TGF-β-signaling with small molecule FKBP12 antagonists that bind myristoylated FKBP12–TGF-β type I receptor fusion proteins

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Background: Growth arrest in many cell types is triggered by transforming growth factor beta (TGF-β), which signals through two TGF-β receptors (type I, TGF-βRI, and type II, TGF-βRII). In the signaling pathway, TGF-β binds to the extracellular domain of TGF-βRII, which can then transphosphorylate TGF-βRI in its glycine/serine (GS)-rich box. Activated TGF-βRI phosphorylates two downstream effectors, Smad2 and Smad3, leading to their translocation into the nucleus. Cell growth is arrested and plasminogen activator inhibitor 1 (PAI-1) is upregulated. We investigated the role of the immunophilin FKBP12, which can bind to the GS box of TGF-βRI, in TGF-β signaling.

Results: Overexpression of myristoylated TGF-βRI and TGF-βRII cytoplasmic tails caused constitutive nuclear translocation of a green-fluorescent-protein–Smad2 construct in COS-1 cells, and constitutive activation of a PAI-1 reporter plasmid in mink lung cells. Fusing FKBP12 to TGF-βRI resulted in repression of autosignaling that could be alleviated by FK506M or rapamycin (two small molecules that can bind to FKBP12). Mutation of the FKBP12-binding site in the FKBP12–TGF-βRI fusion protein restored constitutive signaling. An acidic mutation in the FKBP12–TGFβRI protein allowed FKBP12 antagonists to activate signaling in the absence of TGF-βRII. Further mutations in the TGF-βRI FKBP12-binding site resulted in TGF-β signaling that was independent of both TGF-βRII and FKBP12 antagonists.

Conclusions: Fusing FKBP12 to TGF-βRI results in a novel receptor that is activated by small molecule FKBP12 antagonists. These results suggest that FKBP12 binding to TGF-βRI is inhibitory and that FKBP12 plays a role in inhibiting TGF-β superfamily signals.

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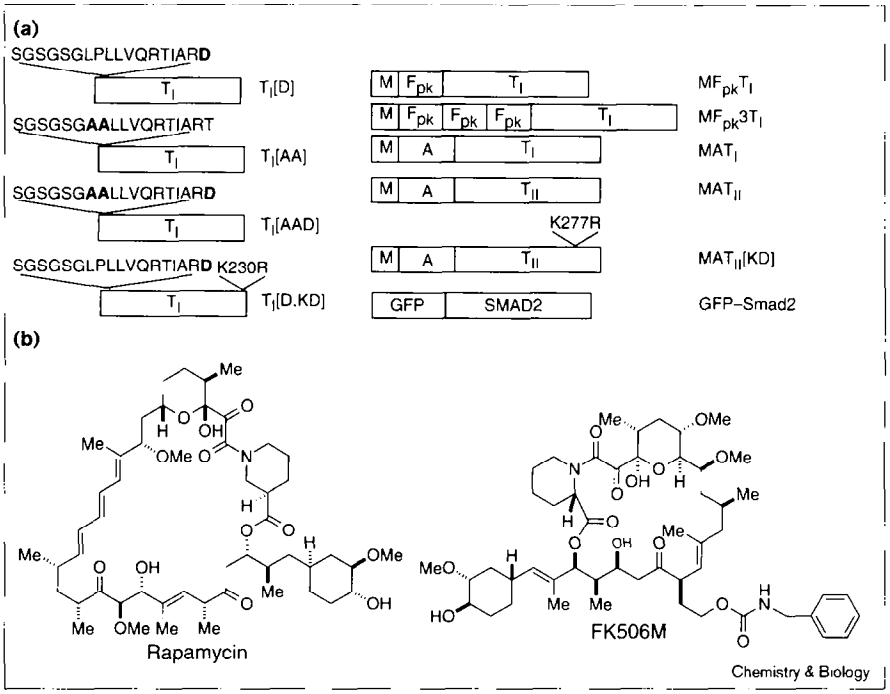
Introduction

The transforming growth factor beta (TGF-β) superfamily consists of numerous secreted dimeric polypeptides with a cysteine-knot fold [1–3]. This diverse superfamily includes factors that regulate sexual development (activins, inhibins, Müllerian-inhibiting substance [MIS]), embryonic patterning (decapentaplegic [DPP], nodal, bone morphogenetic proteins [BMPs], vegetal hemisphere protein [Vg-1], growth and differentiation factors [GDFs] and activin), bone formation (TGF-βs, BMPs) and neuronal survival (GDNF, neurturin and persephin) [4]. TGF-β itself is a multifunctional polypeptide signaling factor that can regulate cell proliferation, differentiation, apoptosis and expression of extracellular matrix proteins, such as plasminogen activator inhibitor 1 (PAI-1) [4]. Two distinct serine/threonine kinase receptors, referred to as type I (TGF-βRI) and type II (TGF-βRII) TGF-β receptors, are necessary and sufficient for transducing most TGF-β-induced signals across the plasma membrane of a target cell [4–6]. TGF-β initially binds to TGF-βRII and forms a complex that can subsequently recruit TGF-βRI [7]. Smad proteins constitute a novel class of signaling proteins that function as downstream effectors of TGF-β signaling by transmitting the TGF-β signal from the plasma membrane to the nucleus of a target cell. Smad1 and Smad5 are downstream of the BMP receptors [8,9], Smad2 and Smad3 are components of the TGF-β and activin signaling pathways [10,11] and Smad4 is a common factor required for TGF-β, activin and BMP signaling [12]. In addition, Smad6 and Smad7 inhibit TGF-β signaling [13–15]. Upon TGF-β stimulation, TGF-βRII phosphorylates TGF-βRI in the GS box, a juxtamembrane region of TGF-βRI rich in glycine and serine residues. Activated TGF-βRI phosphorylates Smad2 and Smad3 [11,16,17], which then associate with each other, as well as with Smad4 [12,18,19]. Smad2, Smad3 and Smad4 all subsequently translocate from the cytoplasm to the nucleus [17,18], where they activate the transcription of TGF-β-responsive genes [10,20–22].

Although the identification of Smad proteins has illuminated a crucial aspect of TGF-β signaling, the role of accessory factors is not fully understood. Three proteins have been shown to associate with the TGF-β receptors in yeast two-hybrid experiments: the $p21^{RAS}$ farnesyltransferase α

Figure 1



Constructs and structures of small molecules used in this study. **(a)** Schematic of fusion proteins with linked immunophilins and TGF-β receptor cytoplasmic tails. The following abbreviations are used: TGF-β, transforming growth factor beta; TGF-βRI, type I TGF-β receptor; TGF-βRII, type II TGF-β receptor; M, 14 amino-acid myristoylation sequence from c-Src [34]; F, wild-type 12 kDa FK506-binding protein (FKBP12); F_{pk}, Gly89→Pro, Ile90→Lys (PK) double mutant of FKBP12 [35]; A, cyclophilin A; GFP, Ser65→Thr mutant of green fluorescent protein; T_I, residues 153–501 of rat TGF-βRI (known as R4); T_I[D], Thr202→Asp mutant of T_I; T_I[AA], Leu191→Ala, Pro192→Ala double mutant of T_I; T_I[AAD], Leu191→Ala, Pro192→Ala, Thr202→Asp triple mutant of T_I; T_I[D,KD], Thr202→Asp, Lys230→Arg kinase-dead double mutant of T_I; T_{II}, residues 193–567 of human TGF-βRII; T_{II}[KD], Lys277→Arg (K277R) kinase-dead mutant of T_{II}. **(b)** Structures of the small molecule FKBP12 antagonists rapamycin and FK506M.

subunit (FTα) [23–25] and the immunophilin FK506-binding protein 12 (FKBP12) interact with TGF-βRI [26], whereas TGF-β receptor-interacting protein-1 (TRIP-1) interacts with TGF-βRII [27]. Because overall protein isoprenylation levels are not affected by TGF-β and L744-832, an inhibitor of FTα activity, does not affect TGF-β-induced gene responses, the enzymatic activity of FTα is not likely to be involved in TGF-β signaling [24]. Thus far, the physiological relevance of TRIP-1 has not been established. The third potential accessory factor, FKBP12, was shown to associate, in a yeast two-hybrid assay, with the cytoplasmic domain of all type I TGF-β superfamily receptors tested [28]. It was also shown that cell-permeable, small molecule FKBP12 antagonists can prevent the association of FKBP12 with TGF-βRI in both yeast two-hybrid [26] and coimmunoprecipitation experiments [28,29]. Several reports [29–31] have shown that mutant type I receptors that do not bind FKBP12 can still transduce some or all of TGF-β's effects, demonstrating that FKBP12 association with TGF-βRI is not required for signaling.

It has been suggested that FKBP12 negatively regulates TGF-βRI function [28,30,31]. Support for this hypothesis comes from the findings that FKBP12 is displaced from TGF-βRI after stimulation by TGF-β [28,31], that FKBP12-binding-defective mutants of TGF-βRI show an increased tendency to be activated spontaneously in the absence of TGF-β [30,31], and that binding of FKBP12 to TGF-βRI correlates with inhibition of TGF-β signaling [31]. The hypothesis that FKBP12 plays an important role in TGF-β signaling remains controversial, however. We show that fusing FKBP12 to myristoylated forms of the type I receptor cytoplasmic tail (Figure 1a; myristoylation is a post-translational modification that adds a fatty acid to a protein, which targets the protein to the cell membrane) results in fusion proteins whose ability to intervene in the TGF-β signaling pathway can be regulated by small molecule FKBP12 antagonists (Figure 1b). The results demonstrate that FKBP12 can repress TGF-β type I receptor signaling activity in fusion proteins and that its repressive actions can be prevented by small molecules that interfere with FKBP12–TGF-βRI interactions.

Results

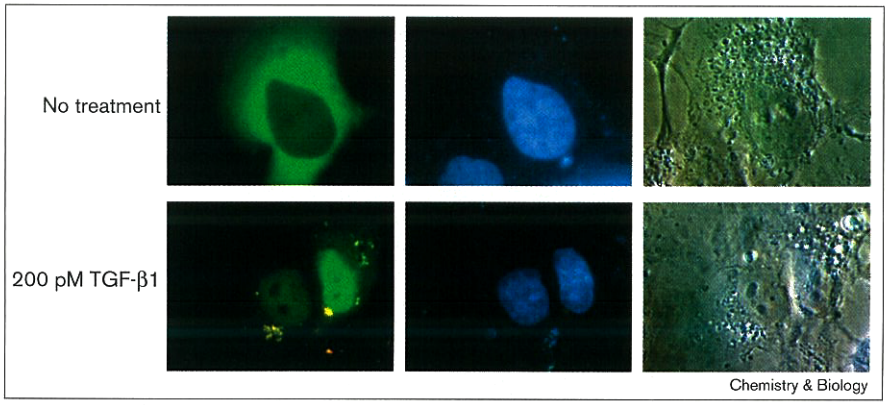
Two assays for TGF-β receptor activation

We used two assays to determine whether chimeric TGF-β receptors had transduced a TGF-β-like signal. First, we used the reporter plasmid p3TPLux, developed by Massague and coworkers [32], which contains a synthetic TGF-β-responsive promoter that consists of three phorbol-response elements and the PAI-1 promoter/enhancer. The plasmid has been used to measure the amount of extracellular matrix protein production induced by TGF-β [32].

We also monitored the subcellular localization of a green fluorescent protein (GFP)–Smad2 fusion protein (Figure 1a). Cotransfection of full-length, wild-type TGF-βRI and TGF-βRII along with GFP–Smad2 into COS-1 cells resulted in a cytoplasmic fluorescent signal that

Figure 2

GFP–Smad2 translocates to the nucleus in response to TGF-β1 in live COS-1 cells. COS-1 cells were transiently transfected with GFP–Smad2, wild-type TGF-βRI and wild-type TGF-βRII, stained with Hoechst 33258 to identify the nucleus and treated with or without 200 pM TGF-β1 for one hour. The left column shows the GFP signal (FITC filters), the center column shows the location of the Hoechst-stained nucleus (no filters) and the right column shows a phase-contrast image of the same cell. The images shown are representative of most transfected cells in each experiment. Magnification is 1000 X.



migrated to the nucleus following treatment with 200 pM TGF-β1 (Figure 2). As expected, cotransfection of both wild-type receptors was required for GFP–Smad2 nuclear translocation (data not shown). GFP alone did not migrate under any of the conditions tested (data not shown), indicating that Smad2 was required for nuclear translocation.

Figure 3

Myristoylated TGF-β receptor cytoplasmic tails signal constitutively. **(a)** GFP–Smad2 translocation is constitutive when myristoylated cyclophilin-A–TGF-βRI and cyclophilin-A–TGF-βRII fusion proteins (MAT_I and MAT_{II}) are coexpressed, but does not occur with a kinase-dead type II receptor construct MAT_{II}[KD]. COS-1 cells were transfected with GFP–Smad2, MAT_I and either kinase-active MAT_{II} or kinase-dead MAT_{II}[KD] and viewed live under a fluorescence microscope. Images were obtained as described in Figure 2. **(b)** Myristoylated TGF-β receptor cytoplasmic domains activate the PAI-1 promoter. Mv1Lu mink lung epithelial cells were transfected with the PAI-1-based reporter p3TPLux, MAT_I and either MAT_{II} or MAT_{II}[KD] and treated with nothing or 1 μM FK506M. Luciferase activity induced from p3TPLux is shown. Error bars represent one standard deviation. **(c)** A model for constitutive TGF-β signaling. Overexpressing the cytoplasmic domains of the receptors results in constitutive TGF-β signaling because of their natural affinity for one another. The cyclophilin domains have no effect on TGF-β signaling. CypA, cyclophilin A; P, phosphate group.

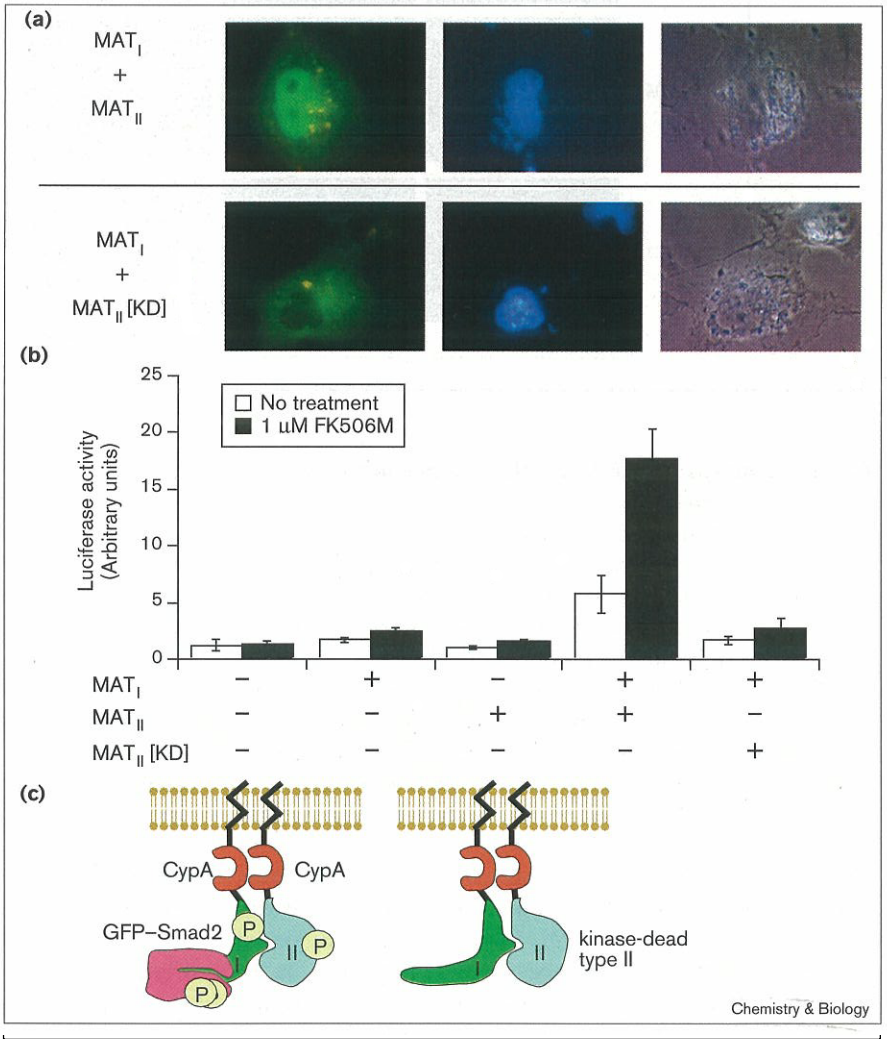
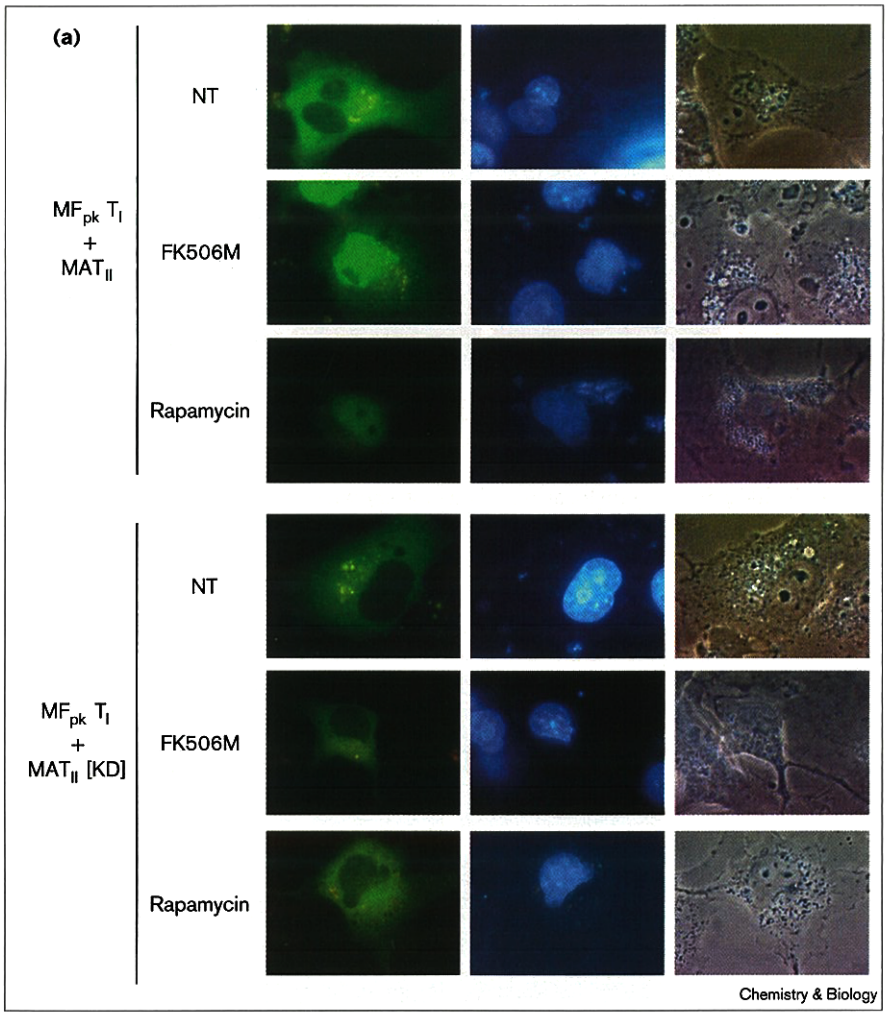


Figure 4



Small molecule FKBP12 antagonists mimic TGF-β in the presence of two fusion proteins: a myristoylated PK mutant of FKBP12 fused to the TGF-βRI cytoplasmic tail and a myristoylated TGF-βRII cytoplasmic tail. **(a)** Nuclear translocation of GFP–Smad2 in response to FK506M or rapamycin. COS-1 cells were transfected with GFP–Smad2, $MF_{pk}T_I$, and either MAT_{II} or MAT_{II}[KD], treated with the indicated compounds for one hour and viewed live under a fluorescence microscope. Images were obtained as described in Figure 2. **(b)** FK506M and rapamycin activate the PAI-1 promoter. Mv1Lu cells were transfected with the PAI-1-based reporter p3TPLux, $MF_{pk}T_I$ and either MAT_{II} (kinase active) or MAT_{II}[KD] (kinase dead) and treated with nothing (NT), 1 μM FK506M or 1 μM rapamycin (rap) for 24 h. Cells were lysed and luciferase activity was measured. **(c)** Dose-response for GFP–Smad2 nuclear translocation in response to rapamycin treatment in the presence of $MF_{pk}T_I$ and MAT_{II}. COS-1 cells were transfected as in (a), treated with the indicated concentrations of rapamycin for one hour and photographed as in Figure 2. **(d)** A model for FKBP12 antagonist-dependent activation of TGF-β signaling. Fusing a PK mutant of FKBP12 to TGF-βRI results in inhibition of what would otherwise be a constitutive signal (see Figure 3a). The association of the PK mutant of FKBP12 with the TGF-βRI cytoplasmic domain can be disrupted by FKBP12 antagonists, allowing TGF-β-independent signaling to occur.

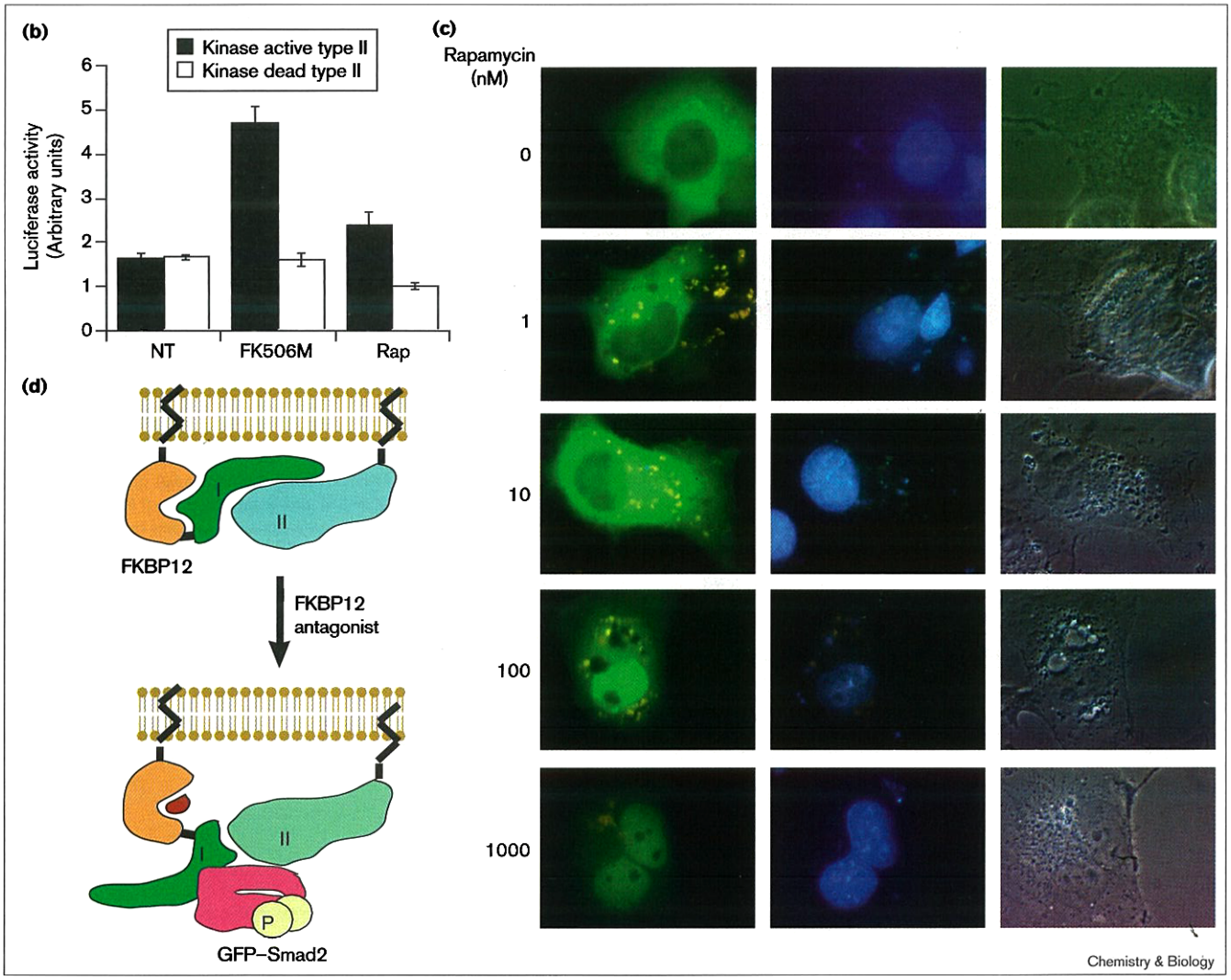
Membrane-localized TGF-β receptor cytoplasmic tails signal constitutively

When we cotransfected myristoylated cyclophilin-A–TGF-β receptor chimeras (MAT_I and MAT_{II}, Figure 1a; cyclophilin A is an enzyme that, like FKBP12, catalyzes the interconversion of *cis* and *trans* isomers of the peptide amide bond at prolyl positions in polypeptides, and because cyclophilin does not bind TGF-βRI, we used this module in place of FKBP12 as a neutral domain that would serve as a control) we found that GFP–Smad2 was constitutively localized to the nucleus (Figure 3a) and p3TPLux was constitutively activated (Figure 3b). These results indicate that overexpression of the receptor cytoplasmic tails at the plasma membrane causes autosignaling (Figure 3c). A kinase-dead mutant of the type II receptor (MAT_{II}[KD], Figure 1a) did not signal constitutively in the presence of MAT_I (Figure 3a,b). Cytosolic, nonmyristoylated constructs did not signal constitutively when coexpressed (data not shown and [33]), indicating either that effective concentrations are higher at the membrane or that receptor activation requires membrane localization. When p3TPLux, MAT_I and MAT_{II} were cotransfected into Mv1Lu mink lung cells, there was some additional reporter gene activation induced by an FKBP12 antagonist (Figure 3b), suggesting that in these cells endogenous FKBP12 could play a role in partially repressing TGF-β-receptor-mediated signaling (see the Introduction section).

A fusion protein consisting of FKBP12 and the TGF-βRI cytoplasmic tail can be activated by FKBP12 antagonists

FKBP12 associates, in a yeast two-hybrid assay, with the cytoplasmic domain of many type I TGF-β superfamily receptors [28]. When we cotransfected MAT_{II} with a myristoylated FKBP12–TGF-βRI fusion protein ($MF_{pk}T_I$, Figure 1a), we observed that the autosignaling described above was repressed (compare Figure 4a and Figure 3a). Two FKBP12 antagonists, rapamycin and FK506M [34],

Figure 4 (cont'd)

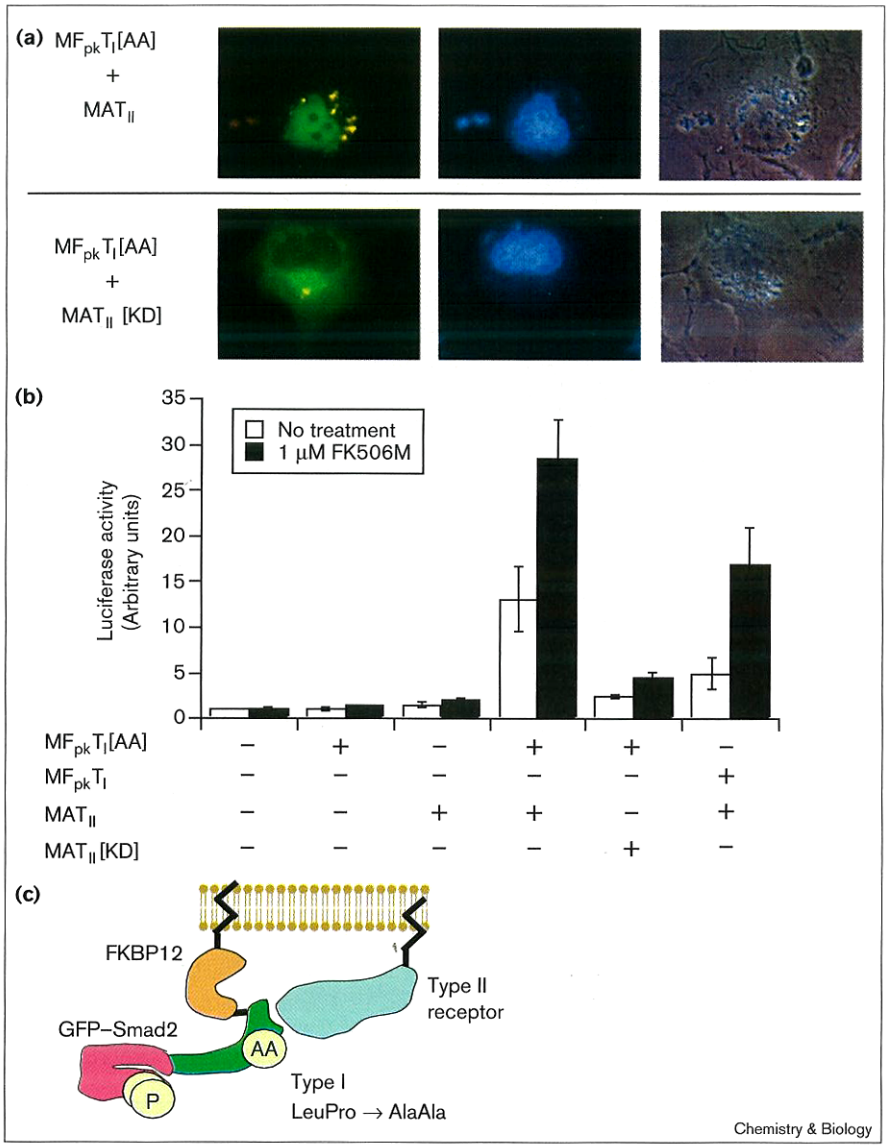


however, activated TGF-β signaling in the presence of $MF_{pk}T_I$ and MAT_{II}. Both rapamycin, which can bind FKBP12 and FKBP12-rapamycin-associated protein (FRAP) simultaneously, and FK506M, a conventional FKBP12 antagonist, induced the nuclear accumulation of GFP–Smad2 within one hour of treatment (Figure 4a). GFP–Smad2 remained in the cytoplasm when a kinase-dead construct (MAT_{II}[KD], Figure 1a) was used (Figure 4a). Leaving out either receptor from the transfection resulted in a cytoplasmic GFP–Smad2 localization that could not be altered using FKBP12 antagonists (data not shown). The EC_{50} for rapamycin-induced GFP–Smad2 translocation was approximately 100 nM (Figure 4c).

We tested whether FK506M and rapamycin could activate the TGF-β-responsive reporter p3TPLux when $MF_{pk}T_I$ and MAT_{II} were cotransfected into Mv1Lu mink lung cells. Both FKBP12 antagonists activated the reporter modestly when the kinase-active construct MAT_{II} was used but neither was able to when the kinase-dead construct MAT_{II}[KD] was used (Figure 4b). As in the GFP–Smad2 translocation assay, both type I and II receptor constructs were required for this effect (data not shown).

A Gly89→Pro, Ile90→Lys (PK) double mutant of FKBP12 (F_{pk}) does not inhibit calcineurin (a phosphatase that is involved in T-cell activation and is not likely to be involved in the TGF-β signaling pathway; see the Discussion section) when bound to FK506, in contrast to wild-type FKBP12 [35]. Also, the only characteristic common to FK506M and rapamycin is their ability to bind FKBP12 potently. These data suggest it is the ability to bind FKBP12 that is responsible for GFP–Smad2 nuclear translocation and activation of p3TPLux. The finding that

Figure 5



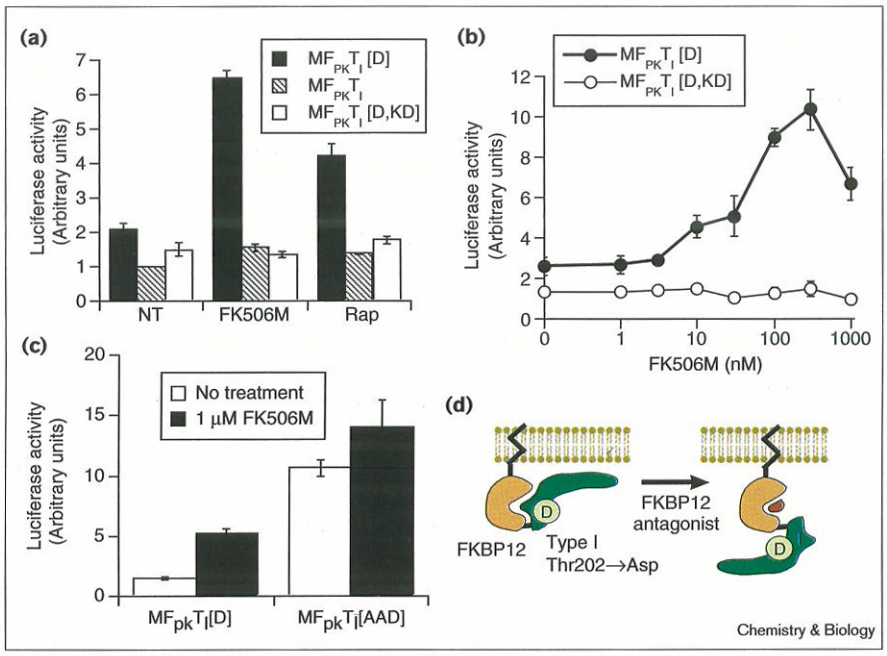
A TGF-βRI cytoplasmic tail mutant (Leu191→Ala, Pro192→Ala [AA]) that does not bind FKBP12 is constitutively active, even when fused to the PK mutant of FKBP12, in the presence of a kinase-active TGF-βRII cytoplasmic tail. **(a)** GFP–Smad2 translocation occurs constitutively when $MF_{pk}T_I$[AA] is cotransfected with the kinase-active construct MAT_{II}, but not a kinase-dead construct MAT_{II}[KD]. COS-1 cells were transfected with GFP–Smad2 and the indicated constructs and photographed as in Figure 2. **(b)** The PAI-1 promoter is activated constitutively when $MF_{pk}T_I$[AA] and MAT_{II}, but not MAT_{II}[KD], are cotransfected. Mv1Lu cells were transfected with p3TPLux and the indicated constructs and treated with nothing or 1 μM FK506M. **(c)** A model for ligand-independent activation of TGF-β signaling with FKBP12-binding-defective TGF-βRI. FKBP12 cannot bind the AA mutant of TGF-βRI and therefore cannot inhibit the constitutive signaling caused by overexpression of the cytoplasmic domains of the two receptor tails (see Figure 3c). A, alanine.

C10-acetamido-FK506, an FK506 derivative defective in FKBP12 binding (D.J. Austin and S.L.S., unpublished results), does not induce GFP–Smad2 translocation or activation of p3TPLux further supports this conclusion (data not shown).

A TGF-βRI cytoplasmic tail mutant that does not bind FKBP12 is constitutively active, even when fused to FKBP12

FKBP12 binds TGF-βRI in yeast two-hybrid [26] and coimmunoprecipitation [29,31] assays, and mutation of the leucine–proline FKBP12-binding site in the GS box of TGF-βRI abolishes the interaction in both assays [30,31]. We investigated whether a similar mutation in $MF_{pk}T_I$ would prevent FKBP12 from repressing TGF-βRI. We made Leu191→Ala, Pro192→Ala (L191A, P192A) mutations in the GS box of TGF-βRI, generating the construct $MF_{pk}T_I$[AA] (Figure 1a). When this construct was cotransfected with MAT_{II}, GFP–Smad2 translocated to the nucleus constitutively and the PAI-1 promoter was activated constitutively (Figure 5a,b). These results indicate that binding of FKBP12 is critical for repression of TGF-βRI signaling activity. There was a modest enhancement of reporter gene activity following addition of FK506M, likely due to displacement of FKBP12 from endogenous TGF-βRI. Furthermore, cotransfection of $MF_{pk}T_I$[AA] with the kinase-dead construct MAT_{II}[KD] did not result in nuclear translocation of GFP–Smad2 or activation of p3TPLux (Figure 5a,b), again indicating that the kinase activity of TGF-βRII is required for constitutive signaling.

Figure 6



An acidic mutation on TGF-βRI allows activation of p3TPLux in response to FKBP12 antagonists in the absence of TGF-βRII. **(a)** DR26 cells were transfected with p3TPLux and MF$_{pk}$T$_I$[D] (kinase active), MF$_{pk}$T$_I$[D,KD] (kinase dead) or MF$_{pk}$T$_I$ (kinase active without acidic mutation) and treated with nothing (NT), 1 μM FK506M or 1 μM rapamycin (rap). **(b)** DR26 cells were transfected with p3TPLux and MF$_{pk}$T$_I$[D] (kinase active) or MF$_{pk}$T$_I$[D,KD] (kinase dead) and treated with the indicated concentrations of FK506M. **(c)** Mutation of the FKBP12-binding site in the context of the acidic mutation results in constitutive activation of p3TPLux in the absence of TGF-βRII. DR26 cells were transfected with p3TPLux and MF$_{pk}$T$_I$[D] or MF$_{pk}$T$_I$[AAD] (FKBP12-binding deficient) and treated with nothing or 1 μM FK506M. **(d)** A model for FKBP12 antagonist-dependent signaling in the absence of TGF-βRII. The acidic (Thr202→Asp) mutant of TGF-βRI does not signal constitutively because the PK mutant of FKBP12 binds intramolecularly to the receptor and inhibits signaling. Addition of FKBP12 antagonists (or mutation of the FKBP12-binding site) causes activation of signaling. D, aspartic acid.

FKBP12 represses and FKBP12 antagonists derepress a TGF-βRII-independent mutant of TGF-βRI

An acidic (Thr204→Asp) mutant of full-length human TGF-βRI is partially active, even in the absence of the TGF-βRII [36]. We tested the effect of the corresponding Thr202→Asp mutation in the rat type I receptor tail, in the context of the construct MF$_{pk}$T$_I$[D] (Figure 1a). This construct was not constitutively active, but was activated by the FKBP12 antagonists FK506M and rapamycin, even in the absence of TGF-βRII (Figure 6d). Treating mink lung cells transfected with MF$_{pk}$T$_I$[D] and p3TPLux with FK506M or rapamycin resulted in activation of p3TPLux (Figure 6a,b). We ensured that endogenous TGF-βRII was not activating MF$_{pk}$T$_I$[D] by using DR26 cells, which have a defective endogenous TGF-βRII [37]. Furthermore, in the absence of TGF-βRII, neither the kinase-dead construct MF$_{pk}$T$_I$[D,KD] nor the wild-type Thr202 version of the kinase-active construct was activated by FK506M or rapamycin (Figure 6a,b).

We were unable to induce complete GFP–Smad2 nuclear translocation with MF$_{pk}$T$_I$[D] alone, or with human TGF-βRI(Thr204→Asp), in the absence of TGF-βRII (data not shown). We did observe partial signaling in some cells transfected with TGF-βRI(Thr204→Asp) or MAT$_I$[D], but GFP–Smad2 translocation was incomplete (data not shown). In contrast, in the luciferase reporter gene assay, activation of the Thr202→Asp mutant was independent of TGF-βRII, and FKBP12 was able to repress this mutant. To confirm that it was the ability of FKBP12 to bind to the cytoplasmic domain of the acidic mutant that was responsible for the repression effect, we mutated the FKBP12-binding site on T$_I$(Thr202→Asp) from leucine–proline to alanine–alanine, generating the triple mutant, MF$_{pk}$T$_I$[AAD] (Figure 1a). This construct constitutively activated p3TPLux (Figure 6c), although again it did not induce complete GFP–Smad2 translocation in COS-1 cells (data not shown).

Discussion

Several reports have shown that TGF-βRI mutants unable to bind FKBP12 can still transduce TGF-β signals, demonstrating that FKBP12 association with TGF-βRI is not necessary for signaling [29–31]. Some reports suggest that FKBP12 is capable of inhibiting TGF-β signaling [28,31]. Wang *et al.* [28] reported that myristoylated wild-type FKBP12 (MFE) inhibited TGF-β-induced activation of p3TPLux and inactivation of the cyclin A promoter (pCAL, cyclin A is a protein whose expression is increased during the DNA synthesis phase of the cell cycle; TGF-β causes a reduced level of transcription from the cyclin A promoter, an effect that has been used as a way of detecting the effect of TGF-β on cell-cycle progression [38]), whereas the myristoylated PK mutant of FKBP12 (MF$_{pk}$E [39]) inhibited p3TPLux activation to a lesser extent and did not inhibit inactivation of pCAL [28]. Because F$_{pk}$ was less effective at inhibiting TGF-β responses, and the F$_{pk}$–FK506 complex is not able to inhibit the phosphatase calcineurin, Wang *et al.* [28] concluded that FKBP12 might be docking an

inhibitory protein, such as calcineurin, to TGF-βRI, and that F_{pk} could bind TGF-βRI but could not recruit the inhibitory protein. In contrast, we have found that the PK mutant of FKBP12 is capable of repressing TGF-β signaling, when fused to TGF-βRI, by binding intramolecularly to TGF-βRI (Figure 4d). It is likely that F_{pk} simply has a decreased affinity for TGF-βRI compared to wild-type FKBP12 and therefore is less effective at binding and repressing TGF-βRI activity when forced to do so intermolecularly. By tethering the FKBP12 PK mutant to the type I TGF-β receptor tail, we appear to have found a proper balance between a binding sufficient to repress signaling yet weak enough to be derepressed in response to small molecule FKBP12 antagonists. Although we cannot rule out the possibility that wild-type FKBP12 and F_{pk} are recruiting an inhibitor of TGF-β signaling to TGF-βRI, the simplest explanation is that FKBP12 and F_{pk} directly inhibit activation of TGF-βRI by binding and occluding the GS box of the receptor, an important element in TGF-β signaling [40].

A report on FKBP12 inhibition of TGF-β signaling by Chen *et al.* [31] suggested that FKBP12 prevents the spontaneous activation of TGF-β receptors, in the absence of TGF-β. TGF-βRI and TGF-βRII have an intrinsic affinity for each other in the absence of TGF-β, as evidenced by the findings that overexpression of either full-length TGF-βRI and TGF-βRII or their cytoplasmic domains causes constitutive activation of p3TPLux and that the cytoplasmic domains of the receptors interact in yeast two-hybrid and coimmunoprecipitation experiments [27]. Chen *et al.* [31] demonstrated that TGF-βRI mutants defective in binding FKBP12 show an increased tendency to be activated spontaneously and that FKBP12 prevents the ligand-independent transphosphorylation of TGF-βRI by TGF-βRII. Because, in a companion paper published elsewhere [33], we have shown that homodimerization of an activated TGF-βRI cytoplasmic domain containing a Thr202→Asp mutation is sufficient to activate TGF-β signaling [33], it is possible that FKBP12 prevents spontaneous autophosphorylation of activated TGF-βRI receptors, and thereby prevents signaling by TGF-βRI. FKBP12 might also prevent the association of downstream signaling components, such as Smad2 and Smad3, with activated TGF-βRI.

An important question involves the natural mechanism by which TGF-β induces the release of FKBP12 from TGF-βRI. It could be that phosphorylation of TGF-βRI by TGF-βRII induces the dissociation of FKBP12, or it could be that a TGF-βRI autophosphorylation event (either in an intermolecular or intramolecular fashion) is responsible for the dissociation. We have demonstrated that the Thr202→Asp activating mutation in TGF-βRI does not remove the repressive actions of the FKBP12 PK mutant on TGF-βRI.

A recent report that E14.5 fibroblasts from FKBP12-null mice show normal TGF-β-dependent activation of p3TPLux concluded that the interaction between FKBP12 and TGF-βRI is not physiologically relevant [41]. The study, however, is not exhaustive in the sense that the authors looked directly at only a single TGF-β response in a cell type that showed just threefold activation of p3TPLux in response to TGF-β. A cell line that responds poorly to TGF-β might be expected to express low levels of the TGF-β receptors. According to the model of Chen *et al.* [31], whereby FKBP12 prevents spontaneous activation of TGF-βRI when the receptors are expressed at a high level, a dramatic effect on TGF-β signaling would not be expected upon removal of FKBP12 from a cell line that expresses low levels of the TGF-β receptors. In addition, the results of Shou *et al.* [41] do not rule out the possibility that other isoforms of FKBP12 still bind to and repress TGF-βRI, or that compensation mechanisms such as receptor downregulation can substitute for FKBP12.

Our original intent was to use FKBP12 as a binding domain for small molecule dimerizers [33]. We fused F_{pk}, and not wild-type FKBP12, to TGF-βRI because, based on the results of Wang *et al.* [28] and our own experiments, we knew that MFE, but not $MF_{pk}E$, inhibits TGF-β signaling. As we have described, however, we observed that when F_{pk} is fused directly to TGF-βRI, TGF-βRI-mediated signaling is repressed and can be derepressed using FKBP12 antagonists. Thus, we found two strategies for activating TGF-β signaling with small membrane-permeable organic molecules. One consists of fusing binding domain modules to the TGF-β receptor tails and using dimeric small molecules to form homo-oligomeric and hetero-oligomeric complexes of the receptor tails [33]. The second strategy, described in this report, consists of fusing the repressor FKBP12 to the type I TGF-β receptor and using FKBP12 antagonists to derepress TGF-β signaling (Figure 6d).

Significance

Transforming growth factor beta (TGF-β) arrests cell growth in many cell types. Two types of TGF-β receptors (type I, TGF-βRI and type II, TGF-βRII) mediate the cell response to TGF-β. TGF-β first binds to TGF-βRII, which can then recruit and phosphorylate TGF-βRI at its GS box (a region rich in glycine and serine residues). Activated TGF-βRI then phosphorylates two downstream effectors, Smad2 and Smad3, allowing them to be translocated to the nucleus. As a result, cell growth is arrested and the plasminogen activator inhibitor (PAI-1) is upregulated. We were interested in exploring the role of the immunophilin FK506-binding protein 12 (FKBP12) in TGF-β signalling, as FKBP12 is able to bind to the GS box of TGF-βRI.

We have demonstrated that fusing a repressor domain to a receptor signaling domain results in a novel receptor that

is activated by small molecule antagonists of the repressor. Although earlier reports found only a synergy between FKBP12 antagonists and TGF-β [28,31], we found that the activity of a myristoylated fusion protein containing the Gly89→Pro, Ile90→Lys (PK) mutant of FKBP12 and the intracellular domain of the TGF-β type I receptor can be directly regulated by FKBP12 antagonists, in the absence of TGF-β. Overexpression of type I and type II receptor cytoplasmic tails causes constitutive signaling, but the PK mutant of FKBP12, when fused to the type I TGF-β receptor, is capable of repressing the autosignaling. Both constitutive and FKBP12-antagonist-dependent signaling require the presence of either both TGF-β receptors or an acidic mutation in the type I TGF-β receptor. The repression by FKBP12 is dependent on its binding to a leucine–proline motif in the type I TGF-β receptor. These results provide further support of the hypothesis that FKBP12 functions as an inhibitor of TGF-β superfamily signaling, and they illustrate a novel method for controlling TGF-β signaling —one that uses simple FKBP12 antagonists rather than small molecule 'dimerizers' [33]. Because FKBP12 binds to other receptors, including the activin and BMP type I receptors, this method of regulating receptor signaling might be applicable to these systems as well.

Materials and methods

DNA constructions

All constructs were expressed from the mammalian expression vector pBJ5.1 [34]. R4, a rat TGF-β type I receptor [42], was obtained from Patricia Donahoe and Tongwen Wang. Human TGF-βRI(Thr204→Asp), human TGF-βRII and p3TPLux were obtained from Joan Massague. pGEX2TK-A_{wt} was generated previously (Karen Liu and S.L.S., unpublished results). The Ser65→Thr mutant of green fluorescent protein (GFP or J), was obtained from the plasmid JC3E [39]. Murine Smad2 (pcsMadR2) was obtained from Richard Harland [10]. MAE_L was generated by ligating the *Xho*I, *Sal*I fragment of pGEX2TK-A_{wt} into *Xho*I, *Sal*I-digested $MF_{pk}E_u$. T_I was obtained by PCR amplification of residues 153–501 of R4 using R4 as template and primers H_p8 (5′-GCC ATA ACC GCA CTG TCA TTC TCG AGC ACC ACC GCG TGC CA-3′) and H_p12 (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 II SK-derivative in which the *Kpn*I site had been 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 MAE_u, $MF_{pk}E_L$, and $MF_{p<}3E_u$ with *Xho*I and *Eco*RI sites, to generate MAT_I, $MF_{pk}T_I$ and $MF_{pk}3T_I$, respectively. The T_I[D] fragment was generated with an amino-terminal flag 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 to generate pBS3-T_I[D]. The T_I[D] fragment was subcloned into $MF_{pk}E_u$, $MF_{pk}E_J$ and MAE_u to generate $MF_{pk}T_I$[D], $MF_{pk}3T_I$[D] and MAT_I[D], respectively. An amino-terminally flag-tagged Leu191→Ala, Pro192→Ala double mutant of T_I was generated by overlap extension PCR using R4 as template and primers B286-1, B298-1 (5′-GCA ATT GTT CTT TGA ACA AGC AGT GCG GCG CCT GAT CCA GAC CCT G-3′), B298-3 (5′-CTG CTT GTT CAA AGA ACA ATT GC-3′) and B286-6 and subcloned into pBS3-T_I using *Kpn*I and *Xho*I. The T_I[AA] fragment was subcloned into $MF_{p<}E_J$ to generate $MF_{pk}T_I$[AA]. An amino-terminally flag-tagged Leu191→Ala, Pro192→Ala, Thr202→Asp triple mutant of T_I was generated by overlap extension PCR using pBS3-T_I[D] as template and primers B286-1, B298-1, B298-3 and B286-6. The PCR product was first subcloned into pBS3-T_I and subsequently the T_I[AAD] fragment was subcloned into $MF_{p<}E_J$ to generate $MF_{p<}T_I$[AAD]. T_I[D, KD] was generated by overlap extension PCR using pBS3-T_I[D] 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 to generate pBS3-T_I[D,KD] and the T_I[D,KD] fragment was subcloned into $MF_{pk}E_u$ and $MF_{pk}3E_u$ with *Xho*I and *Eco*RI to generate $MF_{pk}T_I$[D,KD] and $MF_{p<}3T_I$[D,KD], respectively. A general GFP expression vector pBJ5.1-JE_g was generated from pBJ5.1-$JC3E_u$ 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 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-JE_g using *Sal*I and *Eco*RI to generate pBJ5-JE_gSmad2 (referred to as GFP–Smad2 in the text). TGF-βRII was subcloned into pBluescript IISK$^-$ (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 MAE_u to generate carboxy-terminally flu epitope-tagged MAT_{II}. A kinase-dead Lys277→Arg 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 MAE_u to generate MAT_{II}[KD].

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 eight-well dishes. For transfection of each coverslip, 300 ng GFP–Smad2, 850 ng type I receptor (e.g. $MF_{p<}T_I$) and 850 ng type II receptor (e.g. MAT_{II}) were mixed with 5.5 μl of 2 mg/ml Lipofectamine (GibcoBRL) in 1.0 ml phenol red free and serum free DMEM (VDMEM), incubated at room temperature for 30 minutes and then added to a coverslip that 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 of 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 X 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 (©1996 Integral Technology, Inc.). 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 alanine, aspartic acid, glutamine, glycine, asparagine, and proline. 3.5×10^5 mink lung cells were seeded in each 35 mm well of a six-well dish. After 24 h, cells were transfected using DEAE-dextran. For each well, 250 ng p3TPLux DNA was mixed with 250 ng of 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 NEAA 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. 100 μl of lysate was added to 150 μl of assay mixture (25 mM glycylglycine pH 7.8, 15 mM $MgSO_4$, 4 mM EGTA, 15 mM K_2HPO_4 pH 7.8, 1 mM DTT, 4 mM ATP) and 150 μl of 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 s. The error bars reported represent plus or minus one standard deviation. All experiments were performed multiple times in triplicate.

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