The EMBO Journal Vol.16 No.13 pp.3866–3876, 1997

Mechanism of TGFβ receptor inhibition by FKBP12

Ye-Guang Chen, Fang Liu and Joan Massagué[1]

Cell Biology Program and Howard Hughes Medical Institute, Memorial Sloan-Kettering Cancer Center, New York, NY 10021, USA

[1]Corresponding author
e-mail: j-massague@ski.mskcc.org

Transforming growth factor-β (TGFβ) signaling requires phosphorylation of the type I receptor TβR-I by TβR-II. Although TGFβ promotes the association of TβR-I with TβR-II, these receptor components have affinity for each other which can lead to their ligand-independent activation. The immunophilin FKBP12 binds to TβR-I and inhibits its signaling function. We investigated the mechanism and functional significance of this effect. FKBP12 binding to TβR-I involves the rapamycin/Leu–Pro binding pocket of FKBP12 and a Leu–Pro sequence located next to the activating phosphorylation sites in TβR-I. Mutations in the binding sites of FKBP12 or TβR-I abolish the interaction between these proteins, leading to receptor activation in the absence of added ligand. FKBP12 does not inhibit TβR-I association with TβR-II, but inhibits TβR-I phosphorylation by TβR-II. Rapamycin, which blocks FKBP12 binding to TβR-I, reverses the inhibitory effect of FKBP12 on TβR-I phosphorylation. By impeding the activation of TGFβ receptor complexes formed in the absence of ligand, FKBP12 may provide a safeguard against leaky signaling resulting from the innate tendency of TβR-I and TβR-II to interact with each other.

Keywords: FKBP12/transforming growth factor-β/receptor signaling

Introduction

The growth factor family that includes transforming growth factor-β (TGFβ), activins and bone morphogenetic proteins (BMPs) plays a central role in controlling tissue development and homeostasis (Massagué, 1990; Roberts and Sporn, 1990). Minor differences in the activity of these factors may have major effects on cell fate (Green and Smith, 1990), and so it is not surprising that the levels of these factors and their binding to cell surface receptors are tightly controlled. Thus, TGFβ and related factors are secreted as latent forms that are activated through an intricate process (Massagué, 1990; Roberts and Sporn, 1990). Once activated, the factors interact with soluble or membrane-bound proteins that control their access to signaling receptors either positively, as in the case of the TGFβ-binding protein betaglycan (López-Casillas *et al.*, 1993; Moustakas *et al.*, 1993), or negatively, as in the case of the activin-binding protein follistatin (Nakamura *et al.*, 1990; Hemmati-Brivanlou *et al.*, 1994) or the BMP-binding proteins noggin (Holly *et al.*, 1996; Zimmerman *et al.*, 1996) and chordin (Piccolo *et al.*, 1996). However, little is known about regulation of the TGFβ signaling receptors themselves.

The receptors for the TGFβ family are transmembrane protein serine/threonine kinases that form two structurally and functionally distinct groups known as the type I and the type II receptors, respectively (Massagué, 1996; ten Dijke *et al.*, 1996). Ligand binding induces the formation of a complex in which the type II receptor phosphorylates and activates the type I receptor which then propagates the signal (Wrana *et al.*, 1994). Phosphorylation of the type I receptor occurs at a cluster of serine and threonine residues within a highly conserved region known as the GS domain and located immediately upstream of the kinase domain (Wrana *et al.*, 1994; Wieser *et al.*, 1995). Roles for the type II receptor as a primary receptor and the type I receptor as a signal transducer are also suggested by the ability of constitutively active type I receptor mutants to signal in the absence of ligand or type II receptors (Wieser *et al.*, 1995).

Many of the resulting responses are mediated by the SMADs (Sekelsky *et al.*, 1995; Baker and Harland, 1996; Graff *et al.*, 1996; Hoodless *et al.*, 1996; Lagna *et al.*, 1996; Liu *et al.*, 1996; Savage *et al.*, 1996; Zhang *et al.*, 1996), a family of cytoplasmic proteins that become phosphorylated in response to receptor activation (Eppert *et al.*, 1996; Hoodless *et al.*, 1996; Lagna *et al.*, 1996; Zhang *et al.*, 1996). SMAD phosphorylation is mediated by a receptor-associated kinase activity (Macias-Silva *et al.*, 1996; Zhang *et al.*, 1996; Kretzschmar *et al.*, 1997) and results in the association of SMADs with the related protein DPC4 (Lagna *et al.*, 1996) and accumulation in the nucleus (Baker and Harland, 1996; Hoodless *et al.*, 1996; Liu *et al.*, 1996). In the nucleus, SMADs associate with a DNA-binding protein (Chen *et al.*, 1996) and act as transcriptional regulators (Chen *et al.*, 1996; Liu *et al.*, 1996).

Using a yeast two-hybrid system, three different types of proteins have been shown to interact with the cytoplasmic domain of TGFβ family type I receptors. One of these proteins is the α-subunit of farnesyl transferase (Kawabata *et al.*, 1995b; Ventura *et al.*, 1996; Wang *et al.*, 1996a). However, the physiological relevance of this interaction has been questioned (Ventura *et al.*, 1996). Type I receptors can also interact with type II receptors in a yeast two-hybrid system (Ventura *et al.*, 1994; Chen *et al.*, 1995; Kawabata *et al.*, 1995a; Liu *et al.*, 1995), suggesting that the two receptors have intrinsic affinity for each other. This possibility is also supported by the ability of type I and type II receptors to form ligand-independent, active complexes when overexpressed in insect cells (Ventura

et al., 1994) or in mammalian cells (Chen and Weinberg, 1995; Vivien *et al.*, 1995; Feng and Derynck, 1996). These observations raise the question of what prevents the spontaneous interaction and activation of these receptors under physiological conditions.

The cytoplasmic domain of type I receptors also interacts with FKBP12 (Wang *et al.*, 1994; Kawabata *et al.*, 1995a; Liu *et al.*, 1995), a cytosolic protein previously known to bind the immunosuppressants FK506 and rapamycin (Schreiber, 1991). FKBP12 is highly conserved from yeast through human and is relatively abundant in most tissues and cell types (Siekierka *et al.*, 1990). FKBP12 has peptidyl-prolyl *cis–trans* isomerase (rotamase) activity (Harding *et al.*, 1989) and is thought to have multiple and diverse functions in the cell. In complex with FK506, FKBP12 binds to the Ca^{2+}/calmodulin-dependent protein phosphatase calcineurin and inhibits its ability to mediate T-cell activation (Schreiber, 1992; McCaffrey *et al.*, 1993). In complex with rapamycin, FKBP12 binds to the protein FRAP/RAFT1 (Brown *et al.*, 1994; Sabatini *et al.*, 1994), inhibiting the ability of this kinase to mediate mitogenic responses (Brown *et al.*, 1995). On its own, FKBP12 binds to the ryanodine receptor (Jayaraman *et al.*, 1992) and the inositol 1,4,5-triphosphate receptor (Cameron *et al.*, 1995), stabilizing the calcium channeling activity of these proteins (Brillantes *et al.*, 1994; Cameron *et al.*, 1995). Little is known about other physiological roles of FKBP12.

The FKBP12 interaction with TGFβ family type I receptor was demonstrated recently in mammalian cells (Okadome *et al.*, 1996; Wang *et al.*, 1996b). The FKBP12-binding site was mapped to the activation domain of the TGFβ type I receptor (TβR-I) (Okadome *et al.*, 1996) and, more specifically, to a Leu–Pro motif within this domain (Charng *et al.*, 1996). FKBP12 does not mediate TGFβ signaling (Charng *et al.*, 1996; Okadome *et al.*, 1996) but it rather inhibits it (Wang *et al.*, 1996b). In the present work, we have investigated this inhibitory effect and its mechanism. We provide evidence that FKBP12 prevents the spontaneous, ligand-independent activation of TβR-I by TβR-II. Furthermore, we demonstrate that this effect is based on the ability of FKBP12 to block receptor transphosphorylation. We propose that bound FKBP12 protects TβR-I against ligand-independent activation.

Results

Defective FKBP12 binding causes leaky TβR-I signaling

In order to study the effect of FKBP12 on the signaling activity of TβR-I, we generated several receptor mutants C-terminally tagged with the hemagglutinin (HA) epitope (Figure 1). These constructs were transfected into COS-1 cells together with a FKBP12 construct N-terminally tagged with the Flag epitope. Metabolic labeling of the proteins and immunoprecipitation with anti-Flag monoclonal antibody revealed that wild-type TβR-I was associated specifically with FKBP12 (Figure 2A, lane 3), which is consistent with previous observations (Okadome *et al.*, 1996; Wang *et al.*, 1996b). FKBP12 also co-precipitated TβR-I that had been labeled by cross-linking to bound [^{125}I]TGFβ, demonstrating that FKBP12 interacts with

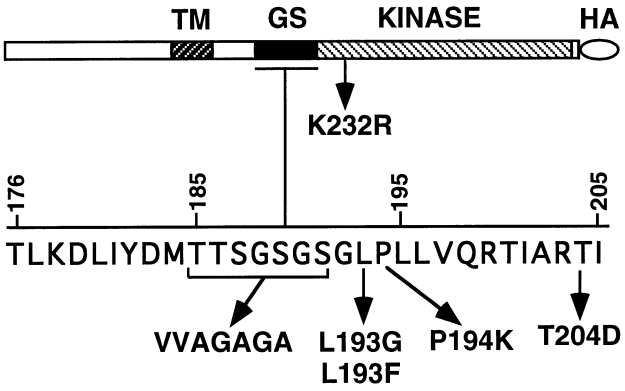

Fig. 1. Schematic representation of the TβR-I mutants used in the present studies. The sequence of the TβR-I GS domain indicates specific mutations. The transmembrane region (TM) and C-terminal HA epitope tag (HA) are indicated.

cell surface TβR-I (data no shown). The level of FKBP12 binding to a kinase-defective mutant receptor, TβR-I(K232R) (Cárcamo *et al.*, 1994), was similar to that of wild-type TβR-I, whereas binding to a constitutively active receptor mutant, TβR-I(T204D) (Wieser *et al.*, 1995), was reproducibly lower (Figure 2A, compare lane 4 with lanes 3 and 5). These results indicate that FKBP12 binding to TβR-I does not require the kinase activity of this receptor but, interestingly, is decreased by an activating mutation in the GS domain.

As a peptidyl-prolyl *cis–trans* isomerase, FKBP12 recognizes a proline preceded by a leucine or similarly branched hydrophobic residue (Albers *et al.*, 1990; Harrison and Stein, 1990). Such a motif (Leu193–Pro) is present in the GS domain of TβR-I (Figure 1) and in other type I receptors. Mutation of this proline to lysine eliminated FKBP12 binding to the receptor (Figure 2A, lane 6). To investigate further the possibility that this is a FKBP12-binding motif, Leu193 was mutated to either phenylalanine or glycine. Phenylalanine can replace leucine as the P1 residue in a substrate peptide without markedly decreasing the catalytic efficiency of FKBP12, whereas leucine replacement with glycine decreases the catalytic efficiency of FKBP12 by 50- to 100-fold (Albers *et al.*, 1990; Harrison and Stein, 1990). Correlating with this specificity profile, the L193F mutation had no discernable effect on FKBP12 binding to TβR-I, whereas the L193G mutation eliminated FKBP12 binding (Figure 2A, lanes 7 and 8). Furthermore, mutation of the adjacent sequence T^{185}TSGSGS to VVAGAGA (Figure 1), which prevents TβR-I phosphorylation and signaling (Wieser *et al.*, 1995), had no effect on FKBP12 binding (data not shown).

To confirm that FKBP12 specifically recognizes the Leu–Pro sequence in the GS domain of TβR-I, we tested the effect of two mutations, F36Y and I90K, at or near the active site of FKBP12. These mutants can still bind macrolide; however, FKBP12(F36Y) lacks peptidyl-prolyl isomerase activity (Wiederrecht *et al.*, 1992) whereas FKBP12(I90K) bound to FK506 fails to associate with calcineurin (Yang *et al.*, 1993; Futer *et al.*, 1995). When tested in our assays, both FKBP12 mutants failed to bind TβR-I (Figure 2B), indicating that the integrity of the substrate interaction site of FKBP12 is essential for the interaction with TβR-I.

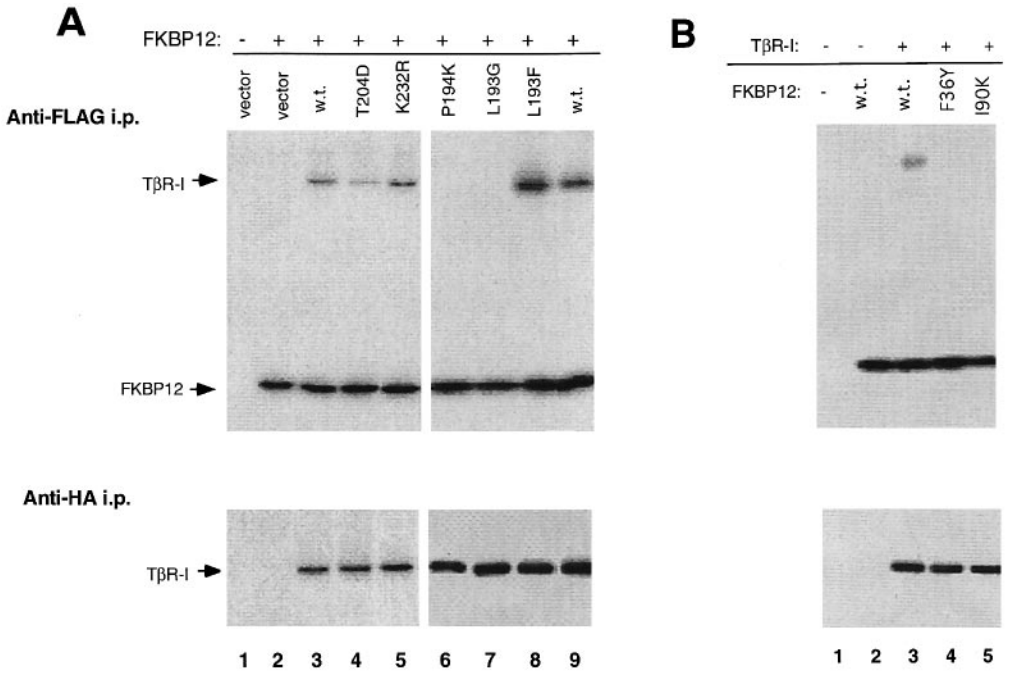

Fig. 2. The interaction between TβR-I and FKBP12. (**A**) The wild-type TβR-I or the indicated TβR-I mutants were HA tagged and co-transfected with Flag-tagged FKBP12 cDNA into COS-1 cell. Cells were labeled with [^{35}S]methionine. Cell lysates were immunoprecipitated with anti-Flag monoclonal antibody to visualize the association of TβR-I with FKBP12 (upper panels), or with the anti-HA monoclonal antibody to monitor the expression level of receptor proteins (lower panels). (**B**) COS-1 cells were co-transfected with HA-tagged TβR-I and wild-type or mutant FKBP12 as indicated. TβR-I–FKBP12 association and the TβR-I expression level were determined as described in (A).

In order to determine the effect of FKBP12 binding on the signaling function of TβR-I, we took advantage of the mutant cell clone R-1B which is derived from the mink lung epithelial cell line Mv1Lu and is defective in TβR-I owing to a marked reduction in TβR-I expression (Boyd and Massagué, 1989; Weis-Garcia and Massagué, 1996). The parental cell line is highly sensitive to TGFβ, and its various responses include cell cycle arrest and activation of extracellular matrix protein expression. The TGFβ responsiveness of Mv1Lu and its mutant derivatives can be evaluated using a previously described reporter construct, p3TP-Lux, that contains a TGFβ-responsive element of the plasminogen activator inhibitor-1 (PAI-1) promoter and three TPA-response elements (Cárcamo *et al.*, 1994). Despite a similar level of receptor expression (Figure 3C), the basal level of luciferase activity in cells transfected with TβR-I mutants defective in FKBP12 binding (referred to as 'FKBP12-blind' mutants), TβR-I(L193G) and TβR-I(P194K), was reproducibly 5- to 8-fold higher than in cells transfected with TβR-I (Figure 3A). The higher signaling activity was still clear at low concentrations of added TGFβ (e.g. 2.5 pM; Figure 3A) but progressively disappeared as the maximal level of luciferase activity (~50-fold over the wild-type basal) was reached at higher (100 pM) TGFβ concentrations. In contrast, the activity of the TβR-I(L193F) mutant was not significantly higher than that of TβR-I. The constitutively active mutant TβR-I(T204D), which was tested in parallel for comparison, produced a 20-fold higher basal activity (Figure 3A), as previously described (Wieser *et al.*, 1995).

Similar results were obtained by measuring the ability of TβR-I to inhibit expression of a cyclin A promoter construct, pCAL2, which serves as a measure of growth inhibition by TGFβ (Feng *et al.*, 1995). All TβR-I constructs tested mediated TGFβ inhibition of pCAL-luciferase expression to ~30% of the basal level in cells transfected with wild-type TβR-I or vector alone (Figure 3B). However, in the absence of ligand, cells transfected with TβR-I(T204D), TβR-I(P194K) or TβR-I(L193G) had significantly lower pCAL-luciferase activity than cells expressing TβR-I or TβR-I(L193F) (Figure 3B). Collectively, these results indicate that FKBP12 binding inhibits receptor activation in the absence of TGFβ or in the presence of low concentrations of the ligand.

FKBP12-blind TβR-I mutants are hypersensitive to TβR-II

Loss of FKBP12 binding might constitutively activate TβR-I by endowing this receptor with a certain level of ligand- and TβR-II-independent activity. Alternatively, loss of FKBP12 binding might hypersensitize TβR-I to activation by TβR-II. To distinguish between these two possibilities, wild-type TβR-I, TβR-I(T204D) or TβR-I(L193G) were transfected either alone or with TβR-II into DR-26 cells. These cells are a mutant Mv1Lu clone that harbor a nonsense mutation in the transmembrane region of the TβR-II and lack this receptor on the membrane (Laiho *et al.*, 1990; Wrana *et al.*, 1992). As shown in Figure 4A, DR-26 cells recover TGFβ responsiveness when transfected with TβR-II (Wrana *et al.*, 1992). Transfection of TβR-I(T204D) into DR-26 cells causes TGFβ-like responses in the absence of TGFβ and TβR-II, whereas transfection of TβR-I, either alone or with co-transfected TβR-II, has no such effect (Figure 4A; Wieser *et al.*, 1995). The TβR-II-independent basal activity of TβR-I(T204D) in DR-26 cells is a demonstration of the constitutive activity of TβR-I(T204D) (Wieser *et al.*, 1995). When TβR-I(L193G) was transfected into

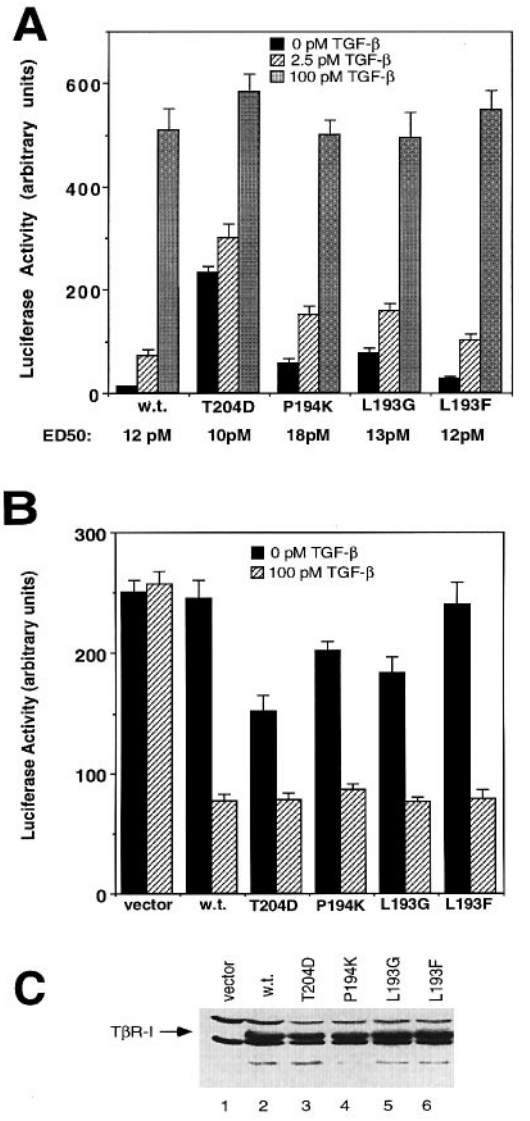

Fig. 3. TβR-I mutants defective in FKBP12 binding have an elevated basal activity. (**A**) R-1B cells were transiently co-transfected with the PAI-1 promoter-driven luciferase reporter p3TP-Lux and vector encoding TβR-I, either wild-type or carrying the indicated mutations. After 24 h, the indicated concentrations of TGFβ1 were added, and luciferase activity was determined 20 h later. Data are the average of triplicate determinations ±SD. The TGFβ1 concentration that caused a half-maximal stimulation (ED_{50}) of luciferase in each case was determined separately in a similar experiment with additional data points, as listed at the bottom. (**B**) R-1B cells were transiently co-transfected with the cyclin A promoter-driven luciferase reporter pCAL2 and the indicated TβR-I constructs. After 24 h, the indicated concentrations of TGFβ1 were added, and luciferase activity was determined 44 h later. (**C**) Aliquots from the same cell lysates used to measure luciferase activity in (A) were analyzed by immunoblotting with anti-HA antibody to monitor TβR-I expression levels.

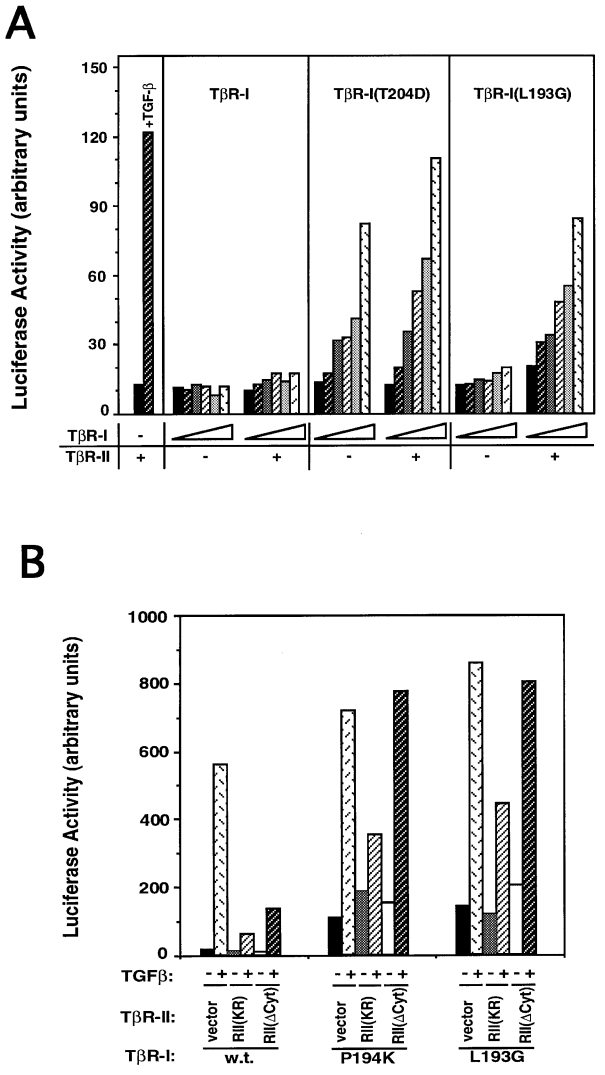

Fig. 4. (**A**) The elevated basal activity of a TβR-I mutant defective in FKBP12 binding requires TβR-II. The TβR-II-defective DR-26 cell line was transiently co-transfected with p3TP-Lux reporter and increasing amount of wild-type or mutant TβR-I DNAs (0.05–2 μg range) alone or with TβR-II DNA (0.5 μg), as indicated. Luciferase activity was determined after 48 h. The left panel shows the ability of DR-26 cells to respond to addition of 100 pM TGFβ when transfected with wild-type TβR-II. Data are the average of triplicate determinations. (**B**) TβR-I defective in FKBP12 binding are partially resistant to the dominant-negative effect of inactive TβR-II mutants. R-1B cells were transiently co-transfected with p3TP-Lux, wild-type or mutant TβR-I, and either TβR-II(K277R), TβR-II(ΔCyt) or empty vector. All constructs were in pCMV5 vector, except TβR-II(ΔCyt) which was in pMEP4. On the second day after transfection, cells were incubated in MEM containing 0.2% FBS and 50 μM $ZnCl_2$ for 4 h in order to induce TβR-II(ΔCyt) expression. Then cells were incubated with 100 pM TGFβ for another 20 h prior to the determination of luciferase activity.

DR-26 cells, its phenotype was unlike those of TβR-I or TβR-I(T204D): TβR-I(L193G) mediated TGFβ-like activation of 3TP-lux in the absence of TGFβ, but only when co-transfected with TβR-II (Figure 4A). Similar results were obtained with TβR-I(P194K) (data not shown). Thus, unlike the constitutively active form TβR-I(T204D), these FKBP12-blind TβR-I mutants require TβR-II in order to display a high basal activity. These mutants therefore appear to be hypersensitive to activation by basal interactions with TβR-II.

Additional evidence that TβR-I(L193G) and TβR-I(P194K) are hypersensitive to a limited source of activator was obtained by testing their activity in the presence of dominant-negative TβR-II constructs that act by limiting TβR-I access to the endogenous TβR-II. Two such constructs were used, TβR-II(ΔCyt) which is truncated right after the transmembrane region and thus lacks most of the cytoplasmic domain (Wieser *et al.*, 1993), and TβR-II(K277R) which is a full-length receptor with a point mutation designed to disrupt coordination of the α and β

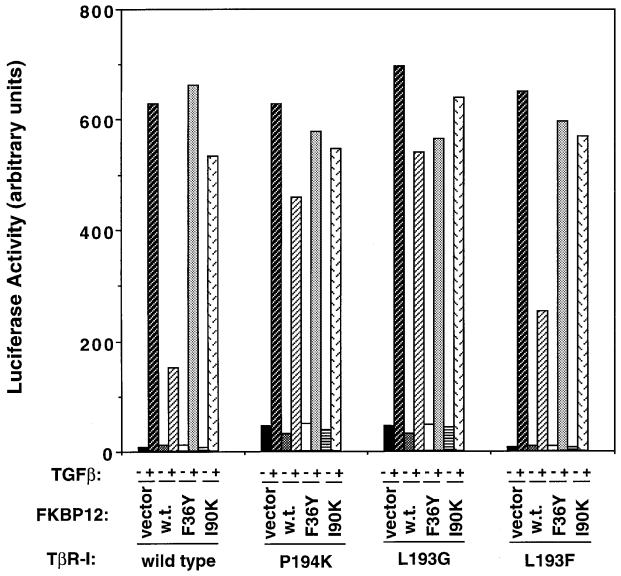

Fig. 5. FKBP12 binding to TβR-I correlates with inhibition of TGFβ action. p3TP-Lux DNA (2 μg), wild-type or mutant TβR-I receptor vector DNA (0.3 μg) and empty, wild-type or mutant FKBP12 vector DNA (1.5 μg), were transiently co-transfected into R-1B cells. After 24 h, cells were incubated with or without 100 pM TGFβ1. Twenty hours later, luciferase activity was determined. Data are the average of triplicate determinations.

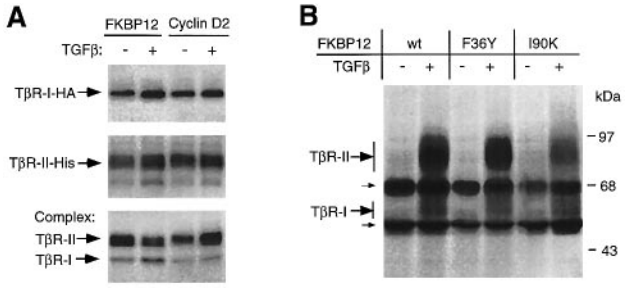

Fig. 6. FKBP12 does not prevent receptor complex formation. (**A**) Insect cells were co-infected with recombinant baculovirus expressing TβR-I-HA, TβR-II-His and either FKBP12 or cyclin D2, as indicated. After 40 h, cells were labeled with [^{35}S]methionine for 2 h and incubated with or without 1 nM TGFβ for the final 20 min. Cells were then lysed and samples of the lysates subjected to HA immunoprecipitation (top panel), binding to Ni-NTA agarose beads (middle panel) or binding to Ni-NTA agarose followed by HA immunoprecipitation of the eluate from these beads (bottom panel). Three times more lysate was used for the bottom panel. (**B**) COS-1 cells were transiently transfected with TβR-I-HA, TβR-II-His and the indicated wild-type or mutant FKBP12 cDNAs. Cells were labeled with 100 μCi/ml of [^{35}S]methionine for 3 h and incubated with or without 1 nM TGFβ for the final 20 min. Cells were then lysed and samples from the lysates subjected to the isolation of TβR-I-associated TβR-II by sequential Ni-NTA agarose incubation and HA immunoprecipitation. Small arrows point to the biosynthetic precursor forms of TβR-I and TβR-II. These forms spontaneously associate in COS-1 cells (Vivien *et al.*, 1995).

phosphate groups in the substrate ATP (Wrana *et al.*, 1992). These two TβR-II mutants can still bind TGFβ and form a complex with TβR-I, hence their dominant-negative effect on the endogenous TβR-II when over-expressed (Wrana *et al.*, 1992; Wieser *et al.*, 1993). TβR-II(K277R) or TβR-II(ΔCyt) were co-transfected with wild-type or mutant TβR-I into R-1B cells. As shown previously (Wieser *et al.*, 1993), signaling by TβR-I was inhibited by both TβR-II(K277R) and TβR-II(ΔCyt), with TβR-II(K277R) being more potent in this respect (Figure 4B). In contrast, the signaling function of the FKBP12-blind TβR-I mutants was less well inhibited by these dominant-negative constructs (Figure 4B). These results are consistent with an increased ability of the FKBP12-blind TβR-I mutants to become activated by a limiting level of endogenous TβR-II, thus resisting the dominant-negative effect of co-transfected inactive TβR-II constructs.

Inhibitory effect of FKBP12 overexpression on TβR-I function

In order to investigate the mechanism of TβR-I inhibition by FKBP12, we sought to induce this effect by over-expressing FKBP12. To this end, various FKBP12 and TβR-I constructs were co-transfected into R-1B cells, and the response of these cells to TGFβ was analyzed. Co-transfection of FKBP12 markedly decreased the TGFβ response in R-1B cells transfected with wild-type TβR-I (Figure 5, left panel). Importantly, FKBP12 had no significant effect on the TGFβ response in cells expressing FKBP12-blind TβR-I mutants (Figure 5, middle panels). Furthermore, neither FKBP12(F36Y) nor FKBP12(I90K) decreased the TGFβ response of R-1B cells transfected with TβR-I (Figure 5). Thus, FKBP12 overexpression recapitulates the inhibitory effect of FKBP12 on TβR-I function and the dependence of this effect on FKBP12 binding to TβR-I. We therefore investigated the mechanism of TβR-I inhibition by overexpressing FKBP12 in various cell systems.

First we tested whether FKBP12 might act as an inhibitor of TGFβ receptor association. Although ligand binding is normally required for TβR-I association with TβR-II, these two proteins have an intrinsic affinity for each other that becomes manifest in their ability to associate spontaneously when overexpressed via baculoviral vectors in insect cells (Ventura *et al.*, 1994) or by transfection in COS-1 cells (Chen and Weinberg, 1995; Vivien *et al.*, 1995; Feng and Derynck, 1996). Insect cells were co-infected with viruses encoding HA-tagged TβR-I, hexahistidine-tagged TβR-II and either FKBP12 or cyclin D2 as a negative control. The expression level of TβR-I and TβR-II was the same under all conditions, as determined by immunoprecipitation from [^{35}S]methionine-labeled cells via the corresponding tags (Figure 6A). The level of receptor complex, as determined by a two-step precipitation protocol, was also the same regardless of the presence of FKBP12 (Figure 6A, bottom panel). In COS-1 cells transfected with HA-tagged TβR-I, His-tagged TβR-II and either wild-type FKBP12 or the mutant FKBP12 forms that do not bind to TβR-I (Figure 6B), TGFβ addition promoted the association of TβR-I with TβR-II equally well in all cases (Figure 6B). Thus, FKBP12 inhibits neither the ligand-independent nor ligand-dependent association of TβR-I and TβR-II.

The converse possibility, namely that ligand binding to TβR-I may cause dissociation of FKBP12, has been examined previously by cross-linking TβR-I to [^{125}I]TGFβ in order to detect the ligand-bound receptor (Okadome *et al.*, 1996; Wang *et al.*, 1996b). These experiments have yielded conflicting results. In one report, FKBP12 interacted with the TGFβ receptor complex only if this complex contained a kinase-inactive TβR-II (Wang *et al.*,

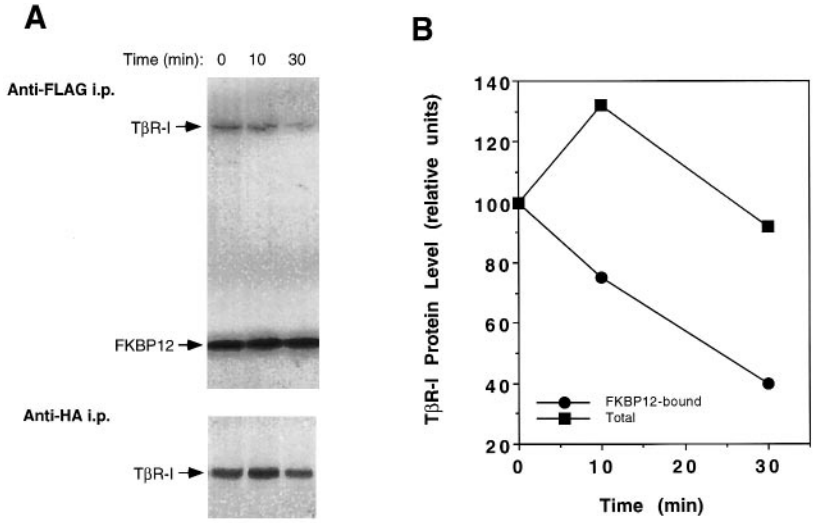

Fig. 7. FKBP12 dissociates from TβR-I upon ligand stimulation. (**A**) COS-1 cells transfected with wild-type HA-tagged TβR-I, TβR-II and Flag-tagged FKBP12 were labeled with [^{35}S]methionine for 1 h and chased in the MEM medium containing excess cold methionine for 2 h. Then, cells were stimulated with 1 nM TGFβ for various times, and cell lysates immunoprecipitated with anti-Flag antibody to determine FKBP12–TβR-I complex (upper panel) and with anti-HA antibody to determine the TβR-I expression level (lower panel). (**B**) Densitometric quantitation of the TβR-I protein level in (A).

1996b), whereas in another report FKBP12 did interact with the wild-type receptor complex (Okadome *et al.*, 1996). We obtained results similar to those of Okadome *et al.* (1996) using either wild-type or kinase-defective receptor complexes (data not shown). The conflicting results obtained by different groups might be due to the fact that receptor cross-linking to [^{125}I]TGFβ is done under media and temperature conditions (Massagué, 1987) that interfere with normal receptor function. As an alternative, we transfected COS-1 cells with HA-tagged TβR-I, TβR-II and Flag-tagged FKBP12 and labeled these cells with [^{35}S]methionine. The label was chased with cold methionine for 2 h, and TGFβ was then added for various periods. The level of TβR-I co-precipitating with FKBP12 from these cells was decreased as a function of time with TGFβ (Figure 7), a result that is consistent with a progressive loss of bound FKBP12 from the TβR-I pool. Note that only a small fraction of the type I receptor is associated with the type II receptor at any time (Chen and Weinberg, 1995; Attisano *et al.*, 1996), which may explain the slow kinetics of FKBP12 loss from the TβR-I pool in our experiment.

FKBP12 inhibition of TβR-I phosphorylation

Next we tested whether FKBP12 might protect TβR-I from activation by preventing its phosphorylation within the receptor complex. We used R-1B cells since in these cells the association and transphosphorylation of transfected TGFβ receptors are dependent on TGFβ (Wrana *et al.*, 1994). R-1B cells were transiently transfected with HA-tagged TβR-I, histidine-tagged TβR-II and either the wild-type FKBP12 or mutant FKBP12 forms that do not bind TβR-I. Cells were labeled with [^{32}P]phosphate and incubated with TGFβ, and receptor complexes were isolated by a sequential precipitation protocol. The recovery of labeled TβR-II was similar in all conditions (Figure 8A). Note that the recovery of TβR-II in this protocol requires its association with TβR-I and, thus, a similar amount of recovered TβR-II reflects the recovery of a similar amount of TβR-I. As previously described (Wrana *et al.*, 1994), TβR-I present in this complex was phosphorylated when recovered from cells expressing wild-type TβR-II but not from cells expressing a kinase-defective TβR-II (Figure 8A, lanes 2 and 3). Co-transfection of wild-type FKBP12 strongly inhibited TβR-I phosphorylation in the complex (lane 4), whereas the binding-defective FKBP12 constructs did not (lanes 5 and 6). These results indicate that FKBP12 binding to TβR-I inhibits its phosphorylation by TβR-II in the receptor complex.

Stimulation of TβR-I phosphorylation by rapamycin

Rapamycin binds to FKBP12 and inhibits its rotamase activity (Schreiber, 1991). Due to its structural resemblance to a Leu–Pro sequence, rapamycin is thought to act as a transition state analog for amide rotation by FKBP12 (Albers *et al.*, 1990). In agreement with previous observations (Okadome *et al.*, 1996), rapamycin decreased FKBP12 binding to TβR-I in a concentration-dependent manner (data not shown). Since the binding of FKBP12 to TβR-I inhibits the activation of this receptor, we determined whether rapamycin can stimulate TβR-I phosphorylation by TβR-II. Rapamycin addition to cells transfected with both receptors slightly increased the phosphorylation level of TβR-I in the receptor complex, as determined by normalization of the [^{32}P]TβR-I signal relative to the associated [^{32}P]TβR-II signal (Figure 8B), which is consistent with the possibility that rapamycin may dissociate endogenous FKBP12 from the receptor. However, the effect of rapamycin was much clearer when tested on cells co-transfected with FKBP12. As described above, FKBP12 overexpression significantly decreased the TGFβ-induced transphosphorylation of TβR-I. This effect of FKBP12 was completely reversed by rapamycin (Figure 8B). Since FKBP12 binding to TβR-I inhibits transphosphorylation by TβR-II, and rapamycin causes the dissociation of FKBP12 from TβR-I, the results in Figure

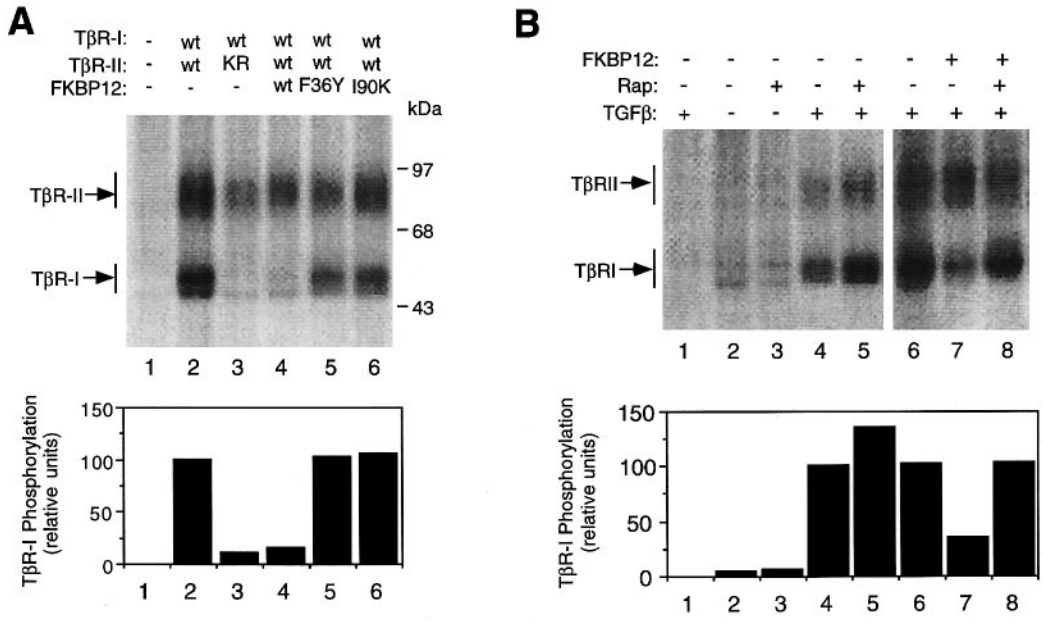

Fig. 8. (**A**) FKBP12 inhibits TβR-I phosphorylation. R-1B cells were co-transfected with wild-type TβR-I, wild-type or kinase-defective TβR-II and wild-type or binding-defective FKBP12 constructs, as indicated. Cells were labeled with [^{32}P]phosphate for 3 h and incubated with 1 nM TGFβ for the final 20 min. Receptor complexes were recovered by sequential precipitation via a hexahistidine tag in the TβR-II constructs and an HA-tag in the TβR-I construct. (**B**) Rapamycin enhances TβR-I phosphorylation. R-1B cells were co-transfected with wild-type TβR-I, TβR-II, FKBP12 constructs or pCMV5 vector alone, as indicated. Cells were labeled with [^{32}P]phosphate for 3 h and incubated with 1 nM TGFβ and/or rapamycin for the final 20 min, as indicated. Receptor complexes were recovered by sequential precipitation via a hexahistidine tag in the TβR-II constructs and an HA tag in the TβR-I construct.

8B suggest that rapamycin stimulates TβR-I transphosphorylation by inducing FKBP12 dissociation.

Discussion

Type I and type II TGFβ family receptors have intrinsic affinity for each other that leads to their association and activation when they are co-incubated *in vitro* (Ventura *et al.*, 1994) or overexpressed in mammalian cells (Chen and Weinberg, 1995; Chen *et al.*, 1995; Vivien *et al.*, 1995; Attisano *et al.*, 1996; Feng and Derynck, 1996; Luo and Lodish, 1996), insect cells (Ventura *et al.*, 1994) or yeast (Ventura *et al.*, 1994; Chen *et al.*, 1995; Kawabata *et al.*, 1995a; Liu *et al.*, 1995). This property could lead to leaky, ligand-independent signaling by the receptors, which probably would be incompatible with the proper function of the TGFβ signaling system. In the present studies, we present evidence that FKBP12 binding to TβR-I prevents leaky signaling by this receptor, and this effect involves an ability of FKBP12 to interfere with TβR-I phosphorylation by TβR-II.

FKBP12 binds adjacent to receptor phosphorylation sites

FKBP12 is a ubiquitous and relatively abundant protein, but knowledge about its normal role is still limited. FKBP12 recently has been shown to interact with many members of the TGFβ type I receptor family (Wang *et al.*, 1996b). This interaction involves the GS domain, or activation domain of TβR-I. This domain contains a cluster of ligand- and TβR-II-dependent phosphorylation sites in the sequence T^{185}TSGSGSGLP (Wrana *et al.*, 1994). This sequence is highly conserved in other type I receptors, and its phosphorylation is thought to lead to receptor activation in all cases (Franzén *et al.*, 1995; Wieser *et al.*, 1995; Attisano *et al.*, 1996; Hoodless *et al.*, 1996; Wiersdorff *et al.*, 1996). We show that FKBP12 binding to TβR-I is abolished by the missense mutations L193G or P194K in this sequence, whereas extensive mutation of the adjacent sequence T^{185}TSGSGS to VVAGAGA does not have this effect. Furthermore, recently it was shown that a P194A mutation disrupts FKBP12 association with TβR-I in a yeast two-hybrid system (Charng *et al.*, 1996). In agreement with this previous report, we conclude that FKBP12 recognizes a specific structure within the GS domain that includes the Leu–Pro motif. Since ActR-I and other type I receptors are also able to interact with FKBP12 in a two-hybrid system (Wang *et al.*, 1996b) and conserve the Leu–Pro sequence, it is likely that this motif mediates FKBP12 binding in every type I receptor.

The FKBP12 binding phenotype of these TβR-I mutations closely matches the substrate specificity of FKBP12 as a peptidyl-prolyl isomerase. FKBP12 shows a strong preference for Leu or Phe preceding the target proline, and shows a strong bias against Gly in that position (Albers *et al.*, 1990; Harrison and Stein, 1990). Indeed, the L193F does not affect the FKBP12 binding activity of TβR-I, whereas the L193G mutation eliminates this activity. Additional evidence that FKBP12 specifically recognizes the Leu–Pro motif is provided by previous reports (Okadome *et al.*, 1996; Wang *et al.*, 1996b) and present observations that rapamycin and FK506 inhibit FKBP12 binding to TβR-I. By virtue of their resemblance to a Leu–(twisted amide)–Pro structure, these agents bind to the active site of FKBP12 (Albers *et al.*, 1990). The involvement of the FKBP12 active site in TβR-I recognition is suggested further by our results with FKBP12 forms that contain mutations near the active site.

Both F36Y mutation, which impairs the isomerase activity of FKBP12 but not its affinity for FK506 (Wiederrecht *et al.*, 1992), and the I90K mutation, which impairs the interaction of the FKBP12–FK506 complex with calcineurin (Yang *et al.*, 1993; Futer *et al.*, 1995), prevent FKBP12 binding to TβR-I.

Taken together, these results indicate that TβR-I interacts with the peptidyl-prolyl-binding site of FKBP12 and adjacent areas encompassing the calcineurin-binding site. The latter contacts probably involve TβR-I sites other than the Leu193–Pro sequence. A Leu–Pro sequence is an optimal substrate for isomerization by FKBP12 in the context of a short peptide (Albers *et al.*, 1990; Harrison and Stein, 1990). However, given that the FKBP12–TβR-I interaction is stable, it seems unlikely that FKBP12 binding would affect isomerization of the Leu193–Pro bond in TβR-I. It seems more likely that contacts involving the catalytic site and additional sites in FKBP12 act cooperatively, generating a tight interaction with TβR-I. The close proximity of the Leu–Pro site to the phosphorylation sites in TβR-I is likely to underlie the inhibitory effect of FKBP12 on TβR-I.

A new role for FKBP12: protection against leaky TGFβ receptor activation

Our results show that TβR-I mutants defective in FKBP12 binding ('FKBP12-blind' mutants) have an elevated signaling activity compared with wild-type TβR-I. The signaling advantage of these mutants is also observed at low concentrations of added TGFβ, and progressively disappears at higher concentrations. Our results also show that overexpression of FKBP12 inhibits signaling by TβR-I. This effect is eliminated by mutations in either FKBP12 or TβR-I that prevent their association. Importantly, the elevated activity of FKBP12-blind TβR-I mutants requires TβR-II since these mutants are inactive when expressed in cells lacking TβR-II. Ligand-induced activation of FKBP12-blind TβR-I mutants is poorly inhibited by dominant-negative TβR-II constructs, further arguing that these mutants can be activated by an otherwise limiting level of TβR-II activity. Therefore, FKBP12-blind TβR-I mutants are hypersensitive to activation by TβR-II. In the absence of FKBP12 binding, the intrinsic affinity of the receptors for each other may be sufficient to generate ligand-independent signals.

Our observation that FKBP12 is a negative regulator of TβR-I is in general agreement with a recent report by Wang *et al.* (1996b). However, there are important differences between the results and conclusions of the two studies. Wang *et al.* concluded that FKBP12 binding to TβR-I was not related to it being a rotamase. However, the identification of Leu193–Pro as a FKBP12-binding site and additional evidence presented here argue that the interaction involves the FKBP12 catalytic site. Based on the lack of calcineurin binding and receptor inhibitory effect of the FKBP12(G89P,I90K) mutant, and the assumption that this mutant binds to the receptor, Wang *et al.* concluded that the ability of FKBP12 to inhibit TβR-I activity is based on its ability to bind calcineurin. However, we show that FKBP12(I90K) fails to bind to TβR-I, which explains the lack of receptor inhibitory activity of this mutant. Signaling by TβR-I is not likely to involve the docking of a mediator to the FKBP12-binding site since mutations in Leu193–Pro that abolish FKBP12 binding do not interfere with signaling. Based on previous observations that the farnesyl transferase α-subunit can interact with TβR-I (Wang *et al.*, 1996a), Wang *et al.* proposed that the receptor signaling process involves the release of farnesyl transferase along with FKBP12 (Wang *et al.*, 1996b). However, it has been shown that the farnesyl transferase holoenzyme does not interact with the TGFβ receptor, and TGFβ action does not affect farnesyl transferase activity or the farnesylation state of proteins such as Ras (Ventura *et al.*, 1996).

Mechanism of TβR-I inhibition by FKBP12

FKBP12 binding could inhibit receptor association, transphosphorylation or recognition of downstream substrates. We have investigated these possibilities. When tested in mammalian or insect cell assay systems, FKBP12 does not inhibit the association of TβR-I with TβR-II. Instead, our evidence suggests that receptor-bound FKBP12 specifically inhibits TβR-I phosphorylation by TβR-II. Several observations support this hypothesis. First, the overexpression of FKBP12 inhibits ligand-induced TβR-I phosphorylation, whereas the overexpression of mutants defective in TβR-I binding does not. Second, rapamycin, which causes FKBP12 dissociation from TβR-I, augments TβR-I phosphorylation and reverses the inhibitory effect of overexpressed FKBP12 on TβR-I phosphorylation.

The ability to inhibit phosphorylation is consistent with the observation that FKBP12 binds to a site adjacent to, and possibly encompassing, the phosphorylation sites of the GS domain. FKBP12 binding to this site is likely to hinder the entry of the phosphorylation sites into the catalytic center of the TβR-II kinase, thus protecting TβR-I from activation during ligand-independent interactions with TβR-II. However, at physiological levels of expression, FKBP12 may not be able to remain bound to TβR-I within the ligand-induced receptor complex. In the presence of ligand, TβR-I and TβR-II form a very tight complex (Wrana *et al.*, 1994), and this interaction may displace the TβR-I-bound FKBP12 thus allowing transphosphorylation by TβR-II. Previous experiments using receptor affinity labeling techniques indeed have provided evidence that TGFβ causes the release of FKBP12 from TβR-I (Wang *et al.*, 1996b), although others using the same experimental approach have obtained discrepant results (Okadome *et al.*, 1996). However, our present results using metabolically labeled receptors support the hypothesis that TGFβ causes the release of FKBP12 from TβR-I. Since FKBP12 is not phosphorylated by TGFβ receptors (Okadome *et al.*, 1996; our unpublished observation), its release may be driven by a ligand-induced structural change in the receptors.

The available evidence suggests a model in which TβR-I-bound FKBP12 may impede the activation of receptor complexes formed in the absence of ligand (Figure 9). In this model, FKBP12 bound to the Leu–Pro site in the TTSGSGSGLP sequence hinders access of TβR-II to phosphorylation sites in this sequence during spontaneous, ligand-independent interactions between the two receptors. In the presence of ligand, the receptors form tight complexes in which FKBP12 dissociation and TβR-I phosphorylation take place. The activated TβR-I kinase domain in turn recruits and phosphorylates SMADs

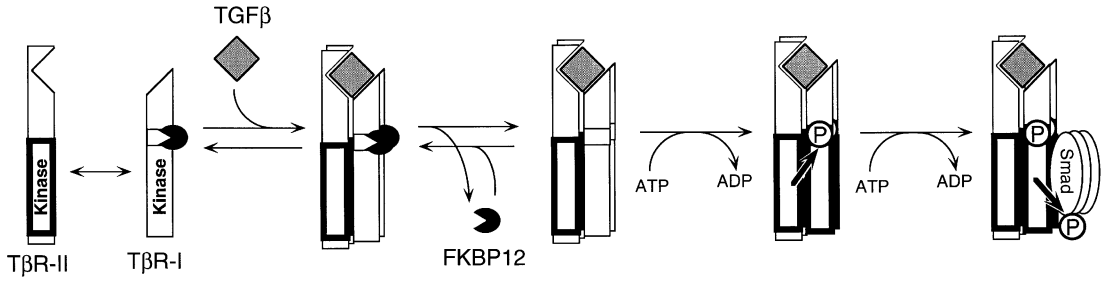

Fig. 9. A model illustrating that FKBP12 may act to prevent signaling during spontaneous TGFβ receptor interactions. In the absence of ligand, FKBP12 bound to the GS domain of TβR-I impedes receptor activation by TβR-II during contacts resulting from the intrinsic affinity of these receptors for each other (left). When tight receptor complexes are induced by the ligand, complete dissociation of FKBP12 allows phosphorylation of the GS domain and therefore activation of TβR-I. Active kinase domains are depicted in thick tracing. See text for additional details and references.

(Macias-Silva *et al.*, 1996; Zhang *et al.*, 1996). We have observed that Smad2 association with the activated receptors is not inhibited by Leu–Pro mutations that eliminate FKBP12 binding (Y.-G.Chen and J.Massagué, unpublished results), suggesting that Leu–Pro is not a SMAD-binding site that FKBP12 occludes.

In summary, we propose that bound FKBP12 may protect TβR-I against phosphorylation and thus activation when spontaneous, ligand-independent encounters with TβR-II occur in the cell. Even if quantitatively limited, the signaling activity resulting from such interactions in the absence of FKBP12 could lead to significant biological effects. Given the ubiquitous presence of TGFβ and related factors in the normal cellular environment, and the many effects of these factors on target cells, keeping the basal activity of their receptors under tight control is probably crucial in the physiological setting. Several highly specialized mechanisms control ligand access to the TGFβ family receptors. FKBP12 binding to type I receptors may provide an additional safeguard against uncontrolled receptor activation.

Materials and methods

DNA constructs

Site-directed mutagenesis was performed by PCR using appropriate primers (see below). For TβR-I mutagenesis, the resulting PCR products were subcloned into TβR-I in the cytomegalovirus promoter-driven mammalian expression vector pCMV5 (Andersson *et al.*, 1989), to replace the corresponding wild-type fragment. All mutations were confirmed by DNA sequencing. TβR-I and its derivatives were tagged at the C-terminus with the influenza virus HA epitope (Cárcamo *et al.*, 1994). The TβR-II cDNA C-terminally tagged with HA or hexahistidine (Wrana *et al.*, 1994), and the TβR-II(K277R) cDNA were subcloned in pCMV5 (Cárcamo *et al.*, 1994). TβR-II(ΔCyt) was subcloned in the Zn^{2+}-inducible mammalian expression vector pMEP4 (Wieser *et al.*, 1993). Human FKBP12 and its mutant derivatives were tagged with the Flag epitope sequence (Hopp *et al.*, 1988) at the N-terminus and subcloned into pCMV5. p3TP-Lux, which contains a luciferase reporter gene under the control of TGFβ-responsive elements, has been described (Cárcamo *et al.*, 1994). pCAL2, which contains a luciferase reporter gene under the control of the cyclin A promoter (Feng *et al.*, 1995), was a gift from Dr R.Derynck.

The PCR primers were 5′-CAAGCAATTTTAAACCTGAGCCAGA-3′ for TβR-I(P194K); 5′-CAATGGTCCACCTGAGCCAGAACCT-3′ for TβR-I(L193G); 5′-CAATGGAAAACCTGAGCCAGAACC-3′ for TβR-I(L193F); 5′-AAGAAATATGATTCCTCCCGGGAC-3′ for FKBP12(F36Y); and 5′-CCCAGGCATCATCCCACCACATGCC-3′ for FKBP12(I90K).

Cell lines and transfections

The TβR-I-defective cell line R-1B (subclone L17) and the TβR-II-defective cell line DR-26 clone were derived from the mink lung epithelial cell line Mv1Lu (CCL-64; American Tissue Culture Collection) by chemical mutagenesis and selection for resistance to TGFβ (Boyd and Massagué, 1989; Laiho *et al.*, 1990). Parental Mv1Lu and its derivatives were maintained in minimal essential medium (MEM, Gibco-BRL) supplemented with 10% fetal bovine serum (FBS), non-essential amino acids, 100 U/ml of penicillin, 100 μg/ml of streptomycin, 20 μg/ml of gentamicin and 0.5 μg/ml of Fungizone (Gibco-BRL) at 37°C in a 5% CO_2 incubator. Monkey kidney COS-1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL) supplemented with 10% FBS and the same antimicrobials. Transient transfection of parental and mutant Mv1Lu cells was carried out by a DEAE–dextran method. Briefly, exponentially growing cells of 50–70% confluency were incubated with MEM containing 1–1.5 μg/ml of DNA, 100 μM chloroquine and 125 μg/ml of DEAE–dextran at 37°C for 3 h. Then, cells were shocked for 2 min with 10% dimethyl sulfoxide in phosphate-buffered saline (PBS) at room temperature. Subsequently, cells were washed and placed in growth medium. Transient transfection of COS-1 cells was performed similarly, except that cells were incubated in DMEM containing 1.5 μg/ml of DNA, 100 μM chloroquine, 400 μg/ml of DEAE–dextran and 10% Nu serum (Becton Dickinson).

Metabolic labeling, immunoprecipitation and cross-linking

Two days after transient transfection, cells were incubated in methionine-free MEM containing 100 μCi/ml of [^{35}S]methionine (Tran^{35}S-label, ICN) for 3 h at 37°C. Cells were then washed once in ice-cold PBS and treated with lysis buffer [20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100 (v/v), and a protease inhibitor mixture]. To isolate HA-tagged receptors, cell lysates were immunoprecipitated for 1 h at 4°C with 10 μg/ml of anti-HA monoclonal antibody 12CA5 (Babco). To isolate Flag-tagged FKBP12 or FKBP12–receptor complexes, cell lysates were incubated with 10 μg/ml of anti-Flag monoclonal antibody M2 (Kodak) for 1 h at 4°C. Protein G–Sepharose beads (Pharmacia) were then added for 1 h at 4°C to collect the immune complexes. 12CA5 immunoprecipitates were washed three times for 10 min at 4°C with 20 mM Tris–HCl, pH 7.4, 0.5% deoxycholate, 0.1% SDS. M2 immunoprecipitates were washed five times for 10 min at 4°C with TNT buffer [20 mM Tris–HCl, pH 7.4, 150 mM NaCl 0.5% (v/v) Triton X-100]. Subsequently, samples were analyzed using SDS–PAGE.

Receptor cross-linking with [^{125}I]TGFβ (NEN) was performed 40 h later after transfection. After being left to sit for 30 min at 37°C in Krebs–Ringer HEPES buffer (KRH) (Lin *et al.*, 1992) plus 0.5% bovine serum albumin (BSA), transfected COS-1 cells were incubated with 100 pM [^{125}I]TGFβ in KRH containing 0.5% BSA for 3.5 h at 4°C, then washed four times with KRH containing 0.5% BSA and once with KRH. Cross-linking was carried out by incubation for 15 min at 4°C with 60 μg/ml disuccinimidyl suberate in KRH, and stopped by washing twice with 0.25 M sucrose, 10 mM Tris–HCl, pH 7.4 and 1 mM EDTA. After cell lysis and immunoprecipitation, samples were analyzed by SDS–PAGE.

Luciferase assay

One day after transfection, cells were seeded into 24-well plates. Four hours later, cells were washed once with MEM containing 0.2% FBS and incubated in the same medium with or without TGFβ1 (100 pM unless otherwise indicated) for 20 h (for p3TP-Lux assay) or for 44 h (for pCAL2 assay). After washing with PBS, cells were harvested, and the luciferase activity of cell lysates was determined using a luciferase assay system (Promega) as described by the manufacturer. Total light

emission during the initial 20 s of the reaction was measured in a luminometer (Berthold Lumat LB 9501). Samples were assayed in triplicate.

Receptor association assays in insect and mammalian cells

Insect High 5 cells were co-infected with recombinant baculoviruses encoding HA-tagged TβR-I (TβR-I-HA), hexahistidine-tagged TβR-II (TβR-II-His) and FKBP12 or cyclin D2 (Ventura *et al.*, 1994). After 40 h, cells were labeled with 100 μCi/ml of [^{35}S]methionine for 2 h. In the last 20 min of labeling, cells were treated with or without TGFβ at a final concentration of 1 nM. Cells were then lysed and the lysates were divided into aliquots for verification of TβR-I expression by anti-HA immunoprecipitation, isolation of hexahistidine-tagged TβR-II with Ni-NTA agarose beads, or purification of the TβR-I–TβR-II complexes (Wrana *et al.*, 1994). To purify the complexes between TβR-I and TβR-II, cell lysates were supplemented with imidazole at a final concentration of 20 mM and incubated with Ni-NTA agarose beads (Qiagen) for 1 h at 4°C. After washing three times with TNT buffer containing 20 mM imidazole, bound proteins were eluted with TNT buffer containing 250 mM imidazole at room temperature for 10 min. The eluates were diluted with 10 volumes of TNT buffer and then subjected to HA immunoprecipitation. The immunoprecipitates were analyzed by SDS–PAGE and fluorography.

To assay receptor association in mammalian cells, COS-1 cells were transiently transfected with pCMV5 vectors encoding TβR-I-HA and TβR-II-His, and labeled with 100 μCi/ml of [^{35}S]methionine for 3 h. In the last 20 min of labeling, cells were treated with or without TGFβ at a final concentration of 1 nM. Cells were then lysed and the complexes between TβR-I and TβR-II subsequently purified.

Acknowledgements

We thank Andrew Marks and Michael Rosen for thoughtful discussions, Nikola Pavletich for help with the depiction of the FKBP12 structure, Frances Weis-Garcia and Jeffrey Wrana for help in the preliminary stages of this work, and Rik Derynck for the pCAL2 construct. This work was supported by NIH grant CA34610 to J.M., and an NIH grant to Memorial Sloan Kettering Cancer Center. Y.-G.C. and J.M. are a Research Associate and an Investigator of the Howard Hughes Medical Institute. F.L. is a fellow of the Jane Coffin Childs Memorial Fund for Medical Research.

References

Albers,M.W., Walsh,C.T. and Schreiber,S.L. (1990) Substrate specificity for the human rotamase FKBP: a view of FK506 and rapamycin as leucine–(twisted amide)–proline mimics. *J. Org. Chem.*, **55**, 4984–4986.

Andersson,S., Davis,D.L., Dahlback,H., Jornvall,H. and Russell,D.W. (1989) Cloning, structure, and expression of the mitochondrial cytochrome P-450 sterol 26-hydroxylase, a bile acid biosynthetic enzyme. *J. Biol. Chem.*, **264**, 8222–8229.

Attisano,L., Wrana,J.L., Montalvo,E. and Massagué,J. (1996) Activation of signalling by the activin receptor complex. *Mol. Cell. Biol.*, **16**, 1066–1073.

Baker,J.C. and Harland,R.M. (1996) A novel mesoderm inducer, Madr2, functions in the activin signal transduction pathway. *Genes Dev.*, **10**, 1880–1889.

Boyd,F.T. and Massagué,J. (1989) Transforming growth factor-β inhibition of epithelial cell proliferation linked to the expression of a 53-kDa membrane receptor. *J. Biol. Chem.*, **264**, 2272–2278.

Brillantes,A.B., Ondrias,K., Scott,A., Kobrinsky,E., Ondriasova,E., Jayaraman,T., Landers,M., Ehrlich,B.E. and Marks,A.R. (1994) Stabilization of calcium release channel (ryanodine receptor) function by FK506-binding protein. *Cell*, **77**, 513–523.

Brown,E.J., Albers,M.W., Shin,T.B., Ichikawa,K., Keith,C.T., Lane,W.S. and Schreiber,S.L. (1994) A mammalian protein targeted by G1-arresting rapamycin–receptor complex. *Nature*, **369**, 756–758.

Brown,E.J., Beal,P.A., Keith,C.T., Chen,J., Shin,T.B. and Schreiber,S.L. (1995) Control of p70 s6 kinase by kinase activity of FRAP *in vivo*. *Nature*, **377**, 441–446.

Cameron,A.M., Steiner,J.P., Sabatini,D.M., Kaplin,A.I., Walensky,L.D. and Snyder,S.H. (1995) Immunophilin FK506 binding protein associated with inositol 1,4,5-trisphosphate receptor modulates calcium flux. *Proc. Natl Acad. Sci. USA*, **92**, 1784–1788.

Cárcamo,J., Weis,F.M., Ventura,F., Wieser,R., Wrana,J.L., Attisano,L. and Massagué,J. (1994) Type I receptors specify growth-inhibitory and transcriptional responses to transforming growth factor beta and activin. *Mol. Cell. Biol.*, **14**, 3810–3821.

Charng,M.-J., Kinnunen,P., Hawker,J., Brand,T. and Schneider,M.D. (1996) FKBP-12 recognition is dispensable for signal generation by type I transforming growth factor-β receptors. *J. Biol. Chem.*, **271**, 22941–22944.

Chen,F. and Weinberg,R.A. (1995) Biochemical evidence for the autophosphorylation and transphosphorylation of transforming growth factor beta receptor kinases. *Proc. Natl Acad. Sci. USA*, **92**, 1565–1569.

Chen,R.H., Moses,H.L., Maruoka,E.M., Derynck,R. and Kawabata,M. (1995) Phosphorylation-dependent interaction of the cytoplasmic domains of the type I and type II transforming growth factor-beta receptors. *J. Biol. Chem.*, **270**, 12235–12241.

Chen,X., Rubock,M.J. and Whitman,M. (1996) A transcriptional partner for MAD proteins in TGF-β signalling. *Nature*, **383**, 691–696.

Eppert,K. *et al.* (1996) MADR2 maps to 18q21 and encodes a TGFβ-regulated MAD-related protein that is functionally mutated in colorectal carcinoma. *Cell*, **86**, 543–552.

Feng,X.H. and Derynck,R. (1996) Ligand-independent activation of transforming growth factor (TGF) β signaling pathways by heteromeric cytoplasmic domains of TGF-β receptors. *J. Biol. Chem.*, **271**, 13123–13129.

Feng,X.H., Filvaroff,E.H. and Derynck,R. (1995) Transforming growth factor-beta (TGF-beta)-induced down-regulation of cyclin A expression requires a functional TGF-beta receptor complex. Characterization of chimeric and truncated type I and type II receptors. *J. Biol. Chem.*, **270**, 24237–24245.

Franzén,P., Heldin,C.H. and Miyazono,K. (1995) The GS domain of the transforming growth factor-beta type I receptor is important in signal transduction. *Biochem. Biophys. Res. Commun.*, **207**, 682–689.

Futer,O., DeCenzo,M.T., Aldape,R.A. and Livingston,D.J. (1995) FK506 binding protein mutational analysis. Defining the surface residue contributions to stability of the calcineurin co-complex. *J. Biol. Chem.*, **270**, 18935–18940.

Graff,J.M., Bansal,A. and Metton,D.A. (1996) *Xenopus* Mad proteins transduce distinct subsets of signals for the TGFβ superfamily. *Cell*, **85**, 479–487.

Green,J.B.A. and Smith,J.C. (1990) Graded changes in dose of a *Xenopus* activin A homologue elicit stepwise transitions in embryonic cell fate. *Nature*, **347**, 391–394.

Harding,M.W., Galat,A., Uehling,D.E. and Schreiber,S.L. (1989) A receptor for the immunosuppressant FK506 is a *cis–trans* peptidyl-prolyl isomerase. *Nature*, **341**, 758–760.

Harrison,R.K. and Stein,R.L. (1990) Substrate specificities of the peptidyl prolyl *cis–trans* isomerase activities of cyclophilin and FK-506 binding protein: evidence for the existence of a family of distinct enzymes. *Biochemistry*, **29**, 3813–3816.

Hemmati-Brivanlou,A., Kelly,O.G. and Melton,D.A. (1994) Follistatin, an antagonist of activin, is expressed in the Spemann Organizer and displays direct neuralizing activity. *Cell*, **77**, 283–295.

Holly,S.A., Neul,J.L., Attisano,L., Wrana,J.L., Sasai,Y., O'Connor,M.B., De Robertis,E.M. and Ferguson,E.L. (1996) The *Xenopus* dorsalizing factor noggin ventralizes *Drosophila* embryos by preventing DPP from activating its receptor. *Cell*, **86**, 607–617.

Hoodless,P.A., Haerry,T., Abdollah,S., Stapleton,M., O'Connor,M.B., Attisano,L. and Wrana,J.L. (1996) MADR1, a MAD-related protein that functions in BMP2 signaling pathway. *Cell*, **85**, 489–500.

Hopp,T.P., Prickett,K.S., Price,V.L., Libby,R.T., March,C.J., Cerretti,D.P., Urdal,D.L. and Conlon,P.J. (1988) A short polypeptide marker sequence useful for recombinant protein identification and purification. *Bio/Technology*, **6**, 1204–1210.

Jayaraman,T., Brillantes,A.M., Timerman,A.P., Fleischer,S., Tempst,P. and Marks,A.R. (1992) FK506 binding protein associated with the calcium release channel (ryanodine receptor). *J. Biol. Chem.*, **267**, 9474–9477.

Kawabata,M., Chytil,A. and Moses,H.L. (1995a) Cloning of a novel type II serine/threonine kinase receptor through interaction with the type I transforming growth factor-beta receptor. *J. Biol. Chem.*, **270**, 5625–5630.

Kawabata,M., Imamura,T., Miyazono,K., Engel,M.E. and Moses,H.L. (1995b) Interaction of the transforming growth facotr-β type I receptor with farnesyl-protein transferase. *J. Biol. Chem.*, **270**, 29628–29631.

Kretzschmar,M., Liu,F., Hata,A., Doody,J. and Massagué,J. (1997) The TGF-β family mediator Smad1 is directly phosphorylated and functionally activated by the BMP receptor kinase. *Genes Dev.*, **11**, 984–995.

Lagna,G., Hata,A., Hemmati-Brivanlou,A. and Massagué,J. (1996) Partnership between DPC4 and SMAD proteins in TGF-β signalling pathways. *Nature*, **383**, 832–836.

Laiho,M., Weis,F.M.B. and Massagué,J. (1990) Concomitant loss of transforming growth factor (TGF)-β receptor type I and II in TGF-β-resistant cell mutants implicates both receptor types in signal transduction. *J. Biol. Chem.*, **265**, 18518–18524.

Lin,H.Y., Wang,X.F., Ng-Eaton,E., Weinberg,R.A. and Lodish,H.F. (1992) Expression cloning of the TGF-beta type II receptor, a functional transmembrane serine/threonine kinase. *Cell*, **68**, 775–785.

Liu,F., Ventura,F., Doody,J. and Massagué,J. (1995) Human type II receptor for bone morphogenic proteins (BMPs): extension of the two-kinase receptor model to the BMPs. *Mol. Cell. Biol.*, **15**, 3479–3486.

Liu,F., Hata,A., Baker,J.C., Doody,J., Cárcamo,J., Harland,R.M. and Massagué,J. (1996) A human Mad protein acting as a BMP-regulated transcriptional activator. *Nature*, **381**, 620–623.

López-Casillas,F., Wrana,J.L. and Massagué,J. (1993) Betaglycan presents ligand to the TGF beta signaling receptor. *Cell*, **73**, 1435–1444.

Luo,K. and Lodish,H.F. (1996) Signaling by chimeric erythropoietin–TGF-β receptors: homodimerization of the cytoplasmic domain of the type I TGFβ receptor and heterodimerization with the type II receptor are both required for intracellular signal transduction. *EMBO J.*, 15, 4485–4496.

Macias-Silva,M., Abdollah,S., Hoodless,P.A., Pirone,R., Attisano,L. and Wrana,J.L. (1996) MADR2 is a substrate of the TGFβ receptor and its phosphorylation is required for nuclear accumulation and signaling. *Cell*, **87**, 1215–1224.

Massagué,J. (1987) Identification of receptors of type β transforming growth factor. *Methods Enzymol.*, **146**, 174–195.

Massagué,J. (1990) The transforming growth factor-β family. *Annu. Rev. Cell Biol.*, **6**, 597–641.

Massagué,J. (1996) TGFβ signaling: receptors, transducers, and Mad proteins. *Cell*, **85**, 947–950.

McCaffrey,P.G., Perrino,B.A., Soderling,T.R. and Rao,A. (1993) NF-ATp, a T lymphocyte DNA-binding protein that is a target for calcineurin and immunosuppressive drugs. *J. Biol. Chem.*, **268**, 3747–3752.

Moustakas,A., Lin,H.Y., Henis,Y.I., Plamondon,J., O'Connor-McCourt, M.D. and Lodish,H.F. (1993) The transforming growth factor beta receptors types I, II, and III form hetero-oligomeric complexes in the presence of ligand. *J. Biol. Chem.*, **268**, 22215–22218.

Nakamura,T., Takio,K., Eto,Y., Shibai,H., Titani,K. and Sugino,H. (1990) Activin-binding protein from rat ovary is follistatin. *Science*, **247**, 836–838.

Okadome,T., Oeda,E., Saitoh,M., Ichijo,H., Moses,H.L., Miyazono,K. and Kawabata,M. (1996) Characterization of the interaction of FKBP12 with the transforming growth factor-β type I receptor *in vivo*. *J. Biol. Chem.*, **271**, 21687–21690.

Piccolo,S., Sasai,Y., Lu,B. and De Robertis,E.M. (1996) Dorsoventral patterning in *Xenopus*: inhibition of ventral signals by direct binding of chordin to BMP-4. *Cell*, **86**, 589–598.

Roberts,A.B. and Sporn,M.B. (1990) The transforming growth factor-betas. In Sporn,M.B. and Roberts,A.B. (eds), *Peptide Growth Factors and Their Receptors.* Springer-Verlag, Heidelberg, pp. 419–472.

Sabatini,D.M., Erdjument-Bromage,H., Lui,M., Tempst,P. and Snyder,S.H. (1994) RAFT1: a mammalian protein that binds to FKBP12 in a rapamycin-dependent fashion and is homologous to yeast TORs. *Cell*, **78**, 35–43.

Savage,C., Das,P., Finelli,A.L., Townsend,S.R., Sun,C.Y., Baird,S.E. and Padgett,R.W. (1996) *Caenorhabditis elegans* genes *sma-2*, *sma-3*, and *sma-4* define a conserved family of transforming growth factor beta pathway components. *Proc. Natl Acad. Sci. USA*, **93**, 790–794.

Schreiber,S.L. (1991) Chemistry and biology of the immunophilins and their immunosuppressive ligands. *Science*, **251**, 283–287.

Schreiber,S.L. (1992) Immunophilin-sensitive protein phosphatase action in cell signaling pathways. *Cell*, **70**, 365–368.

Sekelsky,J.J., Newfeld,S.J., Raftery,L.A., Chartoff,E.H. and Gelbart, W.M. (1995) Genetic characterization and cloning of mothers against dpp, a gene required for decapentaplegic function in *Drosophila melanogaster*. *Genetics*, **139**, 1347–1358.

Siekierka,J.J., Wiederrecht,G., Greulich,H., Boulton,D., Hung,S.H.Y., Cryan,J., Hodges,P.J. and Sigal,N.H. (1990) The cytosolic-binding protein for the immunosuppressant FK-506 is both a ubiquitous and highly conserved peptipyl-prolyl *cis–trans* isomerase. *J. Biol. Chem.*, **265**, 21011–21015.

ten Dijke,P., Miyazono,K. and Heldin,C.H. (1996) Signaling via hetero-oligomeric complexes of type I and type II serine/threonine kinase receptors. *Curr. Opin. Cell Biol.*, **8**, 139–145.

Ventura,F., Doody,J., Liu,F., Wrana,J.L. and Massagué,J. (1994) Reconstitution and transphosphorylation of TGF-beta receptor complexes. *EMBO J.*, **13**, 5581–5589.

Ventura,F., Liu,F., Doody,J. and Massagué,J. (1996) Interaction of transforming growth factor-β receptor I with farnesyl-protein transferase-α in yeast and mammalian cells. *J. Biol. Chem.*, **271**, 13931–13934.

Vivien,D., Attisano,L., Wrana,J.L. and Massagué,J. (1995) Signaling activity of homologous and heterologous transforming growth factor-beta receptor kinase complexes. *J. Biol. Chem.*, **270**, 7134–7141.

Wang,T., Donahoe,P.K. and Zervos,A.S. (1994) Specific interaction of type I receptors of the TGF-beta family with the immunophilin FKBP-12. *Science*, **265**, 674–676.

Wang,T., Danielson,P.D., Li,B.Y., Shah,P.C., Kim,S.D. and Donahoe,P.K. (1996a) The p21(RAS) farnesyltransferase alpha subunit in TGF-beta and activin signaling. *Science*, **271**, 1120–1122.

Wang,T., Li,B.Y., Danielson,P.D., Shah,P.C., Rockwell,S., Lechleider, R.J., Martin,J., Manganaro,T. and Donahoe,P.K. (1996b) The immunophilin FKBP12 functions as a common inhibitor of the TGFβ family type I receptors. *Cell*, **86**, 435–444.

Weis-Garcia,F. and Massagué,J. (1996) Complementation between kinase-defective and activation-defective TGF-β receptors reveals a novel form of receptor cooperativity essential for signaling. *EMBO J.*, **15**, 276–289.

Wiederrecht,G., Hung,S., Chan,H.K., Marcy,A., Martin,M., Calaycay,J., Sigal,N., Kincaid,R.L. and Siekierka,J.J. (1992) Characterization of high molecular weight FK-506 binding activities reveals a novel FK-506-binding protein as well as a protein complex. *J. Biol. Chem.*, **267**, 21753–21760.

Wiersdorff,V., Lecuit,T., Cohen,S.M. and Mlodzik,M. (1996) Mad acts downstream of Dpp receptors, revealing a differential requirement for dpp signaling in initiation and propagation of morphogenesis in the *Drosophila* eye. *Development*, **122**, 2153–2162.

Wieser,R., Attisano,L., Wrana,J.L. and Massagué,J. (1993) Signaling activity of transforming growth factor beta type II receptors lacking specific domains in the cytoplasmic region. *Mol. Cell. Biol.*, **13**, 7239–7247.

Wieser,R., Wrana,J.L. and Massagué,J. (1995) GS domain mutations that constitutively activate TβR-I, the downstream signaling component in the TGF-β receptor complex. *EMBO J.*, **14**, 2199–2208.

Wrana,J.L., Attisano,L., Cárcamo,J., Zentella,A., Doody,J., Laiho,M., Wang,X.F. and Massagué,J. (1992) TGFβ signals through a heteromeric protein kinase receptor complex. *Cell*, **71**, 1003–1014.

Wrana,J.L., Attisano,L., Wieser,R., Ventura,F. and Massagué,J. (1994) Mechanism of activation of the TGF-beta receptor. *Nature*, **370**, 341–347.

Yang,D., Rosen,M.K. and Schreiber,S.L. (1993) A composite FKBP12–FK506 surface that contacts calcineurin. *J. Am. Chem. Soc.*, **115**, 819–820.

Zhang,Y., Feng,X.H., Wu,R.Y. and Derynck,R. (1996) Receptor-associated Mad homologues synergize as effectors of the TGF-β response. *Nature*, **383**, 168–172.

Zimmerman,L.B., De Jesus-Escobar,J.M. and Harland,R.M. (1996) The Spemann Organizer signal noggin binds and inactivates bone morphorgenetic protein 4. *Cell*, **86**, 599–606.

Received on October 31, 1996; revised on March 18, 1997