Probing the role of homomeric and heteromeric receptor interactions in TGF-β signaling using small molecule dimerizers

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Background: Transforming growth factor β (TGF-β) arrests many cell types in the G1 phase of the cell cycle and upregulates plasminogen activator inhibitor 1 (PAI-1). The type I (TGF-βRI) and II (TGF-βRII) TGF-β receptors mediate these and other effects of TGF-β on target cells. TGF-β initially binds to TGF-βRII and subsequently TGF-βRI is recruited to form a heteromeric complex. TGF-βRI phosphorylates the downstream effectors Smad2 and Smad3, leading to their translocation into the nucleus. Here, we explored the role of receptor oligomerization in TGF-β signaling.

Results: We constructed fusion proteins containing receptor cytoplasmic tails linked to binding domains for small-molecule dimerizers. In COS-1 cells, recruitment of a soluble TGF-βRII tail to a myristoylated TGF-βRI tail promoted Smad2 nuclear translocation. In mink lung cells, homo-oligomerization of a myristoylated TGF-βRI tail in the presence of a myristoylated TGF-βRII tail activated the PAI-1 promoter. Oligomerization of an acidic mutant of the TGF-βRI tail in the absence of TGF-βRII activated the PAI-1 promoter and inhibited the growth of mink lung cells.

Conclusions: Non-toxic, small molecules designed to oligomerize cytoplasmic tails of TGF-β receptors at the plasma membrane can activate TGF-β signaling. Although TGF-β normally signals through two receptors that are both necessary for signaling, in one small-molecule system, a dimerizer activates signaling through a single type of receptor that is sufficient to induce TGF-β signaling. These methods of activating TGF-β signaling could be extended to signaling pathways of other TGF-β superfamily members such as activin and the bone morphogenetic proteins.

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Background

Transforming growth factor β 1, 2 and 3 (TGF-β1, β2, β3) are secreted, dimeric polypeptide signaling factors and represent prototypical members of the TGF-β superfamily, which includes activin and the bone morphogenetic proteins (BMPs) [1]. Members of the TGF-β superfamily have numerous activities on different cell types, including in epithelial cells the ability to induce cell cycle arrest in G1 and to upregulate extracellular matrix proteins such as fibronectin and plasminogen activator inhibitor 1 (PAI-1) [1]. The cellular effects of each TGF-β are dependent on their binding to the extracellular domains of two types of transmembrane serine/threonine kinase receptor [2–8], known as type I (TGF-βRI) [9] and type II (TGF-βRII) [10] receptors. Two other types of receptor, type III (TGF-βRIII) [11] and type V (TGF-βRV) [12], may be involved in TGF-β signaling, although an essential role has not been established for either.

TGF-βRII is a constitutively active [13] homodimeric serine/threonine kinase [10] that undergoes autophosphorylation on serine [14] and tyrosine residues [15]. TGF-βRII is the upstream component in the signal transduction cascade — TGF-β first binds to TGF-βRII and then subsequently to TGF-βRI, recruiting it into the complex [13,16,17]. For TGF-β2, binding to either receptor requires the presence of both TGF-βRI and TGF-βRII [18], or both TGF-βRII and TGF-βRIII [19]. TGF-βRII phosphorylates TGF-βRI on serine residues in a glycine and serine-rich intracellular juxtamembrane region known as the GS box [13]. Receptor transphosphorylation is unidirectional in that TGF-βRII phosphorylates TGF-βRI but the converse does not occur [13,18]. Studies using ^{32}P-labeling and phosphopeptide mapping suggest that TGF-βRI does not undergo autophosphorylation *in vivo*, although it does *in vitro* [13,20]. Experiments with chimeric TGF-βRI constructs and a partially constitutively active mutant of TGF-βRI that signals in the absence of TGFβ–RII suggest that TGF-βRII is responsible for activating TGF-βRI and that TGF-βRI determines the specificity of response (that is, whether the cell responds to BMP, TGF-β, or activin) [17,21–24].

Following receptor transphosphorylation, the intracellular TGF-β effectors Smad2 and Smad3 transiently associate

with TGF-βRI and are phosphorylated by this receptor [25–29]. Phosphorylation of Smad2 and Smad3 occurs on an SSXS consensus motif (in single-letter amino acid code) on their carboxyl termini and this phosphorylation probably induces dissociation of the Smad substrate from TGF-βRI [26]. Smad6 and Smad7, both of which lack the carboxy-terminal SSXS consensus sequence, antagonize TGF-β superfamily signaling by associating stably with either type I receptors [30,31] or Smad4 [32]. Phosphorylation of Smad2 and Smad3 is likely to be necessary for at least some of the effects of TGF-β, because mutants of Smad2 or Smad3 that cannot be phosphorylated by TGF-βRI act as dominant-negative inhibitors of TGF-β signaling [25–27,33]. After their phosphorylation, Smad2 and Smad3 then associate with each other [27] and with Smad4, a common Smad required for signaling by TGF-β, activin, and BMP [28,34,35], to form a heteromeric complex that translocates to the nucleus [27,28,36]. Fusion proteins containing the DNA-binding domain of the yeast transcription factor Gal4 fused to the BMP effector Smad1 [29,37,38] or the TGF-β effector Smad2 have latent transcriptional activation activity that is unmasked by stimulation of BMP or TGF-β signaling, respectively [37,39], indicating that Smads can act as transcriptional activators. In the nucleus, Smad2 interacts directly with FAST-1 [40], a winged helix DNA-binding protein [41,42]. In some cases, Smads may directly bind DNA and activate transcription [43]. As FAST-1 binds to the activin-responsive element in the Mix.2 promoter [41], and a fly Smad (named Mad) binds the enhancer region of the *vestigial* gene in response to the TGF-β family member decapentaplegic (Dpp), resulting in *vestigial* expression [43], it is likely that activation and nuclear translocation of Smad2, Smad3, and Smad4 causes upregulation of TGF-β-responsive genes.

As TGF-βRII exists as a preformed homodimer on the cell surface [44,45] and as TGF-β binds to the TGF-βRII extracellular domain with a stoichiometry of 1:2 [46], TGF-β probably binds to a preformed TGF-βRII homodimer. Native gels of the active receptor complex reveal the existence of higher order oligomers of TGF-βRI and TGF-βRII [16]. TGF-βRI may bind to the $TGF\text{-}\beta RII_2$–TGF-β complex as a homodimer. It has been suggested that TGF-βRI is a preformed homodimer and that TGF-β induces the formation of a heterotetramer containing one TGF-βRI homodimer and one TGF-βRII homodimer [47]. Genetic evidence indicates that TGF-βRI has two necessary functions and these functions can be carried out by separate TGF-βRI molecules, consistent with this heterotetramer model [48].

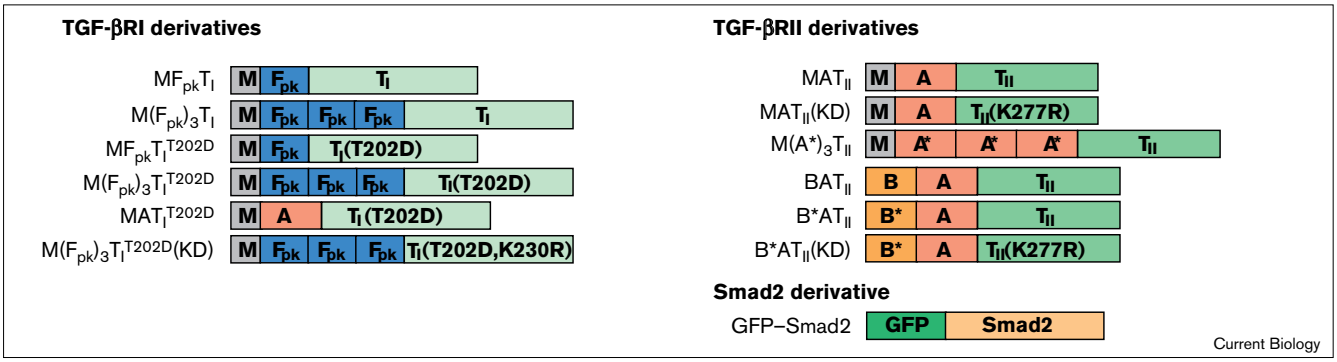
We sought to explore the mechanism of TGF-β receptor activation by inducing the homo-oligomerization and hetero-oligomerization of receptor cytoplasmic tails using cell-permeable chemical inducers of dimerization (CIDs) [49]. We aimed to determine the receptor oligomerization events that are sufficient to activate downstream components of the TGF-β pathway and to develop reagents that can reveal the physiological functions of TGF-β signaling pathways in transgenic organisms.

Results

Recruitment of a soluble TGF-βRII tail to a myristoylated TGF-βRI tail causes Smad2 nuclear translocation

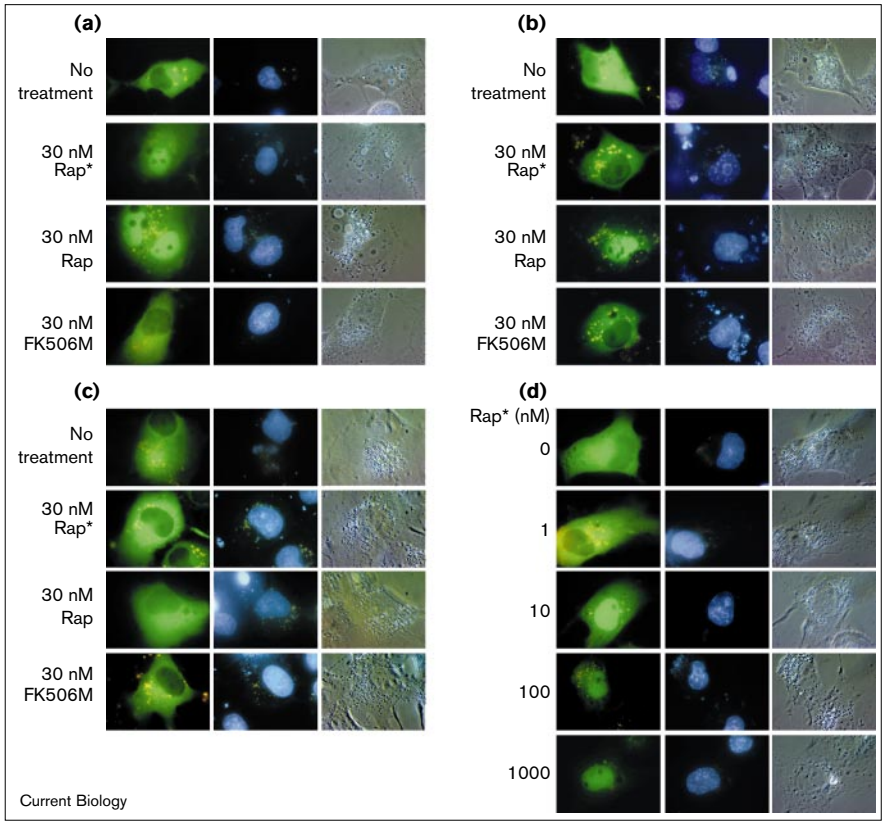
To monitor the level of receptor activation and the state of TGF-β signaling, we used a fusion protein comprising green fluorescent protein (GFP) and Smad2, referred to as GFP–Smad2 (Figure 1). When expressed in COS-1 cells, this protein is localized to the cytoplasm in the absence of a

Figure 1



Schematic representation of constructs encoding small-molecule-binding domain modules fused to TGF-β receptor cytoplasmic tails. The following abbreviations are used: M, 14 amino acid myristoylation sequence from c-Src; F_{pk}, G89P I90K double mutant of the 12 kDa FK506-binding protein, FKBP12; $(F_{pk})_3$, three tandem copies of F_{pk}; A, cyclophilin A; A*, the ATM triple mutant of cyclophilin A that binds cyclosporin and some cyclosporin derivatives [70]; $(A^*)_3$, three tandem copies of A*; B, the FKBP12–rapamycin-binding domain (FRB) [54] (residues 2025–2114) of the FKBP12–rapamycin-associated protein, FRAP; B*, triple mutant of FRB (FRB*) that binds both rapamycin and its non-toxic variant methallylrapamycin [53]; GFP, S65T mutant of green fluorescent protein; T_I, residues 153–501 of the rat TGF-βRI R4; T_I^{T202D}, T202D activated mutant of T_I; T_I^{T202D}(KD), T202D, K230R activated, kinase-dead double mutant of T_I; T_{II}, residues 193–567 of the human TGF-βRII; T_{II}(KD), K277R kinase-dead mutant of T_{II}.

Figure 2

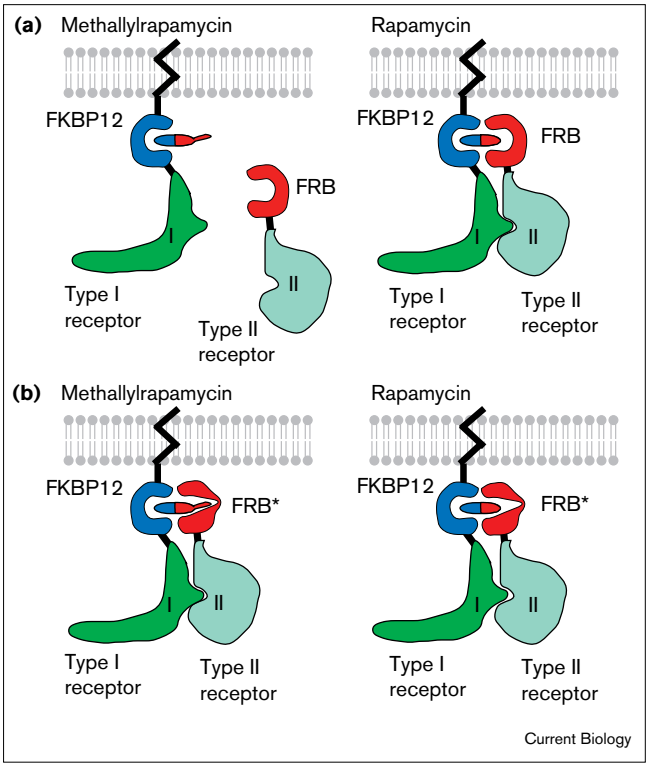


Recruitment of a soluble TGF-βRII cytoplasmic tail to a membrane-localized TGF-βRI cytoplasmic tail results in GFP–Smad2 nuclear translocation. The left column shows the GFP signal (fluorescein isothiocyanate filters), the center column shows the location of the Hoechst-33258-stained nucleus (no filters) and the right column shows a phase contrast image of the same cell. **(a)** With FRB*, both methallylrapamycin and rapamycin induce GFP–Smad2 nuclear translocation. COS-1 cells were transfected with plasmids encoding GFP–Smad2, B*AT_{II} and $MF_{pk}T_I$ and were either left untreated (no treatment) or stimulated for 1 h with 30 nM methallylrapamycin (Rap*), 30 nM rapamycin (Rap) or 30 nM FK506M. **(b)** With wild-type FRB, rapamycin but not methallylrapamycin induces GFP–Smad2 translocation. COS-1 cells were transfected with plasmids encoding GFP–Smad2, BAT_{II} and $MF_{pk}T_I$ and stimulated as for (a). **(c)** Recruitment of a kinase-dead type II receptor does not induce GFP–Smad2 nuclear translocation. COS-1 cells were transfected with plasmids encoding GFP–Smad2, B*AT_{II}(KD) and $MF_{pk}T_I$ and stimulated as for (a). **(d)** Dose-dependent translocation of GFP–Smad2 in response to the heterodimerizer methallylrapamycin. COS-1 cells were transfected with plasmids encoding GFP–Smad2, B*AT_{II} and $MF_{pk}T_I$ as for (a) and stimulated for 1 h with the indicated concentrations of methallylrapamycin.

TGF-β signal and to the nucleus in the presence of a TGF-β signal [50]. We created fusion proteins (Figure 1) containing the TGF-β receptor intracellular domains that could be inducibly associated using CIDs [49]. We constructed a fusion protein containing a myristoylation sequence (M), a G89P,I90K (single-letter amino acid code) double mutant (F_{pk}) [51] of the 12 kDa FK506-binding and rapamycin-binding protein (FKBP12) and the rat TGF-βRI [20] tail (T_I), creating $MF_{pk}T_I$ (Figure 1). We also fused the TGF-βRII cytoplasmic tail to a triple mutant of the FKBP12–rapamycin-binding (FRB) domain of the FKBP12–rapamycin-associated protein (FRAP) [52] (FRB*: the three mutations accommodate the large methallyl substituent of the 'bumped' rapamycin derivative, methallylrapamycin [53]) and to cyclophilin A (A), resulting in the soluble protein B*AT_{II} (Figure 1). We anticipated that these constructs could be conditionally associated using the heterodimerizers rapamycin [54] and its non-toxic and non-immunosuppressive synthetic variant methallylrapamycin [53]. The ability of the CIDs to cause the receptors to intervene in the TGF-β signaling pathway was determined by monitoring the subcellular localization of GFP–Smad2.

Transfection of COS-1 cells with $MF_{pk}T_I$, B*AT_{II} and GFP–Smad2 resulted in a non-nuclear localization pattern for GFP–Smad2 (Figure 2a). Treatment of these cells with 30 nM rapamycin or methallylrapamycin, both of which bind the mutant FRB* domain [53], resulted in translocation of GFP–Smad2 to the nucleus (Figure 2a), by recruiting the soluble TGF-βRII construct to the membrane-localized TGF-βRI construct. Furthermore, treatment with the FKBP12 antagonist FK506M [49] had no effect on the distribution of GFP–Smad2 (Figure 2a). To confirm that binding of the heterodimerizers rapamycin and methallylrapamycin to the FRB* domain is required for the nuclear translocation of GFP–Smad2, we replaced the FRB* domain in B*AT_{II} with the wild-type FRB domain (B), generating the construct BAT_{II} (Figure 1). COS-1 cells cotransfected with plasmids encoding $MF_{pk}T_I$, BAT_{II}, and GFP–Smad2 again showed a cytoplasmic distribution of GFP–Smad2 (Figure 2b). Treatment of these cells with 30 nM rapamycin, but not 30 nM methallylrapamycin or 30 nM FK506M, resulted in translocation of GFP–Smad2 into the nucleus (Figure 2b). This result is consistent with methallylrapamycin having a > 100-fold higher EC_{50} relative to rapamycin in a transcription activation assay using FKBP12 and wild-type FRB [53]. Rapamycin, but not methallylrapamycin, is able to bind BAT_{II} and recruit it to $MF_{pk}T_I$ (Figure 3a). We also tested a kinase-dead mutant of TGF-βRII, B*AT_{II}(KD)

Figure 3



A model for heterodimerizer-induced activation of TGF-β signaling **(a)** Recruitment of a soluble TGF-βRII to a membrane-localized TGF-βRI with rapamycin. Rapamycin is a natural heterodimerizer by virtue of its ability to bind both FKBP12 and FRB. The derivative methallylrapamycin has a synthetic 'bump' that prevents it from binding to wild-type FRB, and so methallylrapamycin cannot recruit the FRB–TGF-βRI fusion protein. **(b)** The triple mutant FRB* binds both methallylrapamycin and rapamycin. Thus, both molecules are able to function as heterodimerizers with the FKBP12 fusion and soluble FRB* fusion proteins.

(Figure 1). In this case, neither rapamycin nor methallylrapamycin treatment induced the nuclear accumulation of GFP–Smad2 (Figure 2c), indicating that the kinase activity of the type II receptor is required for GFP–Smad2 translocation. Finally, when we tested the dose response in this assay, we observed significant nuclear accumulation of GFP–Smad2 with just 10 nM methallylrapamycin (Figure 2d).

Homo-oligomerization of a TGF-βRI tail in the presence of a kinase-active TGF-βRII tail activates the PAI-1 promoter

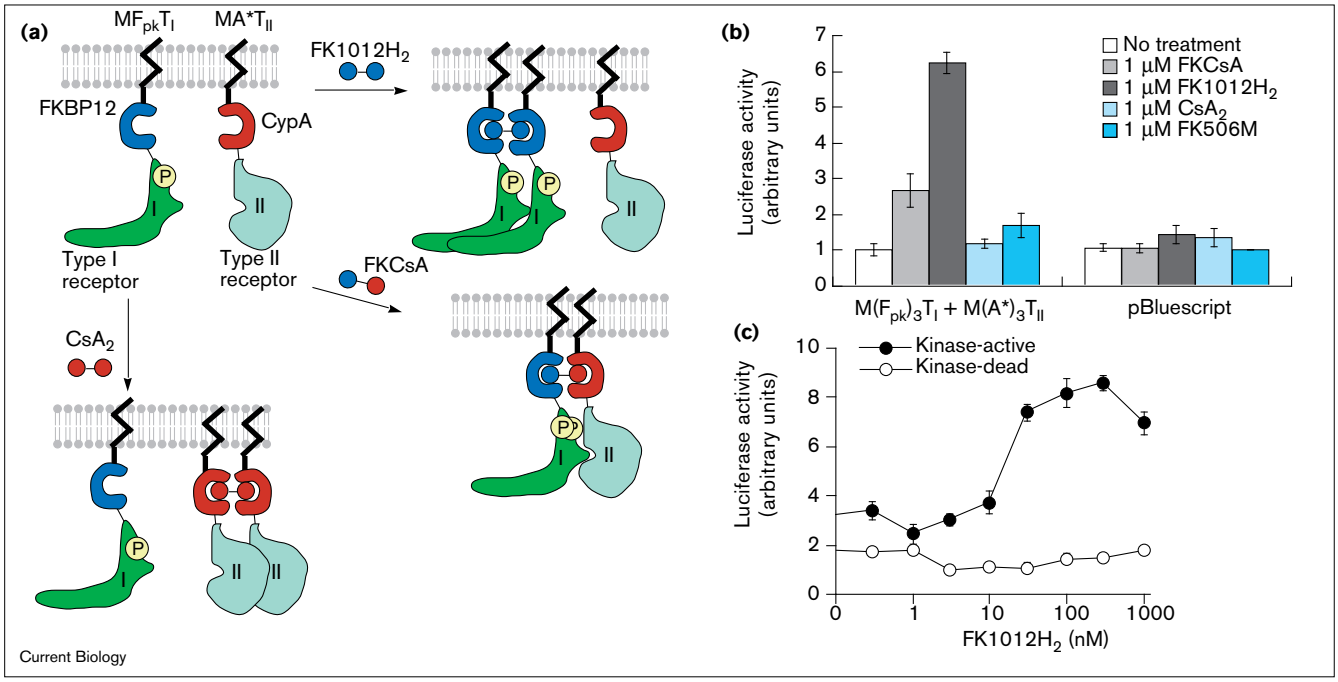
We wanted to homo-oligomerize or to hetero-oligomerize directly the cytoplasmic tails of the TGF-β receptors at the inner leaflet of the plasma membrane using CIDs. To this end, we generated a myristoylated construct containing three tandem F_{pk} domains and the cytoplasmic tail of the type I receptor ($M(F_{pk})_3T_I$) as well as a myristoylated construct with three cyclophilin domains and the cytoplasmic tail of TGF-βRII ($M(A^*)_3T_{II}$; Figure 1). We anticipated that by using the homodimerizer $FK1012H_2$ [55] (for all chemical structures, see Supplementary material published with this paper on the internet), we would oligomerize $M(F_{pk})_3T_I$, whereas using the homodimerizer CsA_2 [56] we would oligomerize $M(A^*)_3T_{II}$ and using the heterodimerizer FKCsA [57] we would form a hetero-oligomeric complex containing both $M(F_{pk})_3T_I$ and $M(A^*)_3T_{II}$ (Figure 4a). Using this strategy, we were able to homo-oligomerize one receptor tail in the presence of the other receptor tail.

To test these constructs, we used a transient transfection assay developed by Wrana *et al.* [58], in which the luciferase reporter gene is fused downstream of three phorbol ester responsive elements and the PAI-1 promoter–enhancer sequence. In mink lung cells, this reporter plasmid (p3TPLux) is activated upon stimulation of transfected cells with TGF-β [58]. We transfected Mv1Lu mink lung epithelial cells with plasmids encoding $M(F_{pk})_3T_I$, $M(A^*)_3T_{II}$ and p3TPLux and tested the effect on reporter gene activity of the dimerizers $FK1012H_2$, CsA_2, FKCsA and the control FKBP12 antagonist FK506M. The heterodimerizer FKCsA modestly activated p3TPLux whereas $FK1012H_2$ more potently activated this reporter and CsA_2 and FK506M had no effect (Figure 4b). The EC_{50} for $FK1012H_2$-induced activation of p3TPLux was 20 nM (Figure 4c). These data suggest that homo-oligomerization of the TGF-βRI cytoplasmic tail activates TGF-β signaling. However, activation of p3TPLux by $FK1012H_2$ required the presence of both TGF-βRI and TGF-βRII kinase-active tails (Figure 4c and data not shown). The nature of the binding domain (FRB, cyclophilin A or FKBP12) on the type II receptor did not affect activation by $FK1012H_2$ (data not shown). These results suggest that the TGF-βRI and TGF-βRII tails might transiently associate to some extent, especially as they bind to each other in the yeast two-hybrid interaction assay [59] and in coimmunoprecipitation experiments [60]. In this case, $FK1012H_2$ would be aggregating both activated and unactivated TGF-βRI tails, although only the activated TGF-βRI oligomers would signal. To simplify the interpretation of this result, we searched for a TGF-βRII-independent method of activating TGF-βRI.

A mutant TGF-βRI tail is activated by oligomerization in the absence of TGF-βRII

Wieser *et al.* [22] reported that a T204D mutant of human TGF-βRI is partially constitutively active, even in the absence of TGF-βRII. Furthermore, erythropoietin (Epo)-induced homodimerization of an Epo receptor extracellular domain fused to the human TGF-βRI(T204D) cytoplasmic domain results in increased growth inhibition of stably transfected Ba/F3 cells [47]. We tested the effect of the corresponding T to D mutation (T202D) in the rat TGF-βRI (known as R4), in the context of $M(F_{pk})_3T_I$, by generating the construct $M(F_{pk})_3T_I^{T202D}$ (Figure 1). This construct was not constitutively active, but was activated

Figure 4



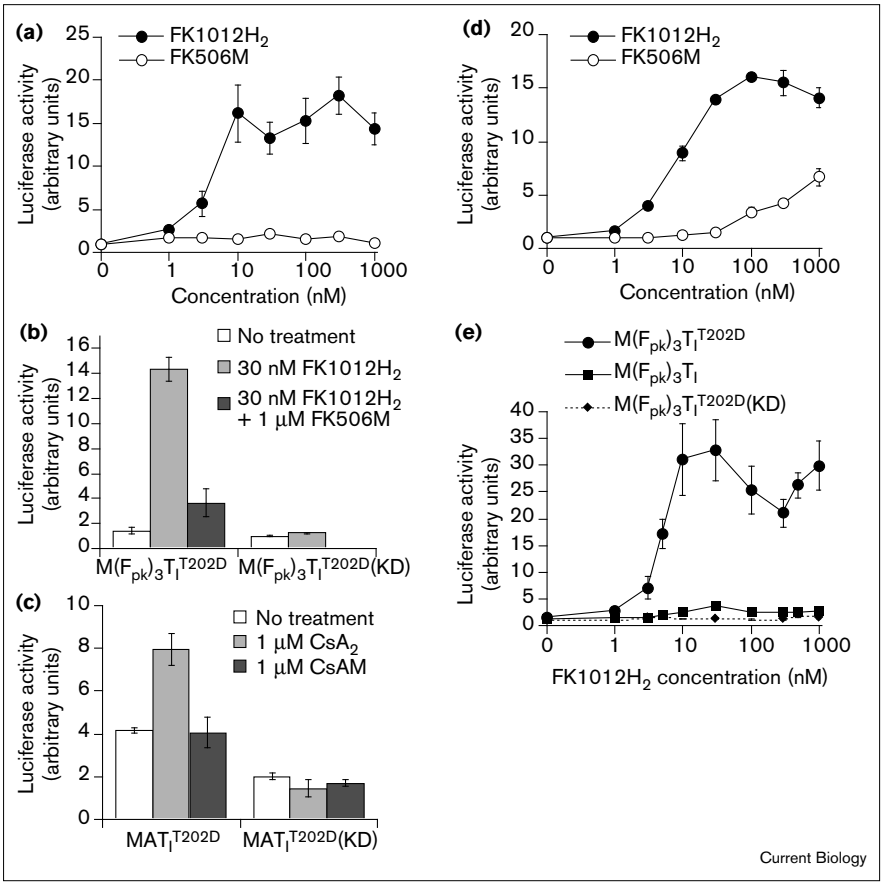
Oligomerization of TGF-βRI in the presence of a kinase-active TGF-βRII activates TGF-β signaling in mink lung cells. **(a)** A cartoon diagram of dimerizer-induced oligomerization of the TGF-β receptor cytoplasmic tails. Coexpression of the two receptor tails results in partial activation and phosphorylation (P) of the type I receptor, possibly because of transient associations between TGF-βRI and TGF-βRII cytoplasmic domains. FK1012H$_2$ oligomerizes MF$_{pk}$T$_I$, CsA$_2$ oligomerizes MA*T$_{II}$ and FKCsA hetero-oligomerizes the two receptor constructs. Single binding domain constructs are shown in the cartoon for simplicity, although some constructs described in the text have three tandem binding domains. **(b)** Type I receptor oligomerization causes activation of the TGF-β-responsive reporter construct p3TPLux. Mv1Lu cells were transiently transfected with the reporter p3TPLux and either plasmids encoding M(F$_{pk}$)$_3$T$_I$ and M(A*)$_3$T$_{II}$ or pBluescript. Transfected cells were treated with the indicated compounds and luciferase activity was measured. **(c)** TGF-β signaling is initiated at low concentrations of the dimerizer FK1012H$_2$. Dose-response curve for FK1012H$_2$-induced activation of the reporter p3TPLux. Mv1Lu cells were transfected with p3TPLux, and plasmids encoding M(F$_{pk}$)$_3$T$_I$ and either kinase-active M(A*)$_3$T$_{II}$ or kinase-dead M(A*)$_3$T$_{II}$(KD); the cells were stimulated with the indicated concentrations of FK1012H$_2$.

by oligomerization with FK1012H$_2$, even in the absence of TGF-βRII (Figure 5a). We ensured that endogenous TGF-βRII was not activating M(F$_{pk}$)$_3$T$_I^{T202D}$ by using DR26 cells, which have a defective endogenous TGF-βRII [58]. DR26 cells transfected with p3TPLux and a plasmid encoding M(F$_{pk}$)$_3$T$_I^{T202D}$ and treated with the dimer FK1012H$_2$, but not the monomeric antagonist FK506M, showed activation of p3TPLux (Figure 5a).

To ensure that activation of M(F$_{pk}$)$_3$T$_I^{T202D}$ by FK1012H$_2$ was due to oligomerization and not simple binding to FKBP12, we performed a competition experiment with FK506M. Simultaneous treatment of M(F$_{pk}$)$_3$T$_I^{T202D}$ and p3TPLux-transfected DR26 cells with FK1012H$_2$ and an excess of FK506M did not activate p3TPLux (Figure 5b), most probably because the monomer can compete with the dimer for binding to FKBP12 and thereby prevent FK1012H$_2$-induced oligomerization of M(F$_{pk}$)$_3$T$_I^{T202D}$. To demonstrate further that TGF-βRI cytoplasmic tail homodimerization resulted in activation of p3TPLux in the absence of FKBP12 ligands, we generated the construct MAT$_I^{T202D}$, in which there are no FKBP12 domains, but simply one cyclophilin domain fused to the T202D mutant of the cytoplasmic tail of TGF-βRI. This construct is somewhat constitutively active [50], but was activated further by treatment with the dimeric molecule CsA$_2$, but not the monomeric antagonist CsAM (Figure 5c).

We have also shown that the construct MF$_{pk}$T$_I^{T202D}$, containing only one F$_{pk}$ domain, can be activated by high concentrations of FKBP12 antagonists such as FK506M [50]. This construct was more potently activated by FK1012H$_2$, a homodimerizer (Figure 5d). However, the single FKBP12 construct MF$_{pk}$T$_I^{T202D}$ showed different dose-response curves for FK506M and FK1012H$_2$ (Figure 5d). The dimerizer FK1012H$_2$ had an EC$_{50}$ of 10–20 nM, as was the case when a TGF-βRII construct was cotransfected with a plasmid encoding M(F$_{pk}$)$_3$T$_I$. This is consistent with a gain-of-function induced by receptor dimerization with FK1012H$_2$, whereas the results with the antagonist FK506M, having an EC$_{50}$ of 300–500 nM, are consistent with a loss-of-function of the

Figure 5



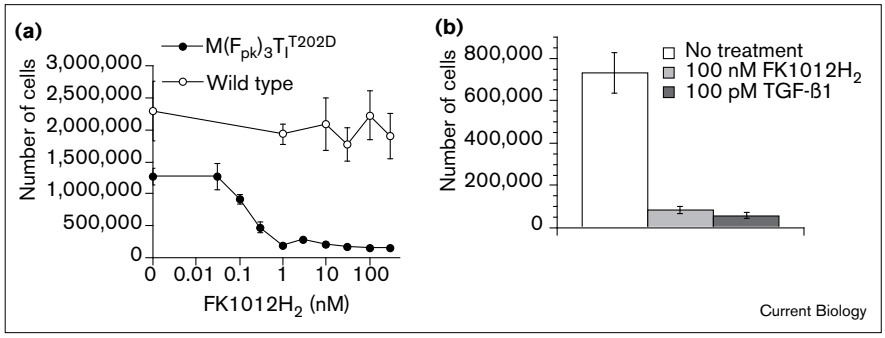
A mutant TGF-βRI is activated by homo-oligomerization in the absence of TGF-βRII. **(a)** The $M(F_{pk})_3T_I^{T220D}$ fusion protein is activated by homo-oligomerization. DR26 mink lung cells, lacking endogenous TGF-βRII, were transfected with plasmids encoding $M(F_{pk})_3T_I^{T202D}$ and p3TPLux and stimulated with the indicated concentrations of FK506M or $FK1012H_2$. Normalized luciferase activity produced is shown. **(b)** Competition with FK506M prevents activation by $FK1012H_2$. DR26 cells were transfected with $M(F_{pk})_3T_I^{T202D}$ or the kinase-dead $M(F_{pk})_3T_I^{T202D}$(KD) and either left untreated or treated with 30 nM $FK1012H_2$ or treated with both 30 nM $FK1012H_2$ and 1 μM FK506M. **(c)** MAT_I^{T202D} is activated by homodimerization. DR26 cells were transfected with p3TPLux and a plasmid encoding MAT_I^{T202D} and then either left untreated or treated with 1 μM CsA_2 or 1 μM CsAM. **(d)** The $MF_{pk}T_I^{T202D}$ fusion protein is potently activated by dimerization and modestly activated by FKBP12 antagonists. DR26 cells were transfected with p3TPLux and a plasmid encoding $MF_{pk}T_I^{T202D}$ and treated with the indicated concentrations of the dimerizer $FK1012H_2$ or the FKBP12 antagonist FK506M. **(e)** In the absence of TGF-βRII, only the mutant TGF-βRI is activated by oligomerization. DR26 cells were transfected with p3TPLux and a plasmid encoding $M(F_{pk})_3T_I^{T202D}$, $M(F_{pk})_3T_I^{T202D}$(KD) or $M(F_{pk})_3T_I$ and stimulated with the indicated concentrations of the dimerizer $FK1012H_2$.

fused FKBP12 as a repressor of T_I. Neither the kinase-dead construct $M(F_{pk})_3T_I^{T202D}$(KD) nor the wild-type construct $M(F_{pk})_3T_I$ was activated by $FK1012H_2$ (Figure 5e). Activation by FKBP12 antagonists, such as FK506M, was not observed with the triple FKBP12 constructs (Figure 5a) possibly because the three intramolecular F_{pk} domains either prevent spontaneous TGF-βRI homomeric interactions or cannot be competed away effectively by FKBP12 antagonists.

We also tested whether the myristoylation sequence was required for activation of $MF_{pk}T_I^{T202D}$ by $FK1012H_2$. We replaced the myristoylation sequence with an amino-terminal FLAG epitope tag, generating the soluble construct $F_{pk}T_I^{T202D}$. In the presence of 100 nM $FK1012H_2$, the soluble construct $F_{pk}T_I^{T202D}$ had half of the activity of the membrane-localized construct $MF_{pk}T_I^{T202D}$ (data not shown). We also found that heterodimerization of soluble TGF-βRI and TGF-βRII constructs with rapamycin could induce partial GFP–Smad2 nuclear translocation in COS-1 cells (data not shown). These results indicate that myristoylation increases the magnitude of dimerizer-induced activation of TGF-β signaling. However, we cannot determine from these experiments whether the soluble receptor constructs spontaneously associate with the plasma membrane or whether a weak TGF-β signal can emanate from the cytoplasm. Similar findings were reported in studies of small-molecule-activated conditional alleles of receptor tyrosine kinases [61].

The hallmark of TGF-β action in mink lung cells is G1 cell cycle arrest. To confirm that small-molecule dimerizers induce the chimeric TGF-β receptors described in this report to inhibit cell division, a mink lung cell line stably expressing $M(F_{pk})_3T_I^{T202D}$ was prepared. Treatment of this cell line with the homodimerizer $FK1012H_2$ resulted in potent growth inhibition with an EC_{50} of 200 pM (Figure 6a). In contrast, the dimerizer had no effect on proliferation of the parent cell line Mv1Lu (Figure 6a). The magnitude of growth inhibition was comparable to that achieved by TGF-β itself in the same cell line (Figure 6b). The lower EC_{50} for growth inhibition compared with p3TPLux activation probably represents the different sensitivities of these two assays, that is, 50% growth inhibition may be observed when 0.1% of receptors are activated whereas 50% reporter gene activation might

Figure 6



The dimerizer $FK1012H_2$ inhibits the growth of a stably transfected mink lung cell line. **(a)** Wild-type mink lung Mv1Lu cells and a stably transfected derivative containing the integrated construct $M(F_{pk})_3T_I^{T202D}$ were sparsely seeded and treated with the indicated concentrations of the homodimerizer $FK1012H_2$ in 1% serum. The number of live cells present at the end of the experiment is plotted. **(b)** The same procedure as for (a) was used, except the stable cell line was either left untreated or treated with 100 nM $FK1012H_2$ or 100 pM TGF-β1.

only be observed when 5% of receptors are activated. TGF-β1 itself has an EC_{50} of 25 pM for p3TPLux activation and 0.2 pM for growth inhibition (data not shown).

Discussion

There has been considerable interest in determining the minimal stoichiometric requirements of an active TGF-β receptor signaling complex. As TGF-βRII transphosphorylates and coimmunoprecipitates with TGF-βRI in a TGF-β-dependent fashion [13,18], and as TGF-βRI can be crosslinked to ^{125}I-labeled TGF-β only in the presence of TGF-βRII [59], it is likely that TGF-β directly dimerizes TGF-βRI and TGF-βRII. In addition, the observations that TGF-βRI and TGF-βRII are both biosynthesized and degraded at different rates [62,63], and that each receptor can be expressed stably on the cell surface in the absence of the other receptor [13], indicate that in mink lung cells, TGF-βRI and TGF-βRII do not exist in stable preformed heteromeric complexes. Through the ability of TGF-β to bind the extracellular domains of the two receptors simultaneously, the intracellular kinase domains of the receptors are brought into close proximity, thereby increasing the rate of the transphosphorylation reaction involving these domains. We have tested this model by directly dimerizing the cytoplasmic effector domains of TGF-βRI and TGF-βRII using the small-molecule heterodimerizers rapamycin and methallylrapamycin. Direct association of the cytoplasmic domains of the two receptors in this manner induced activation of a downstream signaling event — Smad2 nuclear translocation. This effect, as expected, was dependent on the kinase activity of TGF-βRII, indicating that the role of receptor heterodimerization is to increase the rate of the transphosphorylation reaction.

Several reports, including those describing fusion proteins of the cytoplasmic domain of TGF-βRI and TGF-βRII with the extracellular domain of either the granulocyte-macrophage colony stimulating factor α and β receptors [64] or the Epo receptor [47] have demonstrated that heterodimerization is a necessary step in TGF-β signal transduction [65]. Similarly, in our assays, homo-oligomerization of TGF-βRI or TGF-βRII fusion proteins expressed separately did not result in signaling activity (Figures 4c,5e and data not shown). In contrast, in the presence of the TGF-βRII tail, homo-oligomerization of the TGF-βRI tail caused activation of TGF-β signaling (Figure 4b). This effect can be explained by assuming a transient association between TGF-βRI and TGF-βRII tails, at least in the chimeric constructs used in our studies, which results in activation of TGF-βRI. This assumption is consistent with the observation that the cytoplasmic domains of the receptors signal constitutively when overexpressed together [50] and interact in yeast two-hybrid assays [59] and coimmunoprecipitation experiments [60]. The full-length receptors, containing transmembrane and extracellular domains, associate transiently in mink lung cells [13] but do not signal constitutively unless highly overexpressed. The ability to oligomerize one receptor in the presence of the other has allowed us to discern the importance of TGF-βRI oligomerization in TGF-β signaling. As TGF-βRI oligomerization is effective only in the presence of either TGF-βRII or an activating T202D mutation in TGF-βRI, and as it is known that TGF-βRII must phosphorylate and activate TGF-βRI, it is logical to conclude that either subsequent to or concomitant with TGF-βRII transphosphorylation of TGF-βRI, dimerization or oligomerization of TGF-βRI occurs.

We were not able to study the role of homomeric interactions in the GFP–Smad2 translocation assay because of the problem of intrinsic protein–protein interactions described above. We assume that TGF-βRI homomeric interactions are taking place constitutively in this assay, thereby removing the need to induce such interactions specifically. It has been reported that when transiently transfected, both TGF-βRI and TGF-βRII exist as homodimers on the surface of COS-1 cells [16]. However, in mink lung epithelial cells, lower expression levels result from the same transiently transfected plasmids. As a result, homo-oligomerization of a TGF-βRI cytoplasmic domain activated TGF-β signaling in this cell line (Figure 4b,c). We

also demonstrated that $M(F_{pk})_3T_I^{T202D}$ oligomerization, in the absence of a type II TGF-β receptor, is sufficient to activate TGF-β signaling in Mv1Lu cells (Figure 5a,b,e). These results imply that TGF-βRI homomeric interactions are likely to be necessary in the natural system. Although we were not able to observe synergistic activation by adding heterodimerizers and homodimerizers simultaneously (data not shown), our results involving TGF-βRI oligomerization strengthen the hypothesis that TGF-β signals through a heterotetrameric receptor complex rather than a heterodimeric complex [16,47,48].

The T202D mutation in TGF-βRI removed the requirement for co-expression of TGF-βRII in the reporter gene assay (Figure 5a), but not in the GFP–Smad2 translocation assay (data not shown). This seems to imply that the T202D mutation complements TGF-βRII deficiency for PAI-1 upregulation but not for Smad2 translocation. There is already evidence that downstream effects of TGF-β can be uncoupled at the level of TGF-βRI by mutation of specific serine or threonine residues. An S165E mutation in TGF-βRI led to an increase in growth-inhibitory activity and in extracellular matrix protein production, but to a decrease in apoptotic activity, and had no effect on induction of reporter gene activity [66]. Similarly, S172A or T176A mutations in TGF-βRI impaired the growth-inhibitory activity but not the extracellular matrix-producing activity of the receptor [67]. Although it has been reported that a dominant-negative version of Smad2 blocks activation of p3TPLux [26], this does not demand that Smad2 translocation is necessary for p3TPLux activation. To address this question, Smad2-deficient cells are needed to test activation of p3TPLux, similar to the studies in Smad4-deficient cells for investigating the role of Smad4 in p3TPLux activation [34]. However, the inability of the T202D mutation to complement completely TGF-βRII deficiency in the GFP–Smad2 translocation assay may reflect the fact that this mutation is only a partial mimic of TGF-βRII phosphorylation [22] and that these two assays may have different sensitivities and threshold levels. In addition, all of the endogenous Smad2 may translocate in mink lung cells in the presence of the T202D mutant, whereas only a fraction of the GFP–Smad2 protein overexpressed by cotransfection translocates in the COS-1 cell assay. To resolve this issue, we are currently studying stably transfected mink lung cells that express $M(F_{pk})_3T_I^{T202D}$ to determine whether endogenous Smad2 translocates in response to $FK1012H_2$, and to determine the mechanism of $FK1012H_2$-dependent signaling through the $M(F_{pk})_3T_I^{T202D}$ receptor in general. In the current study, these stably transfected cells were examined for their ability to undergo $FK1012H_2$-induced cell growth arrest, in comparison with the actions of TGF-β on the parental cell line. We found that this cell line undergoes growth inhibition in the presence of the dimerizer $FK1012H_2$ with an EC_{50} of 200 pM (Figure 6a) and that the level of growth inhibition is comparable to that achieved with TGF-β in the same cell line (Figure 6b). Luo and Lodish [47] found that Epo-induced homodimerization of a fusion protein comprising the extracellular domain of the Epo receptor and the intracellular domain of human TGF-βRI containing an activating T204D mutation caused growth inhibition of Ba/F3-transfected cells. Together, these results indicate that this acidic mutation can complement the absence of TGF-βRII in TGF-βRI-induced growth inhibition.

An important and unresolved issue is the mechanistic role of TGF-βRI homodimerization or homo-oligomerization. What is the immediate effect of TGF-βRI dimerization? One possibility is that upon dimerization, TGF-βRI autophosphorylates *in trans*, in analogy to the protein tyrosine kinase receptors. However, ^{32}P-labeling experiments have suggested that wild-type TGF-βRI does not undergo autophosphorylation *in vivo* [13], although it does *in vitro* [68]. Another possibility is that TGF-βRI homodimerization does not involve phosphorylation *per se*, but rather creates a docking site for downstream effectors such as Smad2 or Smad3, which must then be phosphorylated by TGF-βRI. Although we have ruled out a requirement for the mink type II TGF-β receptor phosphorylating the $M(F_{pk})_3T_I^{T202D}$ protein by using DR26 cells, we cannot rule out the possibility that other type II receptors, such as the activin type II receptor, are sufficiently promiscuous to phosphorylate the mutant chimeric type I receptor GS box. Nevertheless, we have demonstrated that two of the hallmarks of TGF-β signaling, namely growth inhibition and activation of the PAI-1 promoter, are induced by oligomerization of $M(F_{pk})_3T_I^{T202D}$. These results suggest that oligomerization of this single chimeric protein may activate all downstream effects of TGF-β. We are currently exploring further mechanistic aspects of small-molecule-induced signaling through this receptor.

Conclusions

We have demonstrated that a small-molecule-induced heteromeric interaction between cytoplasmic tails of TGF-βRI and TGF-βRII fusion proteins is sufficient for inducing Smad2 nuclear translocation. This translocation requires both receptors and does not result from two separate pathways each dependent on just one of the receptors. We have also shown that homo-oligomerization of the TGF-βRI cytoplasmic tail induces activation of TGF-β signaling in mink lung cells, either with the wild-type TGF-βRI tail in the presence of a kinase-active TGF-βRII tail, or with a T202D mutant of the TGF-βRI tail in the absence of a TGF-βRII tail. Oligomerization of this mutant TGF-βRI construct resulted in growth inhibition of a stable cell line. This suggests that in the natural system, TGF-βRI homomeric interactions may play an essential role in transducing the TGF-β signal. Furthermore, we have developed several different methods of

conditionally activating TGF-β signaling using small membrane-permeable dimerizers and designed chimeric variants of the TGF-β receptors. These methods of activating TGF-β signaling may be powerful in transgenic organisms expressing the variant receptors, providing a unique means to study the relevance of this pathway to physiological events and disease processes [69].

Materials and methods

DNA constructions

All constructs were prepared and expressed in the mammalian expression vector pBJ5.1. R4 was obtained from Patricia Donahoe. TGF-βRI(T204D), TGF-βRII and p3TPLux were obtained from Joan Massague. Murine Smad2 (pcsMadr2) was obtained from Richard Harland [36]. The plasmids pBJ5.1-E_gBE_u and pBJ5.1-$E_gB^*VE_u$ (E_g and E_u represent FLAG and influenza epitope tags, respectively) were obtained from Stephen Liberles. The construction of $MF_{pk}T_I$, $M(F_{pk})_3T_I$, $MF_{pk}T_I^{T202D}$, $M(F_{pk})_3T_I^{T202D}$, MAT_I^{T202D}, $M(F_{pk})_3T_I^{T202D}$(KD), MAT_{II}, MAT_{II}(KD) and GFP–Smad2 is described elsewhere [50] and in the Supplementary material. BAT_{II}, B^*AT_{II} and B^*AT_{II}(KD) were constructed by digesting pBJ5.1-E_gBE_u, pBJ5.1-$E_gB^*VE_u$ or pBJ5.1-$E_gB^*VE_u$ with *Sal*I and *Eco*RI and ligating in the *Xho*I, *Eco*RI fragment of MAT_{II}, MAT_{II} or MAT_{II}(KD), respectively.

Luciferase and GFP–Smad2 translocation assay

Luciferase and GFP–Smad2 translocation assays were performed as described elsewhere [50] and in the Supplementary material.

Growth inhibition assay

The wild-type Mv1Lu mink lung cell line or the stable cell line containing $M(F_{pk})_3T_I^{T202D}$ was seeded in 6-well clusters (20,000 cells per well) and allowed to attach overnight in 10% serum. Media was changed to 1% FBS with or without TGF-β or $FK1012H_2$. After 4 days, the cells were washed, trypsinized and counted. All experiments were performed in triplicate and repeated at least twice.

Supplementary material

Additional figures depicting the chemical structures of the small molecules used in these experiments and a cartoon diagram of growth inhibition in the stable cell line are published with this paper on the internet. Complete experimental details for assays and plasmid constructions are also published as Supplementary material.

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Supplementary material

Probing the role of homomeric and heteromeric receptor interactions in TGF-β signaling using small molecule dimerizers

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Materials and methods

Plasmid construction

All constructs were prepared and expressed in the mammalian expression vector pBJ5.1. R4, the rat TGF-βRI, was obtained from Patricia Donahoe and Tongwen Wang. The cDNA clones for the T204D mutant of human TGF-βRI and wild-type human TGF-βRII as well as the p3TPLux reporter were obtained from Joan Massague. $M(F_{pk})_3Eu$, MFEu and $MF_{pk}Eu$ were reported previously [14,20]. The pGEX2TK-Awt plasmid was generated previously (Karen Liu and S.L.S., unpublished). The S65T mutant of green fluorescent protein (J), was obtained from the plasmid JC3E (Martin Pruschy and S.L.S., unpublished). The murine Smad2 cDNA (pcsMadR2) was obtained from Richard Harland [23]. MAEu was generated by ligating the *Xho*I–*Sal*I fragment of pGEX2TK-Awt into *Xho*I, *Sal*I-digested $MF_{pk}Eu$. T_I was obtained by PCR amplification of residues 153–501 of R4 using primers Hp8 (5'-GCC ATA ACC GCA CTG TCA TTC TCG AGC ACC ACC GCG TGC CA-3') and Hp12 (5'-GG AAT GTC TAG AGA ATTC TTA CAT TTT GAT GCC TTC CTG TTG G-3'). The PCR product was subcloned into pBS3, a pBluescript IISK- derivative in which the *Kpn*I site was destroyed by Klenow fill-in and blunt-end ligation, to create pBS3-T_I. This and all other PCR products were sequenced using dideoxy sequencing on both strands to ensure that no unwanted mutations had been introduced. The T_I fragment was subcloned into the pBJ5.1-based expression vectors MAEu, $MF_{pk}Eu$, and $M(F_{pk})_3Eu$ with *Xho*I and *Eco*RI sites, to generate MAT_I, $MF_{pk}T_I$ and $M(F_{pk})_3T_I$, respectively. The T_I^{T202D} fragment was generated with an amino-terminal FLAG epitope tag by overlap extension PCR using R4 as template and primers B286-1 (5'-TTA GGT ACC CTC GAG GAT TAT AAA GAT GAC GAT GAT AAA CAC CAC CGC GTG CCA AAT-3'), B286-2 (5'-GCT TTC TTG TAG CAC AAT GTC CCT TGC AAT TGT TCT TTG AAC AAG C-3'), B286-3 (5'-GAC ATT GTG CTA CAA GAA AGC ATC GGC-3') and B286-6 (5'-TTC CTT GGG TAC CAA CAA TCT CCA TG-3') and subcloned into pBS3-T_I with *Xho*I and *Kpn*I to generate pBS3-T_I^{T202D}. The T_I^{T202D} fragment was subcloned into pBJ5.1-$MF_{pk}Eu$, pBJ5.1-$MF_{pk}Eu$ and pBJ5.1-MAEu with *Xho*I and *Eco*RI to generate pBJ5.1-$MF_{pk}T_I^{T202D}$, pBJ5.1-$M(F_{pk})_3T_I^{T202D}$ and pBJ5.1-MAT_I^{T202D}, respectively. T_I^{T202D}(KD) was generated by overlap extension PCR using pBS3-T_I^{T202D} as template and primers B286-1, B286-4 (5'-CTT CTC TAG AAG AGA ATA TCC TCA CGG CAA CTT CTT CTC-3'), B286-5 (5'-AGG ATA TTC TCT TCT AGA GAA GAA CGT TCA TGG-3') and B286-6. The PCR product was subcloned into pBS3-T_I with *Xho*I and *Kpn*I to generate pBS3-T_I^{T202D}(KD) and the T_I^{T202D}(KD) fragment was subcloned into pBJ5.1-$MF_{pk}Eu$ and pBJ5.1-$M(F_{pk})_3Eu$ with *Xho*I and *Eco*RI to generate pBJ5.1-$MF_{pk}T_I^{T202D}$(KD) and pBJ5.1-$M(F_{pk})_3T_I^{T202D}$(KD), respectively. A general GFP expression vector pBJ5.1-JEg was generated from pBJ5.1-JC3Eu by replacing the *Xho*I, *Eco*RI fragment with a new fragment formed by annealing and ligating oligos B92-1 (5'-TCG AGG GGG ATT ATA AAG ATG ATG ATG ATA AAG TCG ACG GGG CAA- CAG-3') and B92-2 (5'-AAT TCT GTT GCC CCG TCG ACT TTA TCA TCA TCA TCT TTA TAA TCC CCC-3'). Murine Smad2 was amplified by PCR using pcsMADr2 as template and primers B214-1 (5'-TTT CTC GTC GAC ATG TCG TCC ATC TTG CCA TTC ACT CCG-3') and B214-2 (5'-TTT CTC GAA TTC TTA CGA CAT GCT TGA GCA TCG CAC TGA AGG-3') and subcloned into pBJ5.1-JEg using *Sal*I and *Eco*RI to generate pBJ5.1-JEgSmad2 (referred to as GFP–Smad2 in the text). TGF-βRII was subcloned into pBluescriptIISK- (Stratagene) with *Bam*HI to generate pBS-TGF-βRII. Residues 193–567 (up to the carboxyl terminus) of TGF-βRII were amplified by PCR using pBS-TGF-βRII as template and primers B5 (5'-CTA CTG CTA CCG CGT TAA CCT CGA GCG GCA GCA GAA GCT GAG-3') and B4 (5'-GGT GAG AGG GGC AGC CTC TCT AGA CAT GCC CAG CCT GCC CCA TAA GAG CTA GTC GAC TTT GGT AGT GTT TAG GG-3'). The *Xho*I, *Sal*I-digested PCR product was first subcloned into pBluescriptIISK to generate pBS-T_{II} and then subcloned into the *Sal*I site of pBJ5.1-MAEu or pBJ5.1-$M(A^*)_3Eg$ to generate pBJ5.1-MAT_{II} or pBJ5.1-$M(A^*)_3T_{II}$, respectively. A kinase-dead K277R mutant of T_{II} (T_{II}(KD)) was generated by PCR amplification using primers B5 and B53-1 (5'-TTC ATC GGA TCC TGA CTG CCA CTG TCT CAA ACT GC-3'), digestion with *Xho*I and *Bam*HI and subsequent ligation into *Xho*I, *Bgl*II-digested pBS-T_{II}. The *Xho*I, *Sal*I fragment T_{II}(KD) was subcloned into pBJ5.1-MAEu or pBJ5.1-$M(A^*)_3T_{II}$ to generate pBJ5.1-MAT_{II}(KD) and pBJ5.1-$M(A^*)_3T_{II}$(KD), respectively.

GFP–Smad2 translocation assay

COS-1 cells were obtained from Patricia Donahoe and maintained in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/ml penicillin G sodium and 100 μg/ml streptomycin sulfate. For the GFP–Smad2 translocation assay, 1.3×10^5 COS-1 cells were seeded on coverslips in 8 well dishes. For transfection of each coverslip, 300 ng GFP–Smad2, 850 ng type I receptor (e.g. $MF_{pk}T_I$) and 850 ng type II receptor (e.g. MAT_{II}) were mixed with 5.5 μl 2 mg/ml Lipofectamine (GibcoBRL) in 1.0 ml phenol red free and serum free DMEM (VDMEM), incubated at room temperature for 30 min and then added to a coverslip which had been washed twice with VDMEM. The cells were incubated with the DNA/Lipofectamine mixture for 4 h at 37° C with 5% CO_2 and then washed twice with VDMEM and incubated 16–20 h in growth medium at 37°C with 5% CO_2. At the end of this time, the cells were washed twice with VDMEM and incubated with the desired reagents (e.g. FK506M, rapamycin or TGF-β) and 500 ng/mL Hoechst 33258 in 1 ml VDMEM. After 1 h, 20 μl VDMEM reagent solution was placed on a microscope slide and the coverslip was inverted onto this droplet. The live cells were viewed through a 100× oil immersion objective on a Leitz Laborlux S 100W Hg fluorescence microscope and images were captured with a Hitachi HV-C12 CCD camera using Flashpoint FPG v2.50 software. Fluorescein filters were used to record GFP images and no filter was used for Hoechst 33258 images. Images shown in the figures are representative of most cells in a given experiment. Each experiment was performed multiple times and the results were consistent.

Luciferase assay

Mv1Lu mink lung epithelial cells were obtained from the American Type Culture Collection (catalog #CCL64) and DR26 cells were obtained from Joan Massague. Both cell lines were cultured in DMEM with 10% FBS, 100 units/ml penicillin G sodium, 100 μg/ml streptomycin sulfate and 100 μM each of the amino acids Ala, Asp, Glu, Gly, Asn, and Pro (three-letter amino acid codes). 2×10^5 mink lung cells (either Mv1Lu or DR26) were seeded in each well of a 6 well dish. After 24 h, cells were transfected using DEAE-dextran. For each well, 250 ng p3TPLux DNA was mixed with 250 ng each receptor DNA (750 ng total DNA), 6 μl 10 mM chloroquine, 7.5 μl 10 mg/ml DEAE-dextran and 600 μl minimal essential medium (MEM) supplemented with the nonessential amino acids (NEAA) and incubated at room temperature for 10 min. The cells were washed twice with MEM and the DNA complexes were added to the cells and incubated at 37°C with 5% CO_2 for 3 h. The cells were then shocked with 1 ml of 10% DMSO in PBS for 2 min, washed twice with Hank's Balanced Salt Solution (HBSS) and incubated in growth media at 37 °C with 5% CO_2. After 20 h, the cells were washed once and incubated in DMEM containing 0.2% FBS and 100 μM each of the amino acids Ala, Asp, Glu, Gly, Asn, and Pro and

the reagent of interest (e.g. FK506M, rapamycin or TGF-β) for 25–30 h. Cells were incubated on ice for 15 min, washed three times with HBSS and lysed in extraction buffer (25 mM glycylglycine, pH 7.8, 15 mM $MgSO_4$, 4 mM EGTA, 1% Triton X, 1 mM DTT, 1 mM PMSF) by shaking gently at 4 °C for 30 min. The lysates were centrifuged for 5 min at 10,000 × *g* at 4 °C and stored on ice. Lysate (100 µl) was added to 150 µl assay mixture (25 mM glycylglycine pH 7.8, 15 mM MgSO4, 4 mM EGTA, 15 mM K_2HPO_4 pH 7.8, 1 mM DTT, 4 mM ATP) and 150 µl luciferin buffer (25 mM glycylglycine pH 7.8, 15 mM $MgSO_4$, 4 mM EGTA, 10 mM DTT, 167 µM D-luciferin). This mixture was placed in a 500 µl microfuge tube inside a glass scintillation vial, and luminescence was detected by counting in single photon mode (SPM) on a Beckman LS 6500 liquid scintillation counter for 15 sec. The error bars reported represent plus or minus one standard deviation. All experiments were performed multiple times in triplicate

Figure S1

Structures of the homodimerizers $FK1012H_2$ and CsA_2, the heterodimerizers FKCsA and rapamycin, and the FKBP12 and cyclophilin antagonists FK506M and CsAM, respectively.

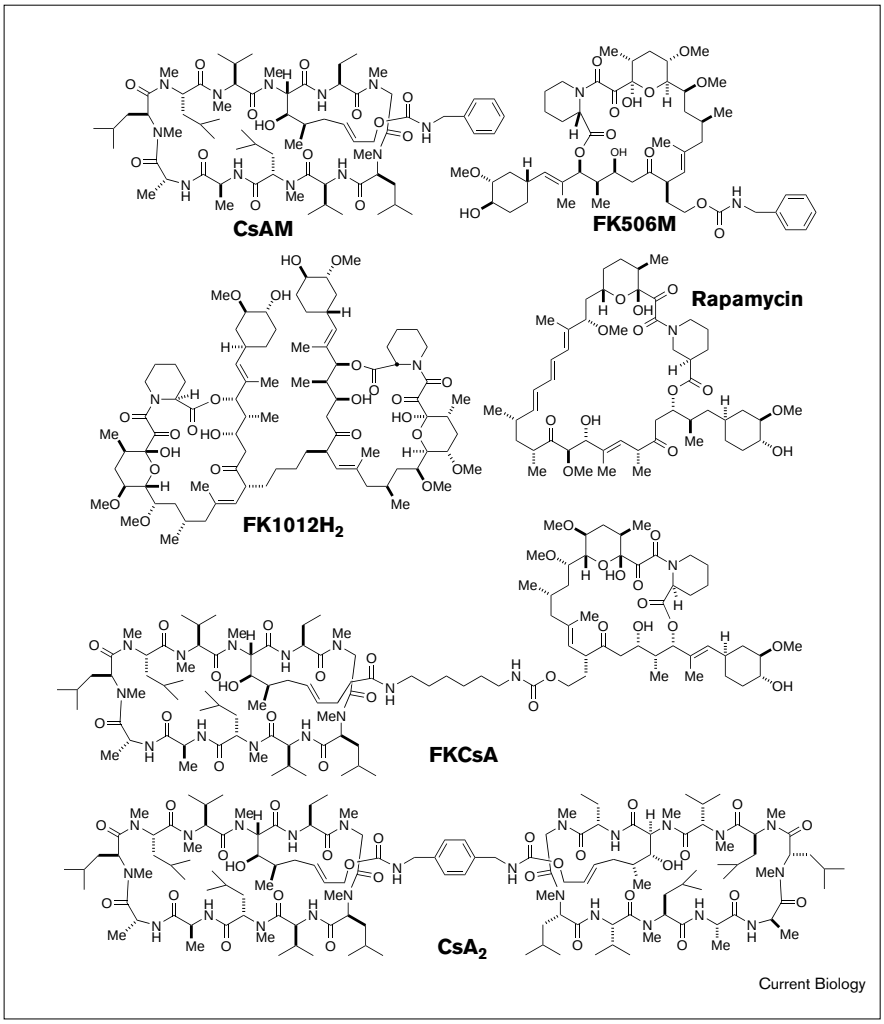

Figure S2

A cartoon diagram of FK1012H_2-induced oligomerization of the $MF_{pk}3T_I^{T202D}$ protein stably expressed in mink lung cells. Oligomerization of the type I receptor tail inhibits the growth of the stable cell line. For simplicity, a single FKBP12 domain is shown, although the construct $M(F_{pk})_3T_I^{T202D}$ contains three tandem FKBP12 domains.