Buffer control (orange bars) contained only 2 nM Tb-anti-His and 30nM SA-665. D. TGF-β luciferase reporter activity for TGF-β1, TGF-β3 C77S, and mmTGF-β2-7M. The solid lines, colored red and blue, correspond to the fitted curves to derive the EC_{50} (green line for mmTGF-β2-7M was not fit due to the lack of signaling activity for this variant). E. TGF-β luciferase reporter activity for cells treated with a sub-saturating concentration of TGF-β1 (8 pM) with increasing concentration of mmTGF-β2-7M added. The solid red line corresponds to the fitted curve for mTGF-β2-7M to derive the IC_{50}. Figure is adapted and reproduced with permission from Kim, et. al, *J. Biol. Chem.*, 292, 7173-7188 (2017).