

TGF- β signalling is mediated by two autonomously functioning T β RI:T β RII pairs

Tao Huang¹, Laurent David², Valentín Mendoza³, Yong Yang⁴, Maria Villarreal¹, Keya De⁵, LuZhe Sun⁵, Xiaohong Fang⁴, Fernando López-Casillas³, Jeffrey L Wrana^{2,6} and Andrew P Hinck^{1,*}

¹Department of Biochemistry, University of Texas Health Science Center at San Antonio, San Antonio, TX, USA, ²Center for Systems Biology, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Ontario, Canada, ³Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, Ciudad de México, México, ⁴Beijing National Laboratory for Molecular Sciences, Institute of Chemistry, Key Laboratory of Molecular Nanostructures and Nanotechnology, Chinese Academy of Sciences, Beijing, China, ⁵Department of Cellular and Structural Biology, University of Texas Health Science Center at San Antonio, San Antonio, TX, USA and ⁶Department of Molecular Genetics, University of Toronto, Ontario, Canada

Transforming growth factor (TGF)- β s are dimeric polypeptides that have vital roles in regulating cell growth and differentiation. They signal by assembling a receptor heterotetramer composed of two T β RI:T β RII heterodimers. To investigate whether the two heterodimers bind and signal autonomously, one of the TGF- β protomers was substituted to block receptor binding. The substituted dimer, TGF- β 3 WD, bound the T β RII extracellular domain and recruited the T β RI with affinities indistinguishable from TGF- β 3, but with one-half the stoichiometry. TGF- β 3 WD was further shown to retain one-quarter to one-half the signalling activity of TGF- β 3 in three established assays for TGF- β function. Single-molecule fluorescence imaging with GFP-tagged receptors demonstrated a measurable increase in the proportion of T β RI and T β RII dimers upon treatment with TGF- β 3, but not with TGF- β 3 WD. These results provide evidence that the two T β RI:T β RII heterodimers bind and signal in an autonomous manner. They further underscore how the TGF- β s diverged from the bone morphogenetic proteins, the ancestral ligands of the TGF- β superfamily that signal through a RI:RII heterotrimer.

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Introduction

Transforming growth factor- β (TGF- β) isoforms are secreted signal ligands that have vital roles in coordinating wound healing, modulating immune cell function, maintaining the extracellular matrix, and regulating epithelial and endothelial cell growth and differentiation (Massagué, 1998). The importance of the TGF- β s is underscored by their conservation among vertebrates and their demonstrated roles in a variety of human diseases, including tissue fibrosis (Blobe *et al*, 2000) and cancer (Derynck *et al*, 2001). TGF- β s are members of an extended signalling superfamily that arose in early metazoans (Kingsley, 1994). The superfamily has greatly diversified, with >30 known members in vertebrates, including the prototypical TGF- β s, the bone morphogenetic proteins (BMPs), the closely related growth and differentiation factors (GDFs), and the activins and inhibins (Massagué, 1998).

TGF- β s are disulphide-linked dimers of identical 112-residue protomers. The protomers include four disulphide bonds, three of which form a conserved structure known as a cystine knot (Sun and Davies, 1995). BMPs, GDFs, activins, and most other ligands of the TGF- β superfamily share a similar structure, though the cysteine that forms the inter-chain disulphide bond is lacking in three family members, GDF-3, GDF-9, and BMP-15 (McPherron and Lee, 1993; Dube *et al*, 1998). The ligands of the superfamily signal by binding and bringing together two single pass transmembrane receptor kinases, known as receptor types I and II (Derynck, 1994). This initiates a transphosphorylation cascade where the type II kinase phosphorylates and activates the type I (Wrana *et al*, 1994). The type I kinase phosphorylates Smad proteins (Shi and Massagué, 2003) and other effectors (Zhang, 2009), which regulate the transcription of target genes (Massagué and Wotton, 2000).

TGF- β s have been shown to assemble a receptor heterotetramer on the cell surface comprising two molecules of its type I receptor, T β RI, and two molecules of its type II receptor, T β RII, based on differential receptor tagging (Moustakas *et al*, 1993; Henis *et al*, 1994; Wells *et al*, 1999), two-dimensional gel electrophoresis (Yamashita *et al*, 1994), and genetic complementation (Weis-Garcia and Massagué, 1996). T β RI and T β RII have been further shown to form stable homodimers in the absence of TGF- β (Chen and Derynck, 1994; Gilboa *et al*, 1998; Rechtmann *et al*, 2009), suggesting a two-step mechanism for assembly of a receptor heterotetramer.

The recently reported structures of TGF- β 1 and - β 3 bound to the T β RI and T β RII extracellular domains support the binding stoichiometry deduced on the basis of the cell-based experiments, with two molecules of each receptor symmetrically bound, T β RII at the ‘fingertips’, and T β RI directly adjacent on the underside of the ‘fingers’ (Groppe *et al*, 2008; Radaev *et al*, 2010) (Figure 1A). T β RI and T β RII directly contact one another in the complex and these receptor-receptor contacts are responsible for the pronounced

*Corresponding author. Department of Biochemistry, MC 7760, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Dr, San Antonio, TX 78229-3900, USA. Tel.: +1 210 567 8780; Fax: +1 210 567 8778; E-mail: hinck@uthscsa.edu

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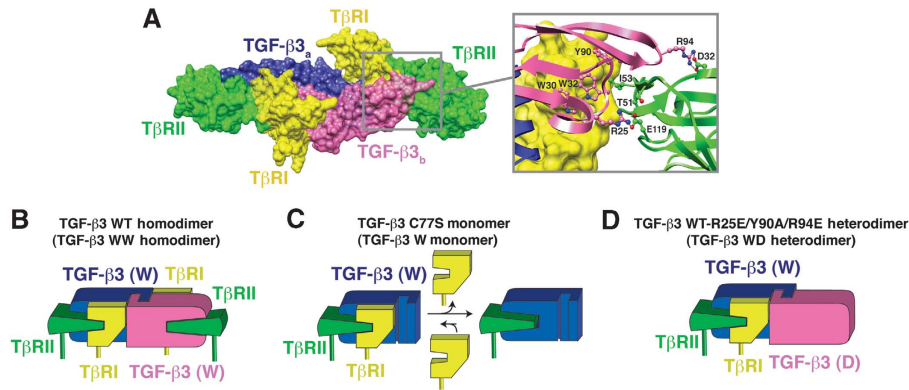


Figure 1 Structure of the TGF- β receptor complex and effect of ligand monomerization. (A) Surface representation of the TGF- β 3 homodimer bound to the T β RI and T β RII extracellular domains (PDB 2PJY). The two protomers of TGF- β 3 are depicted in pink and dark blue. T β RII binds to the fingertips of each protomer and is depicted in green, whereas T β RI binds to the underside of the fingers and is depicted in yellow. The residues in the interface between TGF- β 3 and T β RII are shown in the expanded view shown on the right. (B) Schematic representation of the TGF- β 3 receptor complex where high affinity T β RI binding is dependent upon interactions with both TGF- β 3 protomers and T β RII. (C) Schematic representation of the TGF- β 3 monomer, which binds T β RII with the same affinity as wild-type TGF- β 3, but which is impaired in its ability to bind and recruit T β RI. (D) Schematic representation of a TGF- β 3 heterodimer comprising a wild-type protomer (blue) and a variant (pink) bearing substitutions that block T β RI and T β RII binding.

stepwise manner with which TGF- β s bind T β RII and recruit T β RI (Groppe *et al*, 2008) first determined based on genetic complementation studies with receptor-deficient mink lung epithelial cells (Laiho *et al*, 1991). The additional constraint imposed by the receptor-receptor contact is thought to be further important for enhancing the specificity with which T β RI and T β RII bind TGF- β s and preventing activation of TGF- β responses by other ligands of the superfamily (Groppe *et al*, 2008; Massagué, 2008).

The binding of TGF- β by two well-separated T β RI:T β RII heterodimeric pairs suggests that the two heterodimers might bind and signal independently of one another. This is further suggested by the finding that low but measurable signalling was induced when T β RI and T β RII were artificially dimerized with small immunophilin domains (Stockwell and Schreiber, 1998) or when TGF- β responsive cells are treated with monomeric TGF- β 1 or - β 3 (Amatayakul-Chantler *et al*, 1994; Zúñiga *et al*, 2005). Monomeric TGF- β 3, even though impaired 10–15-fold in its affinity for binding and recruiting T β RI (Groppe *et al*, 2008), retains significant reporter gene activity with a reduction in potency of just 10-fold relative to wild-type homodimer (Zúñiga *et al*, 2005). Other studies, such as one in which the T β RI and T β RII kinases were fused to the extracellular domain of the erythropoietin receptor (Luo and Lodish, 1996) or another in which the T β RI kinase domain was fused to the T β RII extracellular domain (Okadome *et al*, 1994), do not however support independent signalling. Monomeric TGF- β 3 has been further shown to possess an intrinsic propensity to non-covalently dimerize, especially in the presence of T β RI and T β RII (Zúñiga *et al*, 2005), suggesting that the retention of activity by the monomers might reflect their propensity to non-covalently dimerize and assemble T β RI:T β RII heterotetramers, not assemble and signal through T β RI:T β RII heterodimers.

The objective of this study was to thoroughly investigate if TGF- β s signal through two independently functioning T β RI:T β RII heterodimers. This was accomplished by isolating a disulphide-linked TGF- β 3 dimer composed of a wild-type protomer and a variant bearing substitutions of Arg25, Tyr90,

Arg94, residues previously shown (De Crescenzo *et al*, 2006; Baardsnes *et al*, 2009) or implicated (Groppe *et al*, 2008) to be critical for binding of T β RI and T β RII. Using a series of complementary biochemical techniques, the substituted TGF- β 3 dimer was shown to bind the T β RII extracellular domain and recruit the T β RI with affinities indistinguishable from the wild-type homodimer, but with one-half the stoichiometry. Using three established assays for TGF- β function, the substituted dimer was further shown to retain one-quarter to one-half the signalling activity of the wild-type homodimer. Collectively, these results show that the two T β RI:T β RII heterodimers bind and signal nearly independently of one another.

Results

Design and isolation of TGF- β 3 WD

The objective was to generate a form of TGF- β that bound T β RII and recruited T β RI with affinities comparable to TGF- β 1 or - β 3, but with one-half the stoichiometry. This necessitated that a dimeric form of TGF- β 1 or - β 3 be used as T β RI binds across the dimer interface and requires both protomers, as well as T β RII, to bind with high affinity (Zúñiga *et al*, 2005; Groppe *et al*, 2008; Radaev *et al*, 2010) (Figure 1B and C). This was accomplished by generating a heterodimer with one wild-type protomer and one protomer in which Arg25 and Arg94 were substituted with glutamate and Tyr90 was substituted with alanine (Figure 1D). The importance of Arg25 and Arg94 for high affinity T β RII binding was first suggested based on the fact that these, along with Val92, are the only residues in the interface with T β RII that are substituted in TGF- β 2 (Hart *et al*, 2002), the isoform that binds T β RII weakly (Cheifetz *et al*, 1990). This was later confirmed by TGF- β 3- β 2 and TGF- β 2- β 3 chimeras in which swaps of these residues between isoforms, Arg25 and Arg94 in TGF- β 3 and Lys25 and Lys94 in TGF- β 2, decreased or increased affinity several hundred fold to that of the other isoform (De Crescenzo *et al*, 2006; Baardsnes *et al*, 2009). The hydrogen-bonding arrangement of these arginines with the sidechain carboxylates of Asp32 and Glu119 on T β RII

(Figure 1A, right), together with the dramatic decrease in affinity when conservatively replaced, led to the idea that substitution with glutamate would abolish T β RII binding altogether.

The blockade of T β RII binding would be expected to greatly impair the binding of T β RI due to the loss of receptor-receptor contacts essential for binding and recruiting T β RI (Groppe *et al*, 2008). This alone would probably be sufficient based on the weak apparent affinity of the T β RI extracellular domain for TGF- β 1, - β 2, and - β 3 ($K_d \sim 30\text{--}100 \mu\text{M}$) (Groppe *et al*, 2008; Radaev *et al*, 2010), though to further diminish binding, Tyr90 was substituted. This residue is centrally located within the T β RI interface and was replaced with a less bulky alanine sidechain, with the goal to reduce T β RI binding based on its substantial contact with T β RI (Groppe *et al*, 2008; Radaev *et al*, 2010).

The heterodimer was prepared by first producing wild type and R25E, Y90A, R94E triply substituted human TGF- β 3 monomers (designated, WT or W and dead or D, respectively) in bacteria. These were reconstituted from inclusion bodies, purified to near homogeneity in 8 M urea, and then diluted, in a 1:1 molar ratio, into refolding buffer (Cerletti, 2000). The folding mixture, which contained the desired heterodimer, TGF- β 3 WD, as well as wild-type and substituted homodimers, TGF- β 3 WW and TGF- β 3 DD, respectively, was then fractionated using high-resolution cation-exchange chromatography at pH 4.0 (Figure 2A).

This separation yielded five major species, and as anticipated, three of these, b, d, and e, corresponded to reductant-sensitive 25 kDa dimers (Figure 2B). The other two, a and c, corresponded to 12.5 kDa monomers (Figure 2B). The three dimers, as well as the two monomers were predicted to be positively charged under the experimental conditions, though reductions in the positive charge were expected for each arginine to glutamate substitution. Thus, peaks e, d, and b were predicted to correspond to the TGF- β 3 WW, WD, and DD dimers, respectively, while peaks c and a, the TGF- β 3 W and D monomers. To confirm this, TGF- β 3 W and TGF- β 3 D monomers were folded and fractionated under identical conditions. This yielded the anticipated chromatograms, with peaks e and c corresponding to dimeric and monomeric forms of wild-type TGF- β 3, peaks b and a to the dimeric and monomeric forms of dead TGF- β 3, and peak d, the purported wild-type-dead heterodimer, TGF- β 3 WD, with no matching counterpart. To confirm the identity of the TGF- β 3 WD, the protein was reduced and applied to a reverse phase C18 column. This led to two peaks, the ESI-MS determined masses of which were within 1.0 Da of the predicted masses of the W and D monomers, 12722.5 and 12576.2 Da, respectively.

TGF- β 3 C77S, a variant of TGF- β 3 in which the cysteine residue that forms the inter-chain disulphide has been substituted, was also produced. This variant is covalently monomeric (Figure 2B) and as shown previously binds T β RII with

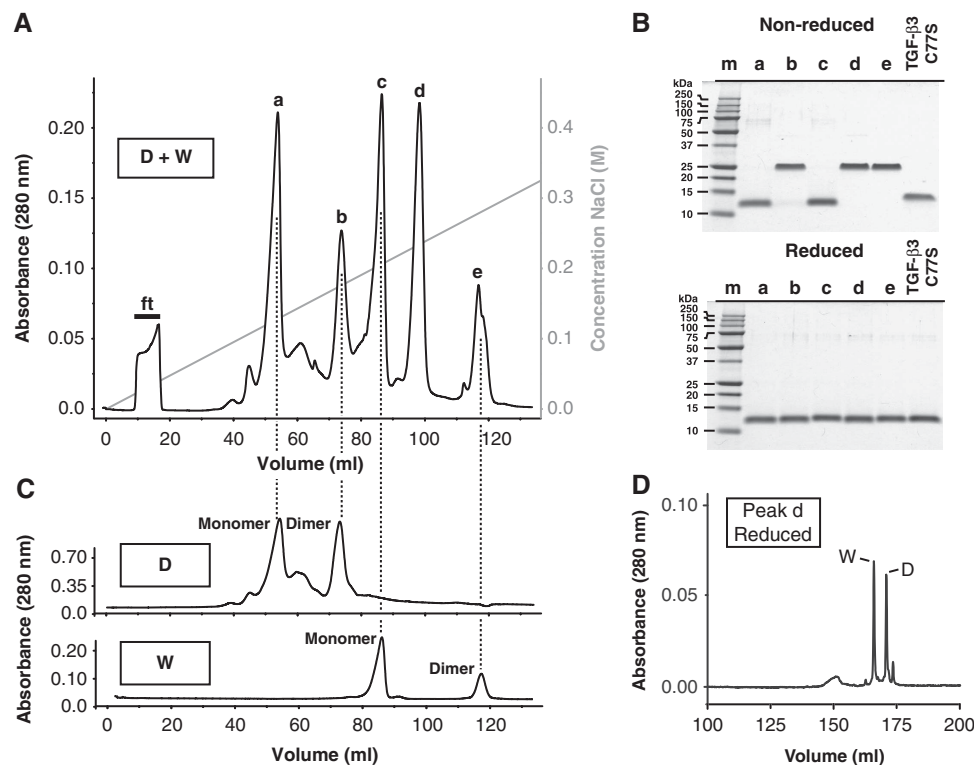


Figure 2 Isolation of the TGF- β 3 heterodimer. (A) HPLC cation-exchange elution profile of an oxidative folding reaction including equal amounts of TGF- β 3 WT and TGF- β 3 R25E, Y90A, R94E monomers. Five major peaks are eluted (a, b, c, d, and e) as a function of increasing concentration of NaCl. Flow through following loading is indicated by 'ft'. (B) SDS-PAGE analysis of the peaks eluted in panel (A) and TGF- β 3 C77S under both non-reducing (top) and reducing conditions (bottom). Peaks b, d, and e are shown to be reductant-sensitive dimers, whereas a and c are monomers. (C) HPLC cation-exchange elution profiles, exactly as in panel (A), except for refolding reactions of TGF- β 3 WT ('W', bottom) or TGF- β 3 R25E, Y90A, R94E monomer ('D', top) alone. (D) C18 reverse phase chromatogram of reduced peak d gives two major peaks, one of which has an intact ESI mass that matches the predicted mass for the WT monomer ('W', 12722.5 Da) and one that matches the predicted mass for the R25E Y90A R94E monomer ('D', 12576.2 Da).

nearly the same affinity as the wild-type dimer, but is impaired in its ability to bind and recruit T β RI (Ilangovan *et al*, 2004; Zúñiga *et al*, 2005; Groppe *et al*, 2008).

Qualitative analysis of receptor binding by native gel electrophoresis

The relative affinities and stoichiometries of receptor binding by the isolated ligands were assessed by analysing the complexes formed with the purified T β RI and T β RII extracellular domains using native gel electrophoresis. The initial experiments focused on T β RII binding and were performed by titrating a fixed amount of T β RII extracellular domain, or T β RII-ED, with increasing molar amounts of the isolated TGF- β 3 WW, WD, and DD dimers and a TGF- β 3 WT dimer control (there in principle should be no difference between TGF- β 3 WW and WT, though these are designated differently to emphasize that TGF- β 3 WW was isolated from the folding reaction in which both wild-type and dead monomers were added, whereas TGF- β 3 WT was isolated from a folding reaction in which only the wild-type monomer was added). The results showed that TGF- β 3 WT, WW, and WD dimers formed detectable complexes with T β RII-ED, whereas TGF- β 3 DD did not (Figure 3A). The fact that TGF- β 3 DD failed to yield a detectable complex was consistent with expectations regarding its reduced affinity for T β RII. Though less convincing, the results also support the anticipated stoichiometry, with the intensity of the complex bands maximizing in intensity at a 2:1 T β RII-ED:TGF- β dimer ratio for TGF- β 3 WT and WW, and a 2:2 ratio (same as 1:1) for TGF- β 3 WD.

The subsequent experiments focused on T β RI recruitment and were performed by titrating a fixed amount of T β RI

extracellular domain, or T β RI-ED, with increasing amounts of T β RII-ED:TGF- β dimer complex. The T β RII:TGF- β complex was always added in a 2:1 molar ratio, regardless of whether the T β RI-ED was needed or not, to ensure that binding of T β RI-ED was not limited by T β RII-ED. The ligands that bound T β RII, TGF- β 3 WT, WW, and WD, also bound and recruited T β RI-ED. TGF- β 3 DD, which did not detectably bind T β RII, also failed to bind and recruit T β RI (Figure 3B). The stoichiometries in this case are more convincing, with the T β RI-ED:T β RII-ED:TGF- β complex appearing neither undertitrated (i.e. excess T β RI present) nor overtitrated (i.e. excess T β RII:TGF- β complex present) at a 2:1 T β RI-ED:TGF- β dimer ratio for TGF- β 3 WT and WW and a 2:2 (same as a 1:1) ratio for TGF- β 3 WD. These results also support the binding stoichiometry with T β RII as excess T β RII is present when TGF- β 3 WD, T β RII-ED, and T β RI-ED are combined in a ratio of 2:4:2 (same as 1:2:1), but not when TGF- β 3 WT (or WW), T β RII-ED, and T β RI-ED are combined in this same ratio. These results, though qualitative, indicate that TGF- β 3 WD binds and assembles a 1:1:1 TGF- β 3:T β RII:T β RI complex, not a 1:2:2 as does TGF- β 3 WT or TGF- β 3 WW.

Quantitative analysis of receptor-binding affinities using SPR

TGF- β 3 WW, WD, and DD were quantitatively characterized in terms of their ability to bind T β RII-ED and recruit T β RI-ED using SPR. To accomplish this, the ligands were biotinylated in the presence of excess of T β RI-ED and T β RII-ED, separated away from the bound receptors by HPLC, and captured at moderate-to-low density, 100–150 resonance units (RU), on a streptavidin surface. Two sets of measurements were made,

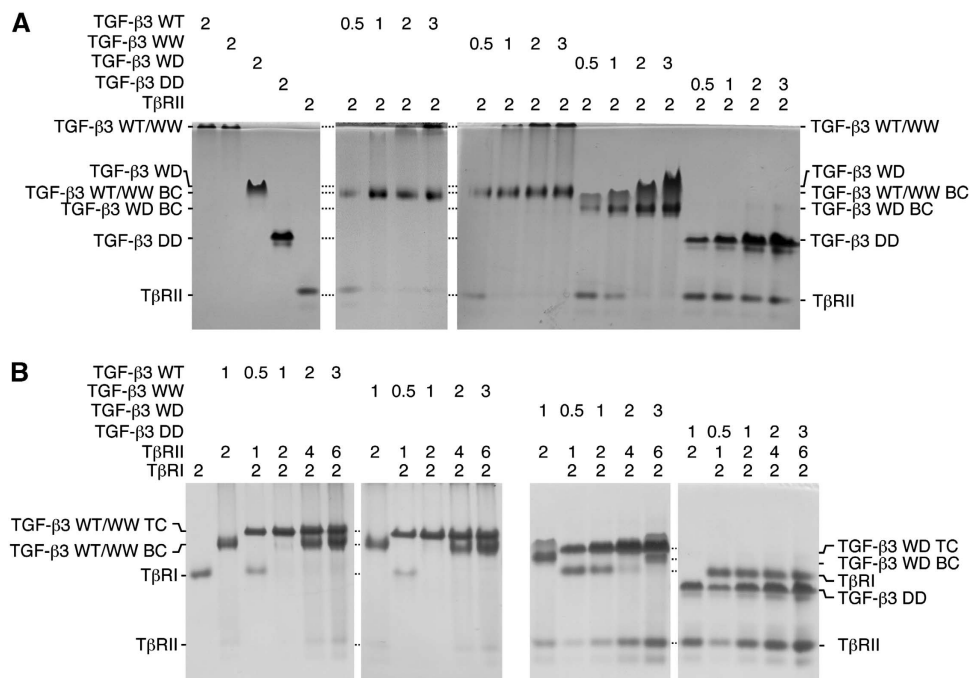


Figure 3 Qualitative native gel analyses of receptor-binding properties. (A) TGF- β 3:T β RII binary complexes were formed by adding 0.5–3.0 molar equivalents of ligand dimers (WT, WW, WD, and DD) to 2.0 molar equivalent of T β RII, then electrophoresed through a native 12% polyacrylamide gel at room temperature and stained with Commassie brilliant blue. Positions of ligands alone and TGF- β 3:T β RII binary complexes (BC) are labelled. TGF- β 3 WD runs with a pronounced smile, and while the underlying basis for this is not known, it may be related to alternate conformational states that it adopts (see also Figure 6D and accompanying explanation). (B) TGF- β 3:T β RI:T β RII ternary complexes were formed by adding 0.5–3.0 molar equivalents of ligands (WT, WW, WD, and DD), together with 2.0 molar equivalents of T β RII (relative to ligands), to 2.0 molar equivalents of T β RI and analysed as above. Positions of T β RI and T β RII alone and the corresponding TGF- β 3:T β RII binary (BC) and TGF- β 3:T β RII:T β RII ternary complexes (TC) are labelled.

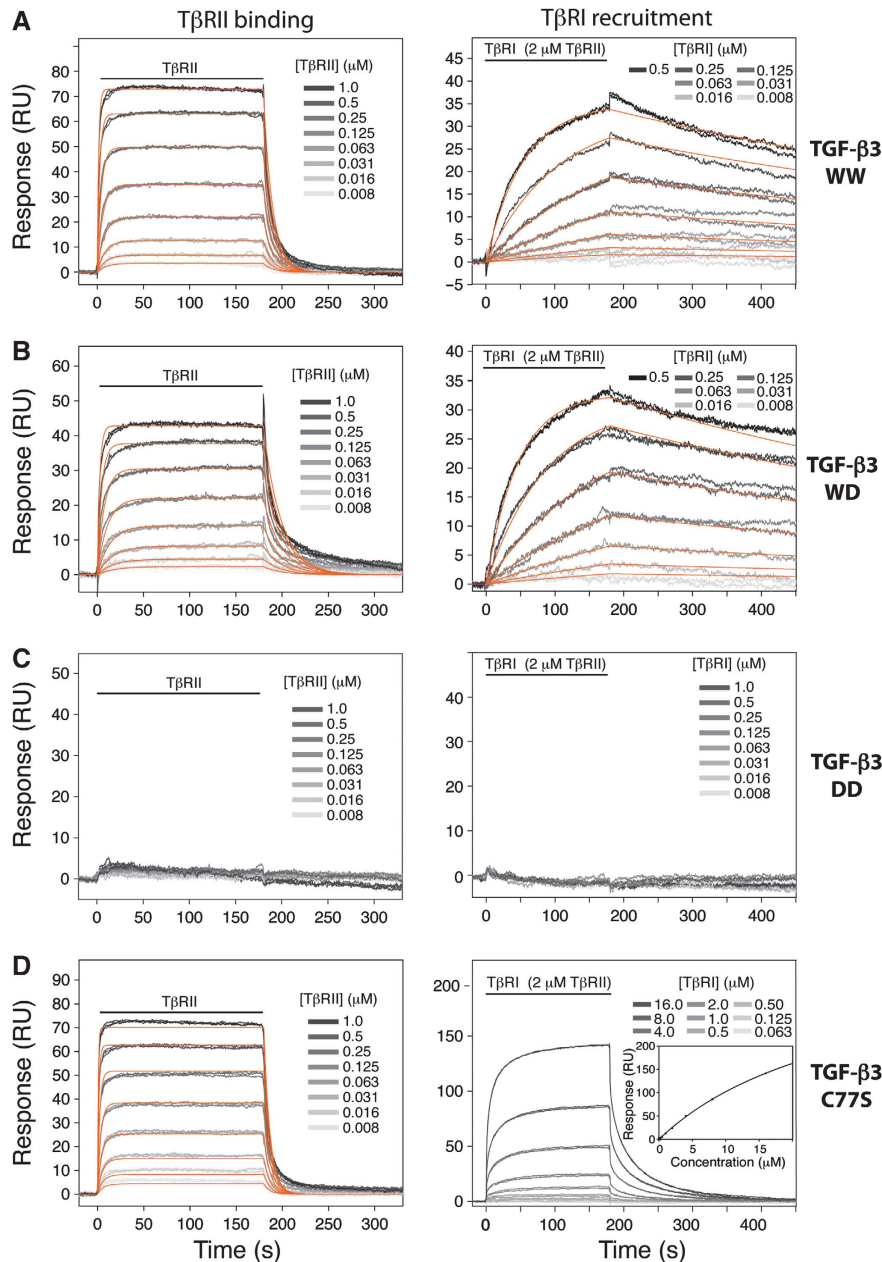


Figure 4 Quantitative SPR measurements of receptor-binding properties with biotinylated ligands. **(A)** Sensorgrams obtained as T β RII alone was injected (left) or T β RI was injected in the presence of 2.0 μ M T β RII (right) over a TGF- β 3 WW surface. The traces shown (grey) correspond to triplicate measurements of a two-fold serial dilution of the receptor over the concentration range shown. The orange curves correspond to global fits of each data set to a 1:1 binding model. The horizontal line indicates the time during which the analyte was injected. **(B–D)** Same SPR experiments as in panel **(A)**, except over TGF- β 3 WD, TGF- β 3 DD, and TGF- β 3 C77S (W) surfaces, respectively. The sensorgrams for T β RI recruitment by TGF- β 3 C77S in the presence of 2.0 μ M T β RII was analysed by fitting the equilibrium response as a function of concentration to a standard binding curve (panel **D**, right panel, inset).

one in which increasing concentrations of T β RII-ED was injected and another in which the running buffer was supplemented with a near-saturating concentration of T β RII and increasing concentrations of T β RI-ED were injected. The former provided information about T β RII binding, while the latter, T β RI recruitment (Groppe *et al*, 2008; Radaev *et al*, 2010).

The series of sensorgrams obtained from these two sets of measurements are presented in Figure 4 (left and right panels, respectively). Through visual inspection, the results are consistent with expectations: TGF- β 3 WW and WD

robustly bind T β RII and recruit T β RI, while TGF- β 3 DD is neither capable of binding T β RII nor recruiting T β RI. The low surface density, together with the uniformity of the immobilized ligands, allowed the sensorgrams to be globally fit to a simple kinetic model (orange lines), yielding the association (k_a) and disassociation (k_d) rate constants as well as the dissociation constant (K_d) (Table I). These data show that TGF- β 3 WW and WD are indeed indistinguishable in their ability to bind T β RII and recruit T β RI, with K_d s of 0.18 ± 0.02 and 0.16 ± 0.01 μ M, respectively for binding T β RII, and K_d s of 0.031 ± 0.002 and 0.027 ± 0.001 μ M, respectively, for T β RI

Table I Binding constants for TGF- β 3 and variants to the signalling receptors

Surface	Analyte	Dissociation constant (μ M)				
		Buffer suppl.	k_{on} ($M^{-1} s^{-1}$)	k_{off} (s^{-1})	K_d (μ M)	R_{max} (RU)
TGF- β 3 WT	T β RII	None	$7.4 (\pm 0.4) \times 10^5$	$0.10 (\pm 0.01)$	0.14 ± 0.01	99 ± 9
TGF- β 3 WW	T β RII	None	$5.5 (\pm 0.1) \times 10^5$	$0.10 (\pm 0.00)$	0.18 ± 0.02	86 ± 2
TGF- β 3 WD	T β RII	None	$3.3 (\pm 0.1) \times 10^5$	$0.052 (\pm 0.002)$	0.16 ± 0.01	50 ± 1
TGF- β 3 DD	T β RII	None	ND ^a	ND ^a	ND ^a	ND ^a
TGF- β 3 C77S	T β RII	None	$1.1 (\pm 0.1) \times 10^6$	$0.15 (\pm 0.01)$	0.14 ± 0.01	80 ± 3
TGF- β 3 WT	T β RI	2 μ M T β RII	$3.5 (\pm 0.1) \times 10^4$	$1.2 (\pm 0.1) \times 10^{-3}$	0.034 ± 0.002	59 ± 2
TGF- β 3 WW	T β RI	2 μ M T β RII	$3.5 (\pm 0.1) \times 10^4$	$1.1 (\pm 0.1) \times 10^{-3}$	0.031 ± 0.002	37 ± 2
TGF- β 3 WD	T β RI	2 μ M T β RII	$4.2 (\pm 0.1) \times 10^4$	$1.1 (\pm 0.1) \times 10^{-3}$	0.027 ± 0.001	35 ± 1
TGF- β 3 DD	T β RI	2 μ M T β RII	ND ^a	ND ^a	ND ^a	ND ^a
TGF- β 3 C77S	T β RI	2 μ M T β RII	ND ^b	ND ^b	30 ± 2	400 ± 20

^aNot determined due to weak binding.

^bNot determined due to large systematic deviations in the fits to a simple (1:1) kinetic model.

recruitment. These values are further shown to be equivalent to those of TGF- β 3 WT (Supplementary Figure S1; Table I). TGF- β 3 DD did not yield any detectable response, indicating it either binds T β RII and recruits T β RI very weakly or is non-native. The reason for the systematic deviation in the kinetic fits during the dissociation phase for T β RII binding to TGF- β 3 WT, WW, and WD is not known, but does not alter our conclusions as near identical K_d values were obtained by fitting the equilibrium response, R_{eq} , as a function of receptor concentration to a standard binding isotherm (not shown).

TGF- β 3 C77S was reexamined in terms of its ability to bind T β RII-ED and recruit T β RI-ED (Figure 4D). The sensorgrams, together with the fitted parameters, confirmed that TGF- β 3 C77S bound T β RII with nearly the same affinity as TGF- β 3 WT, WW, and WD (Table I). TGF- β 3 C77S, in contrast, was significantly impaired in its ability to bind and recruit T β RI. The K_d in this case could not be obtained by kinetic analysis using a simple (1:1) model due to large systematic deviations in both the association and disassociation phases. This is likely because the T β RI-binding site was partially modified during the biotinylation reaction. To derive the K_d , the data were therefore analysed by fitting the equilibrium response, R_{eq} , as a function of receptor concentration to a standard binding isotherm (not shown). This yielded a K_d nearly 100-fold greater than TGF- β 3 WT, WW, and WD (Table I), consistent with the reduced affinity previously reported (Groppe *et al*, 2008). These data show that the TGF- β 3 WD dimer, unlike the TGF- β 3 C77S monomer, has not altered its affinity for the signalling receptors.

Quantitative analysis of receptor-binding stoichiometries using SPR

To determine the stoichiometry with which T β RI and T β RII bind, the endoglin-like domain of betaglycan, or BG_e, was studied. BG_e binds all three TGF- β isoforms with high affinity (Mendoza *et al*, 2009) and its binding site does not overlap with T β RII (Verona *et al*, 2008). The rationale was that the maximal SPR response attained with BG_e should reflect the amount of immobilized binding-competent TGF- β and allow one to infer stoichiometry based on the normalized maximal SPR response for binding of T β RI and T β RII.

The measurements were made using surfaces in which TGF- β 3 WW, WD, and DD were immobilized using standard

Table II Binding constants for TGF- β 3 and variants to the beta-glycan endoglin-like domain

Surface	Analyte	Dissociation constant (μ M)			
		Buffer suppl.	K_d (μ M)	R_{max} (RU)	Norm R_{max}
TGF- β 3 WW	BG _e	None	3.5 ± 0.4	700 ± 30	1.00 ± 0.05
TGF- β 3 WD	BG _e	None	2.0 ± 0.2	1000 ± 100	1.00 ± 0.04
TGF- β 3 DD	BG _e	None	5.6 ± 0.2	1600 ± 100	1.00 ± 0.02
TGF- β 3 WW	T β RII	None	1.2 ± 0.1	330 ± 10	0.47 ± 0.02
TGF- β 3 WD	T β RII	None	0.60 ± 0.10	190 ± 10	0.19 ± 0.02
TGF- β 3 DD	T β RII	None	ND ^a	ND ^a	ND ^a
TGF- β 3 WW	T β RI	4 μ M T β RII	0.27 ± 0.05	270 ± 50	0.39 ± 0.02
TGF- β 3 WD	T β RI	4 μ M T β RII	0.30 ± 0.05	150 ± 10	0.15 ± 0.01
TGF- β 3 DD	T β RI	4 μ M T β RII	ND ^a	ND ^a	ND ^a

^aNot determined due to weak binding.

carbodiimide-based amine coupling. The rationale for this was to ensure that all three ligands were uniformly modified, which may not have been so with the biotinylated ligands described earlier since these were prepared in the presence of excess T β RI and T β RII and may have been differentially modified. The sensorgrams obtained upon injection of increasing concentrations of BG_e over these surfaces are provided as Supplementary data (Supplementary Figure S2, panels A–C). To derive the dissociation constant, K_d , and maximal response, R_{max} , the data were analysed by fitting the equilibrium response, R_{eq} , as a function of concentration to a simple binding model. The derived parameters show that the K_d s are similar, with all three ligands binding in the low micromolar range (Table II). The same surface was used to assess T β RII binding and T β RI recruitment by injecting increasing concentrations of T β RII-ED alone or T β RI-ED in the presence of a near-saturating concentration of T β RII-ED. The sensorgrams show that TGF- β 3 WW and WD exhibit robust concentration-dependent responses, but TGF- β 3 DD does not (Supplementary Figure S2, panels D–F and G–I, respectively). The fact that TGF- β 3 DD failed to bind T β RII and recruit T β RI, but bound BG_e in a manner essentially indistinguishable from TGF- β 3 WW and WD, showed that its inability to bind T β RI and T β RII is a consequence of the R25E Y90A R94E substitutions, not conformational changes or misfolding.

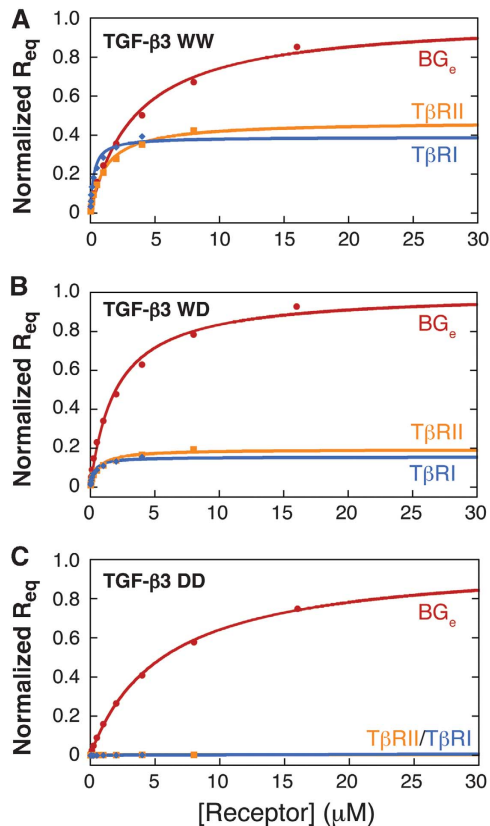


Figure 5 SPR equilibrium analysis to determine receptor-binding stoichiometries. (A) Normalized equilibrium response curves for binding of BG_e (red) and T β RII (orange) to TGF- β 3 WW, or T β RI to TGF- β 3 WW in the presence of 4 μ M T β RII (blue). The normalized equilibrium responses shown were obtained by dividing the observed equilibrium responses from the raw sensorgrams (Supplementary data; Figure 2, panels A–C) by the fitted maximal response value (R_{\max}) for BG_e. The latter was obtained by fitting the equilibrium response for BG_e (the region of the sensorgram near the end of the injection period) as a function of concentration to a standard binding equation. (B, C) Normalized equilibrium response as in panel (A), except for binding of the three receptors to TGF- β 3 WD (panel B) or TGF- β 3 DD (panel C).

The amplitudes of the responses at the highest concentration of injected receptor over the TGF- β 3 WW surface are each lower than BG_e, which is expected, even for a 2:1 receptor:ligand stoichiometry, as BG_e is 38 kDa in size whereas T β RII-ED is 14 kDa and T β RI-ED is 11 kDa. The responses at the highest receptor concentration over the TGF- β 3 WD surface are decreased even further relative to BG_e, presumably due to the reduced stoichiometry. To quantify this effect, the equilibrium response as a function of concentration for T β RII binding and T β RI recruitment were normalized by the corresponding maximal response for BG_e and then fitted to a standard binding equation as before. The fits for T β RII binding and T β RI recruitment yielded normalized R_{\max} values of 0.47 ± 0.02 and 0.39 ± 0.02 for TGF- β 3 WW and 0.19 ± 0.02 and 0.15 ± 0.01 for TGF- β 3 WD, respectively (Figure 5; Table II). The normalized R_{\max} values for T β RII binding and T β RI recruitment differ by a factor 2.47 ± 0.37 and 2.05 ± 0.21 , respectively, providing the first quantitative demonstration of the reduced stoichiometry with which TGF- β 3 WD binds T β RII and recruits T β RI.

Isolation of ligand–receptor complexes and direct determination of stoichiometries

To directly demonstrate the reduced stoichiometry, an excess of T β RI-ED and T β RII-ED were added to TGF- β 3 WW and WD and the complexes were isolated using size exclusion chromatography. The elution profiles, and corresponding SDS gel, show that the TGF- β 3 WW complex elutes before the TGF- β 3 WD complex and both elute before the uncomplexed receptors (Figure 6A and B). The isolated complexes were analysed using native gel electrophoresis to ascertain that they were fully saturated with T β RI and T β RII. This was accomplished by challenging the isolated complexes with additional T β RII-ED, T β RI-ED, or both T β RII-ED and T β RI-ED (Figure 6C). This resulted in no apparent changes, indicating that the ligands were bound by their full complement of receptors.

To analyse the stoichiometry, the isolated complexes were separated using high-resolution ion-exchange chromatography in the presence of 8 M urea. The UV absorption profiles, recorded at 280 nm, included three components as anticipated (Figure 6D). The split T β RII peak is a consequence of deamidation of Asn19 and has no effect on T β RII's ability to bind TGF- β (Hinck *et al*, 2000). The splitting of the TGF- β 3 WD peak is unexpected, but is not due to contamination of TGF- β 3 WD with either TGF- β 3 WW or TGF- β 3 DD as reanalysis of the TGF- β 3 WD peak from Figure 6D in the absence of urea yields a single peak well resolved from either TGF- β 3 WW or DD (Supplementary Figure S3, panel A). The splitting may instead arise from alternate slowly converting conformations under the conditions used to dissociate the complex, as reanalysis of material from the leading edge of the split peak in the presence of 8 M urea yields an identical split peak (Supplementary Figure S3, panel B).

To quantitate stoichiometries, the areas under the peaks were measured and compared with those for 2:2:1 and 1:1:1 T β RI:T β RII:TGF- β 3 dimer complex calculated from the corresponding molar extinction coefficients at 280 nm. The results show that the relative integrated HPLC peak areas uncorrected for differences in extinction coefficients for the T β RI:T β RII:TGF- β 3 WW complex, 0.099:0.45:1.00, closely match those expected for a 2:2:1 T β RI:T β RII:TGF- β 3 complex, 0.085:0.41:1.00, whereas those for TGF- β 3 WD complex, 0.043:0.16:1.00, match those expected for a 1:1:1 T β RI:T β RII:TGF- β 3 complex, 0.043:0.20:1.00 (Table III). These results unambiguously demonstrate that the TGF- β 3 WD heterodimer binds T β RII-ED and recruits T β RI-ED with an affinity indistinguishable from the TGF- β 3 WT homodimer, but with one-half the stoichiometry.

Biological activities of the heterodimer

The biological activity of the TGF- β 3 WD heterodimer was compared with TGF- β 3 WT and TGF- β 3 WW to determine the effect of its altered stoichiometry on signalling. The first assay involved measuring the induction of phospho-Smad3, a direct downstream target of T β RI. MCF10A human breast epithelial cells were treated with 40 and 80 pM TGF- β s for 30 min and the cell lysates were analysed by western blotting with a phospho-Smad3 antibody (Figure 7A, upper panel). The results show that the activity of TGF- β 3 WD was partially compromised compared with TGF- β 3 WT and TGF- β 3 WW, whereas the other two variants tested, TGF- β 3 DD and TGF- β 3 C77S, were fully compromised. Time-dependent

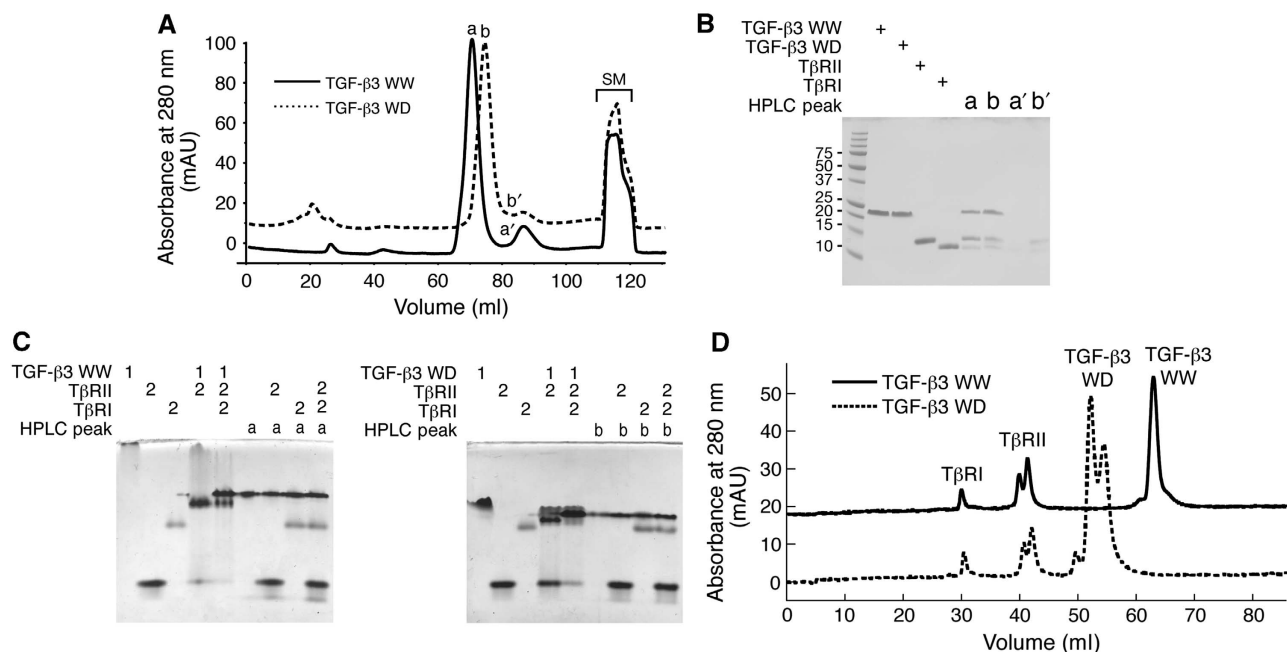


Figure 6 Stoichiometry of TGF- β receptor complexes determined by HPLC analysis. (A) Isolation of complexes of TGF- β 3 WW (solid line) and TGF- β 3 WD (dashed line) with the T β RI and T β RII extracellular domains using size exclusion chromatography. Small molecules that elute at the total volume are indicated by 'SM'. (B) Peaks from panel (A) were analysed by SDS-PAGE gel under non-reducing conditions; peaks a and b correspond to the complexes with T β RI and T β RII, whereas a' and b' correspond to excess unbound receptors. (C) Native gel analysis of the isolated TGF- β 3 WW and WD receptor complexes (left and right panels, respectively) either alone (lane 6) or with 2.0 molar equivalents of added T β RII (lane 7), T β RI (lane 8), or T β RI and T β RII (lane 9). (D) HPLC cation-exchange analysis of the isolated TGF- β 3 WW and WD receptor complexes (solid and dashed lines, respectively) under denaturing conditions (8M urea, pH 4.0). Identity of the eluted peaks is indicated.

Table III Relative HPLC peak areas at 280 nm for T β RI:T β RII:TGF- β dimer complexes

Complex	Relative integrated HPLC peak areas ^a		
	T β RI	T β RII	TGF- β dimer
Theoretical, 2:2:1	0.085	0.41	1.00
Theoretical, 1:1:1	0.043	0.20	1.00
HPLC, TGF- β 3 WW	0.099	0.45	1.00
HPLC, TGF- β 3 WD	0.043	0.16	1.00

^aIntegrated HPLC peak areas are uncorrected for differences in molar extinction coefficients.

Smad3 phosphorylation assays were performed to compare the rate and amplitude of signalling (Figure 7B). The appearance and disappearance of phospho-Smad3 upon stimulation with TGF- β 3 WD, WW, and WT had similar overall kinetics, but the amplitude at all time points for TGF- β 3 WD was reduced by roughly a factor or four compared with TGF- β 3 WW and TGF- β 3 WT (Figure 7B, lower panel).

The ligands were further tested in a luciferase-reporter gene assay by transfecting cultured mink lung epithelial (Mv1Lu) cells with a CAGA₁₂ luciferase reporter and by treating them with the TGF- β s over a range of concentrations for 18 h. The dose-response curves, presented in Figure 7C, show that TGF- β 3 WD is essentially indistinguishable from TGF- β 3 WW and TGF- β 3 WT, with EC₅₀ values in the range of 10–16 pM (Table IV). TGF- β 3 C77S is ~11-fold less potent, with an EC₅₀ of ~140 pM, and TGF- β 3 DD is significantly less potent, with no detectable activity at 200 pM, the highest concentration tested (Table IV).

The ligands were also characterized in terms of their ability to growth inhibit mink lung epithelial cells by treating them with the TGF- β s over a range of concentrations for 24 h. The dose-response curves, presented in Figure 7D, show that TGF- β 3 WD exhibits comparable potency to TGF- β 3 WW and TGF- β 3 WT, especially at lower concentrations, though at higher concentrations, TGF- β 3 WD appeared approximately two-fold less potent, with an IC₅₀ close to 0.8 ± 0.2 pM versus 0.4 ± 0.1 and 0.6 ± 0.1 pM for TGF- β 3 WT and WW, respectively (Table IV). TGF- β 3 C77S, in contrast, weakly inhibited growth, with an IC₅₀ close to 17 ± 2 pM, and TGF- β 3 DD exhibited marginal inhibition, with an IC₅₀ of ~1300 pM (Table IV).

These results show that TGF- β 3 WD heterodimer possesses one-quarter to one-half the biological activity of the wild-type homodimer, TGF- β 3 C77S is significantly compromised, and TGF- β 3 DD is severely compromised. These results, together with the studies presented above, show that the diminishment of receptor binding, either partially as in TGF- β 3 C77S or fully as in TGF- β 3 DD, attenuates biological activity, but eliminating the binding of one of the two T β RI:T β RII pairs reduces the activity by no more than a factor of four.

Receptor complex assembly on the cell surface

TGF- β 3 WD may not bind the cell surface receptors in the same overall manner as the purified receptor extracellular domains due to interactions between the transmembrane or cytoplasmic domains that promote assembly of a T β RI:T β RII heterotetramer. To investigate this possibility, single-molecule TIRF-based fluorescence imaging was used. This method measures the proportion of receptors that are monomeric or

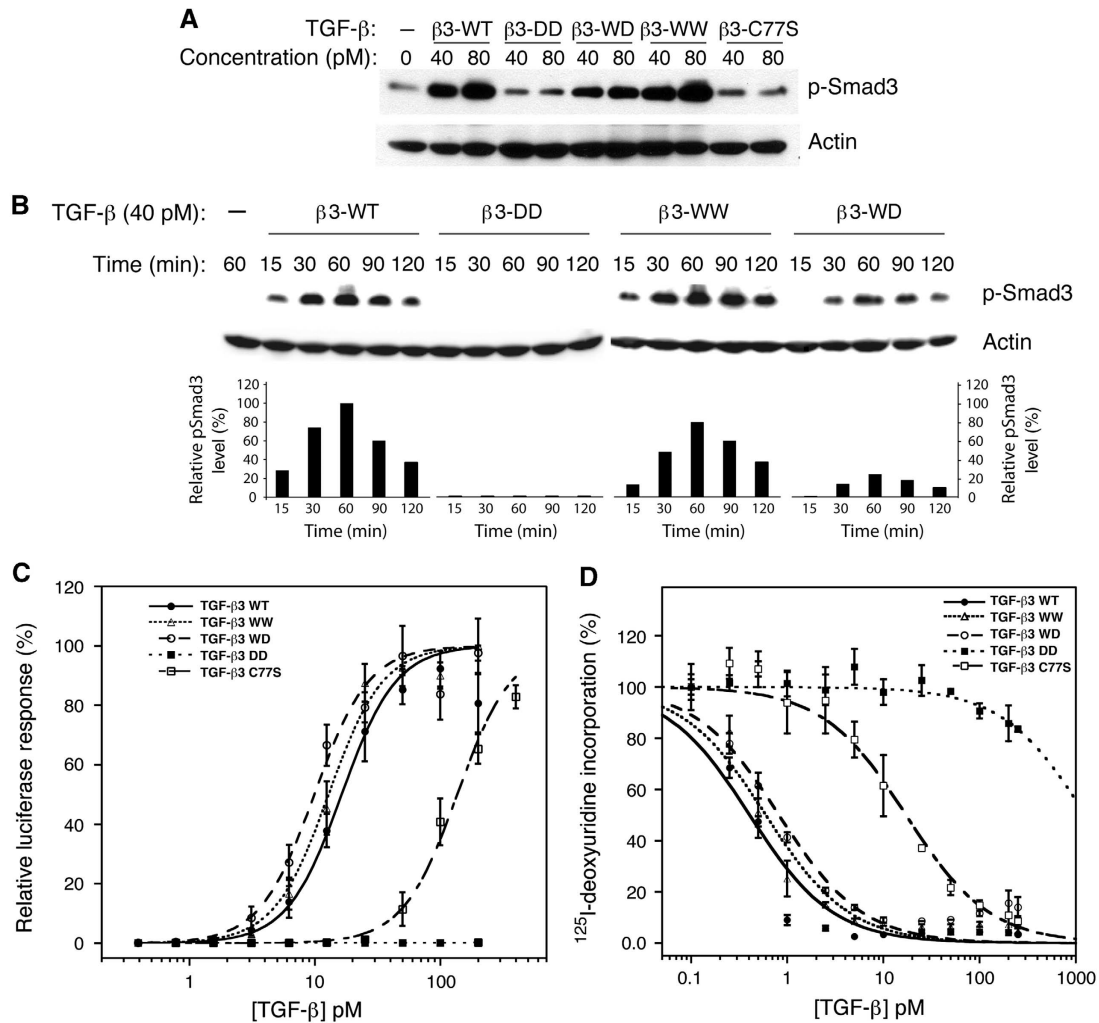


Figure 7 Cell-based assays of TGF- β function. (A) Induction of Smad3 phosphorylation in human breast epithelial cells as a function of increasing concentrations of added TGF- β s. Control for equal loading (actin) is aligned below. (B) Induction of Smad3 phosphorylation as in panel (A), but as a function of treatment time as shown. Relative amounts of pSmad3 are plotted below. Values shown are normalized relative to actin and are expressed as a percentage of the maximum detected with TGF- β WT at 60 min. (C) Luciferase-reporter gene activity in mink lung epithelial cells transiently transfected with a fixed amount of CAGA₁₂-Luc and β -galactosidase reporters as a function of increasing concentrations of added TGF- β s. Luciferase are expressed as a percentage of the maximum value attained by the wild-type protein. Lines shown correspond to the best fit to an equation describing the EC₅₀. (D) Growth inhibitory activity examined by measuring the incorporation of 5-[¹²⁵I]-iodo-2'-deoxyuridine into total DNA in cultured mink lung epithelial cells as a function of increasing concentrations of added TGF- β s. Values shown are expressed as percentage relative to untreated cells. Lines shown correspond to the best fit to an equation describing the IC₅₀.

Table IV Biological activity of TGF- β ligands

Ligand	Reporter gene assay EC ₅₀ (pM)	Growth inhibition assay IC ₅₀ (pM)
TGF- β WT	16 ± 2	0.4 ± 0.1
TGF- β WW	13 ± 1	0.6 ± 0.1
TGF- β WD	10 ± 1	0.8 ± 0.2
TGF- β DD	ND ^a	1300 ± 200
TGF- β C77S	140 ± 10	17 ± 2

^aNot determined due to the absence of a detectable response.

dimeric based on an analysis of the bleaching statistics, that is the fraction of molecules that photobleach in a single step versus those that bleach in two (Iino *et al*, 2001). This technique revealed that TGF- β treatment leads to a significant increase in the proportion of dimeric receptors on the cell surface, with both T β RI and T β RII being about 90%

monomeric and 10% dimeric in the absence of TGF- β and about 65% monomeric and 35% dimeric in its presence (Zhang *et al*, 2009, 2010).

The same procedure was employed to determine whether TGF- β WD led to any significant dimerization of T β RI or T β RII on the cell surface. This involved transiently transfecting cultured HeLa cells with either C-terminally GFP-tagged T β RI or T β RII, expressing these for a limited time to ensure expression at endogenous levels, treatment with TGF- β WT or WD, and analysis of the fixed cells using single-molecule TIRF-based imaging. Typical TIRF images and bleaching patterns for cells transfected with T β RII-GFP and T β RI-GFP are shown in Figure 8 (panels A–C and E–G, respectively). These, as well as the corresponding bleaching statistics, are similar to those reported earlier, with TGF- β treatment increasing the proportion of T β RI and T β RII dimers from 11.8 ± 1.3 to 36.1 ± 2.6% and 8.5 ± 0.9 to 37.2 ± 2.5%,

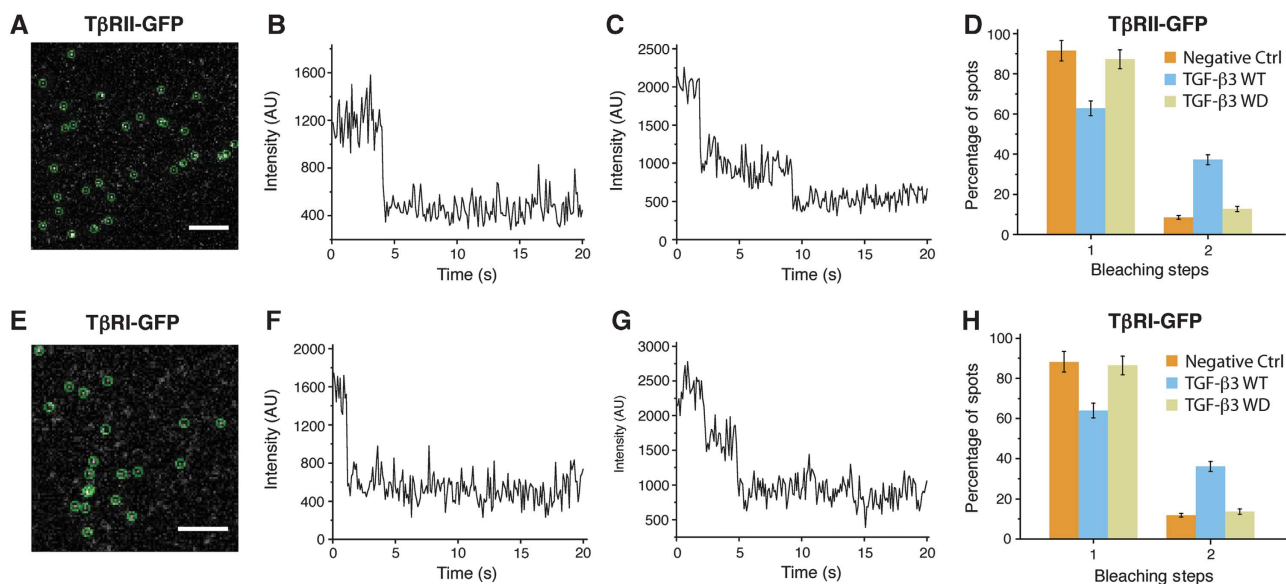


Figure 8 Analysis of bleaching steps of single T β RII and T β RI spots imaged with fixed HeLa cells. (A) A typical image with diffraction-limited fluorescent spots of T β RII-GFP on the cell membrane of transfected HeLa cells. The spots enclosed with the green circles (3×3 pixels) were chosen for single-molecule bleaching analysis. Scale bar: $4 \mu\text{m}$. (B, C) Two representative time courses of T β RII-GFP emission after background correction show one-step (B) and two-step bleaching (C). (D) Frequency of one- and two-step bleaching events for T β RII-GFP in the absence (yellow) or presence of TGF- β 3 WT (blue) or TGF- β 3 WD (grey) with fixed HeLa cells. (E–H) As in panels (A–D) but for T β RI-GFP. The data presented were obtained from three independent experiments.

respectively (Figure 8D and H). This readily measurable increase in dimers was not however apparent upon treatment with TGF- β 3 WD, with the proportion of T β RI and T β RII dimers essentially within the error limits of the control, 13.6 ± 1.2 and $12.7 \pm 1.3\%$, respectively. These results show that the TGF- β 3 WD heterodimer is incapable of assembling a T β RI:T β RII heterotetramer on the cell surface.

Discussion

The objective of this study was to thoroughly investigate if TGF- β s signal through two independently functioning T β RI:T β RII heterodimers. This was accomplished by investigating a heterodimeric form of TGF- β 3 bearing substitutions in one of its protomers to block T β RII binding and T β RI recruitment. The heterodimer was shown using a series of complementary biochemical techniques to bind the T β RII extracellular domain and recruit the T β RI with affinities indistinguishable from the wild-type homodimer but with one-half the stoichiometry. TGF- β 3 C77S bound T β RII-ED indistinguishably from the wild-type homodimer, but was impaired nearly 100-fold in its ability to bind and recruit T β RI-ED. TGF- β 3 DD, though native, as shown by its ability to bind the betaglycan endoglin-like domain, was reduced at least 200-fold in its ability to bind T β RII-ED and recruit T β RI-ED.

The functional data showed that TGF- β 3 WD, which bound the receptor extracellular domains with affinities indistinguishable from wild-type homodimer, but with one-half the stoichiometry, had four-fold lower activity compared with TGF- β 3 in the Smad phosphorylation assay, a two-fold lower IC_{50} in the growth inhibition assay, and an indistinguishable EC_{50} in the reporter gene assay. TGF- β 3 C77S, which was significantly impaired in its ability to bind and recruit T β RI-ED, had a nine-fold higher EC_{50} in the reporter gene assay and

a 43-fold higher IC_{50} in the growth inhibition assay. TGF- β 3 DD, which did not detectably bind T β RII-ED or recruit T β RI-ED, had no detectable activity in the reporter gene assay and an IC_{50} three to four orders of magnitude higher than TGF- β 3 in the growth inhibition assay.

The fact that TGF- β 3 WD exhibits a small, but detectable decrease in activity compared with wild-type dimer in the Smad phosphorylation assay and growth inhibition assay, but not the reporter gene assay is likely due to lower intrinsic sensitivity of this assay compared with the others. This is illustrated by the data of Amatayakul-Chantler *et al* (1994) who showed that monomeric TGF- β 1 (TGF- β 1 C77S) was reduced in its potency eight-fold compared with dimeric (WT) TGF- β 1 in a reporter gene assay, but >100 -fold in a growth inhibition assay. Thus, it is not surprising that TGF- β 3 WD, which is reduced in its growth inhibitory activity by no more than two-fold, exhibits no detectable difference in its reporter gene activity.

The four-fold reduction in Smad phosphorylation activity for the TGF- β 3 WD heterodimer shows that the two T β RI:T β RII pairs bind TGF- β and function in a nearly autonomous manner. The diminishment in activity of the heterodimer compared with the wild-type homodimer by an additional factor of two beyond that anticipated for independent binding and signalling may be a consequence of increased apparent affinity of the wild-type homodimer for the cell surface receptors. This could occur by membrane-localization effects, where the apparent affinity of the wild-type homodimer for cell surface T β RII is increased after it binds T β RII through one of its two sites and becomes localized on the membrane surface. The other possible explanation is that the two T β RI:T β RII pairs functionally interact, but this seems unlikely given the limited magnitude of the effect.

The conclusion that the two T β RI:T β RII pairs bind and function in a near-autonomous manner presumes that

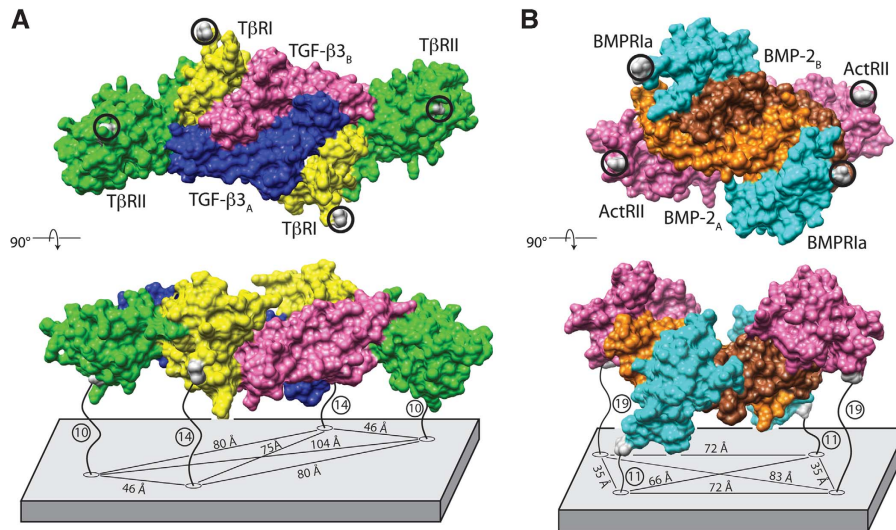


Figure 9 Positioning of the TGF- β 3 and BMP receptor complexes on a membrane surface. **(A)** Surface representation of the TGF- β 3 homodimer bound to the T β RI and T β RII extracellular domains (PDB 2PJY). The two protomers of TGF- β 3 are depicted in magenta and blue, T β RII in green, and T β RI in yellow. C-terminal residues of the receptor extracellular domains are shaded grey. Distances between the C-terminal residues of the receptor extracellular domains are shown on the projection onto the cell surface. Circled numbers correspond to the number of amino acids between the last structurally ordered residue in receptor extracellular domain and the first predicted residue of the transmembrane domain. **(B)** Surface representation of the BMP-2 homodimer bound to the BMPRIa and ActRII extracellular domains (PDB 2GOO) shown in a similar manner to the TGF- β complex in panel **(A)**. The two protomers of BMP-2 are depicted in orange and brown, ActRII in magenta, and BMPRIa in cyan.

TGF- β 3 WD binds the cell surface receptors in the same manner as the purified receptor extracellular domains. This seems likely because the TIRF-based single-molecule fluorescence data obtained with C-terminally GFP-tagged T β RI and T β RII showed that treatment with TGF- β 3 WD leads to a negligible increase in the proportion of dimeric T β RI and T β RII on the cell surface, whereas treatment with TGF- β 3 leads to more than a three-fold increase (Figure 8).

The current results indicate that receptor transactivation occurs exclusively within T β RI:T β RII heterodimers, not between. This is probably determined by the arrangement of the receptors as the last structurally ordered residue on the C-terminus of T β RI and T β RII are separated by just 46 Å within a T β RI:T β RII heterodimer, but 80 Å between heterodimers (Figure 9A). The current results stand in contrast to results obtained in earlier studies in which the receptors were artificially dimerized. Two such studies employed T β RI and T β RII variants bearing dimerization domains, one employing the extracellular domain of the erythropoietin receptor (Luo and Lodish, 1996) and the other small immunophilin domains inserted between the kinase and transmembrane domains (Stockwell and Schreiber, 1998). The erythropoietin-dimerized receptors led to T β RII-T β RI transactivation, but not activation of downstream signalling, while the immunophilin-dimerized receptors led to the activation of downstream signalling, though at a significantly reduced level compared with wild-type homodimer. A third study employed TGF- β as the dimerizer, but utilized a chimeric receptor comprising the T β RI kinase domain and the T β RII extracellular domain (Okadome *et al*, 1994). This construct, designated T β RII-I, yielded no detectable signalling when transfected into a T β RI-deficient cell line.

The discrepancy between these results and the previous emphasizes that the precise positioning of the receptors is important, with wild-type-like signalling when T β RI and

T β RII are arranged natively, but diminished or no detectable signalling when arranged non-natively. The spacing between the last structurally ordered residue on the C-terminus of the two bound T β RIIs in the TGF- β receptor complex is 104 Å (Figure 9A) and that between those of the two bound Epo receptors in the Epo receptor complex is 30 Å (Syed *et al*, 1998). The 104-Å spacing, which would be expected for signalling with the combination of T β RII and T β RII-I, is likely too large compared with the 46-Å spacing when T β RI and T β RII are arranged within a T β RI:T β RII heterodimer. The 30-Å spacing, which would be expected for the chimeric Epo-T β RI and Epo-T β RII receptors, enables efficient transactivation, but perhaps brings the T β RI and T β RII kinases too closely together so that Smad phosphorylation is inhibited. These observations suggest that the separation between the kinases is a critical factor for efficient signalling, though it should be emphasized that this conclusion is tentative given that orientation effects might also be important and that both T β RI and T β RII include 10 to 14 structurally disordered residues bridging the last structurally ordered residue of their extracellular domain and the first predicted residue of the transmembrane domain (Figure 9A). Thus, further studies are required to ascertain whether receptor activity is determined by distance alone, or whether orientation or other effects might also have a role.

The isolated T β RI and T β RII kinase domains have previously been shown to weakly interact using yeast two hybrid and other methods (Ventura *et al*, 1996). Thus, it seems likely that the close proximity between the T β RI and T β RII extracellular domains, as in TGF- β 3 or TGF- β 3 WD complexes, promotes this otherwise intrinsically weak interaction, and positions the kinases for optimal transactivation and signalling. The current results therefore emphasize that binding of T β RI and T β RII adjacent to one another and with direct contact in the TGF- β receptor complex has roles beyond T β RI

recruitment and enhancing specificity (Groppe *et al*, 2008), but also promoting transphosphorylation that leads to downstream signalling.

The finding that TGF- β s signal through T β RI:T β RII heterodimers is of interest in light of recent single-molecule fluorescence imaging studies that show that both T β RI and T β RII are predominantly monomeric in the absence of ligand (Zhang *et al*, 2009, 2010). The discrepancy between these findings and the previous findings that showed that the receptors are dimeric (Chen and Derynck, 1994; Gilboa *et al*, 1998; Rechtman *et al*, 2009) is likely a consequence of differences in expression levels, as the single-molecule studies showed that T β RI and T β RII were predominantly monomeric when expressed at endogenous levels, but dimeric when overexpressed. The findings from the single-molecule study, together with the current findings, therefore show that the TGF- β s have adapted to bind and assemble T β RI:T β RII heterodimers, not T β RI:T β RII heterotetramers.

The data presented, even though they show that the two T β RI:T β RII pairs bind and function in an autonomous manner, does not imply that one of the two T β RI:T β RII pairs is dispensable. Thus, as explained above, one important function of the two pairs may be to increase potency by enhancing the apparent affinity for binding TGF- β *via* membrane-localization effects. The four-fold increase in pSmad levels with the wild-type homodimer versus the heterodimer might be vital *in vivo* where the local concentration of active dimer can lead to very different biological outcomes (McKarns *et al*, 2003).

The fact that TGF- β s activate the Smad pathway through two near-autonomously functioning T β RI:T β RII pairs stands in contrast to the BMPs, which are unable to activate the Smad pathway when one of the type II receptor-binding sites is blocked (Knaus and Sebald, 2001; Isaacs *et al*, 2010). This suggests that BMPs have a minimal requirement for a type I:type II:type II heterotrimer. The type I and type II receptor extracellular domains do not contact one another in the BMP receptor complex (Figure 9B) and thus the requirement for a heterotrimer in the BMP system must be a consequence of direct or indirect interactions between the transmembrane or kinase domains of the receptors. One possible role for the type I:type II:type II heterotrimer is to promote efficient receptor transactivation and signalling. Another is to enhance potency *via* multivalent binding, perhaps overcoming the low intrinsic affinity that many BMPs have for their type II receptors (Nickel *et al*, 2009).

The requirement for a heterotrimer in the BMP system, but not the TGF- β , may be related to differences in the manner by which these two subfamilies of ligands bind their receptors. The TGF- β s bind the type I and type II receptors as two well-separated heterodimeric pairs, whereas the BMPs bind their type I and type II receptors without any direct contact, but in much closer spatial proximity to one another (Figure 9). Thus, in analogy to the TGF- β s, where direct contact between the extracellular domains promotes recruitment of the low affinity receptor and signalling, so too may the close proximity between the transmembrane and/or kinase domains of the receptors in the BMP system promote functions critical for ligand binding and signalling. Though speculative, it might be this function is related to enhancement of ligand binding by dimerization of the type II receptor, rather than signalling, as the type II receptors are somewhat closer together in the

BMP system (84 Å) compared with the TGF- β (104 Å) and the two type I—type II receptor distances are not that different from that in the TGF- β system, with one ‘short’ distance compatible for transactivation, 35 Å, and one ‘long’ distance that is not, 72 Å.

Materials and methods

Protein preparation

Human TGF- β 3 was expressed, refolded, and purified as previously described (Cerletti, 2000) with modifications (Supplementary data). TGF- β 3 WW, WD, and DD were prepared by expressing and purifying the wild-type (W) and R25E, Y90A, R94E (D) monomers separately and by combining them in a 1:1 ratio for refolding. The three dimers that formed, WW, WD, and DD, were separated from one another, as well as non-dimerized W and D monomers, using cation-exchange chromatography as described (Supplementary data). Other proteins were produced as described (Supplementary data).

Native gel assay

Native gel assays to assess the binding and stoichiometry of TGF- β 3 WT, WW, WD, and DD to the T β RI and T β RII extracellular domains, T β RI-ED and T β RII-ED, respectively, were performed as previously described (Zúñiga *et al*, 2005)

SPR-binding assays

Binding studies were performed with BIAcore 3000 instrument (GE Healthcare) and were analysed using the software package Scrubber2 (Biologic Software). For kinetic experiments, TGF- β s were biotinylated and captured on streptavidin chip surfaces (GE Healthcare). TGF- β 3 WT, WW, WD, and C77S were biotinylated in the presence of excess T β RI and T β RII, whereas TGF- β 3 DD was biotinylated in the presence of excess soluble betaglycan, using sulfo-NHS-LC-LC-Biotin (Pierce) at pH 7.5. Singly biotinylated TGF- β s were separated from receptors and doubly and multiply biotinylated forms by Source S cation-exchange column (GE Healthcare) in the presence of 30% isopropanol at pH 4.0. Surface densities of captured TGF- β s were kept at 50–300 RU to minimize rebinding artifacts. For equilibrium experiments, TGF- β 3 DD, WD, and WW were covalently attached to flow cells using an amine coupling kit (GE Healthcare). Binding assays were performed by injecting two-fold serial dilutions of the receptors in triplicate in HBS-EP buffer (GE Healthcare) at a flow rate of either 10 μ l per min (equilibrium experiments) or 50 μ l per min (kinetic experiments). Surfaces were regenerated by a brief injection of 4 M guanidine hydrochloride (10 s contact time at a flow rate of 100 μ l per min). Instrument noise was removed by double referencing. Equilibrium analyses were performed by averaging the equilibrium binding response near the end of the injection. Kinetic analyses were performed by global fitting with a simple 1:1 model. Standard errors were obtained from the variation among the derived parameters.

Stoichiometry by HPLC

TGF- β 3 WW and WD ternary complex with T β RI and T β RII were formed and purified as before (Groppe *et al*, 2008). Compositional analysis of the isolated complexes was determined by separating the components using high-resolution ion-exchange chromatography (Mono S HR 5/5 column, GE Healthcare) in the presence of 8 M urea and 50 mM sodium formate at pH 4.0 (0–400 mM NaCl in 50 mM sodium formate over 80 column volumes). Chromatograms were recorded at 280 nm and peaks areas of individual components were determined using the program Peakfit (Systat Software Inc., San Jose, CA).

Smad3 phosphorylation assays

MCF10A human breast epithelial cells were treated with 40 and 80 pM TGF- β ligands for 30 min. Western blot was carried out as previously described (Zúñiga *et al*, 2005) using a rabbit polyclonal anti-phospho-Smad3 antibody (Upstate Biotechnology) or an anti-actin antibody (Ambion). Time-dependent Smad3 phosphorylation was performed in the same manner, but with different treatment times.

Growth inhibition assay

The effect of TGF- β isoforms on the proliferation of cultured mink lung epithelial cells was tested as described (Cheifetz *et al*, 1990; Zúñiga *et al*, 2005).

Luciferase-reporter gene assays

The induction of a TGF- β CAGA₁₂ luciferase-reporter construct was performed as previously described (Dennler *et al*, 1998) with modifications (Supplementary data).

Single-molecule fluorescence

HeLa cells were cultured and transfected with T β RRII-GFP or T β RI-GFP plasmid and expressed at endogenous levels or lower as previously described (Zhang *et al*, 2009, 2010). For the ligand stimulation experiments, the transfected cells that were ready for fluorescence imaging, were added with 200 pM TGF- β 3 (WT) or TGF- β 3 (WD) in DMEM for 15 min at 4°C. Single-molecule fluorescence imaging was performed and analysed as previously reported (Zhang *et al*, 2010). The experiments were performed three times and 200–300 spots were selected and analysed every time.

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Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.

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