Cell, Vol. 86, 435–444, August 9, 1996, Copyright ©1996 by Cell Press

The Immunophilin FKBP12 Functions as a Common Inhibitor of the TGFβ Family Type I Receptors

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Summary

The immunophilin FKBP12 is an evolutionarily conserved abundant protein; however, its physiological roles remain poorly defined. Here we report that FKBP12 is a common cytoplasmic interactor of TGFβ family type I receptors. FKBP12 binds to ligand-free TGFβ type I receptor, from which it is released upon a ligand-induced, type II receptor mediated phosphorylation of the type I receptor. Blocking FKBP12/type I receptor interaction with FK506 nonfunctional derivatives enhances the ligand activity, indicating that FKBP12 binding is inhibitory to the signaling pathways of the TGFβ family ligands. Overexpression of a myristylated FKBP12 in Mv1Lu cell specifically inhibits two separate pathways activated by TGFβ, and two point mutations on FKBP12 (G89P, I90K) abolish the inhibitory activity of FKBP12, suggesting that FKBP12 may dock a cytoplasmic protein to the type I receptors to inhibit TGFβ family mediated signaling.

Introduction

The transforming growth factor β (TGFβ) family consists of many structurally related small peptides, which regulate a wide range of critical cell growth and differentiation events (Moses et al., 1990; Border and Ruoslahti, 1992; Roberts and Sporn, 1993; Attisano et al., 1994; Kingsley, 1994; Massagué et al., 1994). Two types of single transmembrane serine/threonine kinase receptors, the type I and type II, have been found to mediate the cellular effects of the TGFβ family ligands. Molecular characterization of the type I and the type II receptors of the prototypical TGFβ revealed that the two types of receptors play different roles in mediating downstream signaling. The type II receptor is a constitutively active serine/threonine kinase receptor that can bind TGFβ independently, but cannot signal without the type I receptor (Wrana et al., 1992). The type I receptor can only bind to TGFβ in the presence of the type II receptor (Ebner et al., 1993; Wrana et al., 1994a). The recent discovery of a mutant type I receptor with a constitutively active kinase, able to signal in the absence of the type II receptor and TGFβ, suggests that the type I receptor is responsible for initiating the downstream signaling pathway subsequent to its activation by the type II receptor (Wieser et al., 1995).

Little is known about how the type II receptor activates the type I receptor. Upon TGFβ binding, the type II receptor forms heteromeric complexes with and is known to phosphorylate the type I receptor within a serine/threonine rich domain located immediately amino-terminal to the kinase domain of the type I receptor (the GS box), although all phosphorylation sites on the type I receptor have yet to be mapped (Wrana et al., 1994a, 1994b). Type II receptor-mediated type I receptor phosphorylation could activate the type I receptor kinase by either inducing a conformational change of the type I receptor to directly activate the kinase activity, or by creating binding sites for activators or abolishing binding sites for inhibitors. To fully understand the molecular details involved in the activation of the type I receptor, it is therefore essential to identify and characterize the cytoplasmic interactors of the type I receptors before and after ligand binding.

We previously isolated the immunophilin FKBP12 as a specific cytoplasmic interactor of a member of the TGFβ family type I receptors (Wang et al., 1994). FKBP12 is known to mediate the immunosuppressive activities of two macrolides, FK506 and rapamycin, by binding to the macrolides and then recruiting, and thereby inactivating, the serine/threonine phosphatase calcineurin and the serine kinase FRAP (or RAFT1), respectively, resulting in the blockage of the signaling pathways mediated by calcineurin or FRAP (Harding et al., 1989; Siekierka et al., 1989; Liu et al., 1991, 1992; Clipstone and Crabtree, 1992; Brown et al., 1994; Sabatini et al., 1994; Brown et al., 1995; Zheng et al., 1995). Since FKBP12 is ubiquitous in virtually all mammalian cell types and is highly conserved from plants to mammals, its physiological role is likely to be very important.

Here we provide evidence that one physiological role of FKBP12 is to inhibit the TGFβ family type I receptor mediated signaling pathways, possibly by serving as a docking protein for a cytoplasmic inhibitor, e.g. calcineurin, to the cytoplasmic domains of these receptors and thereby regulating their signaling activities. Further, we demonstrate that in the presence of ligand, the TGFβ type II receptor phosphorylates the type I receptor within an undefined site, leading to the release of FKBP12, which may be essential for the activation of the type I receptors. The serine-threonine phosphorylation-dependent release of FKBP12, and another cytoplasmic interactor (FNTA) (Wang et al., 1996), suggests that TGFβ family members signal via a novel mechanism distinct from those known to be employed by the members of the receptor tyrosine kinase family.

‡Both contributed equally to the project, as co-first authors.

Table 1. Summary of Results of Three Yeast Two-Hybrid Screens Using Different Cytoplasmic Domains of Type I Receptors as Bait

Baits	Library	FKBP12 (#/total positives)
R1C	rat neonatal heart	rFKBP12 (57/76)
R4C	human fetal brain	hFKBP12 (54/109)
TKV-C	Drosophila imaginal disc	Dr FKBP12 (38/38)

The entire cytoplasmic domain of R1 (He et al., 1993), R4 (He et al., 1993, Bassing et al., 1994), or Thickvein (Tkv) (Brummel et al., 1994) was fused in frame with the DNA binding domain LexA to serve as the baits in the yeast two-hybrid screens. h, human; r, rat; Dr FKBP12, a novel cDNA isolated from a Drosophila imaginal disc library.

Results and Discussion

FKBP12 Is a Specific Interactor for Multiple Type I Receptors of the TGFβ Family

Using a modified yeast two-hybrid system, we previously isolated the immunophilin FKBP12 as a candidate cytoplasmic interactor of one member (R1) of the type I receptor family (Wang et al., 1994). In order to isolate interactors specific for each of the known type I receptors, we screened two additional yeast expression libraries using cytoplasmic domains of two different type I receptors as baits. FKBP12 was again isolated as the predominant interactor for each type I receptor. A novel cDNA that is 66% identical to mouse FKBP12 was isolated from a Drosophila imaginal disc cDNA library as the only interactor for the decapentaplegic (DPP) type I receptor Thickvein (Tkv) (Brummel et al., 1994), and was thus designated the Drosophila FKBP12 (Dr FKBP12) (T. W. et al., unpublished data). The results of the three screens are summarized in Table 1.

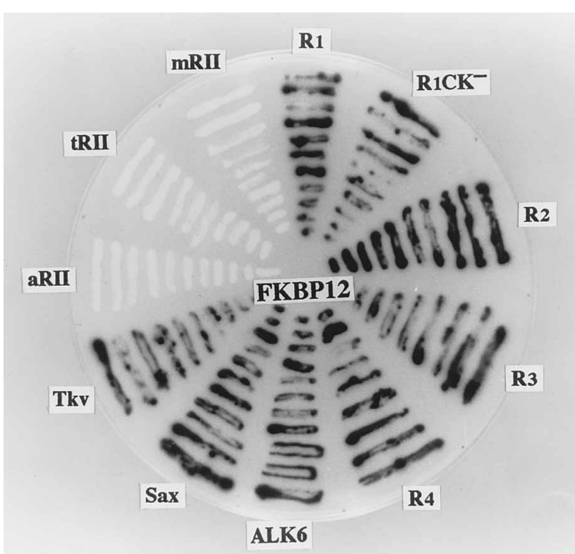

Figure 1. Specific Interaction between TGFβ Family Type I Receptors and FKBP12

A yeast selective plate (Ura^- His^- Trp^- galactose plate with X-Gal) containing yeast colonies expressing two fusion proteins, B42 fused to FKBP12 and LexA fused to cytoplasmic domains of different type I and type II receptors, as indicated. aRII, activin type II receptor; tRII, TGFβ type II; and mRII, a putative MIS type II receptor (Teixeira et al., 1996).

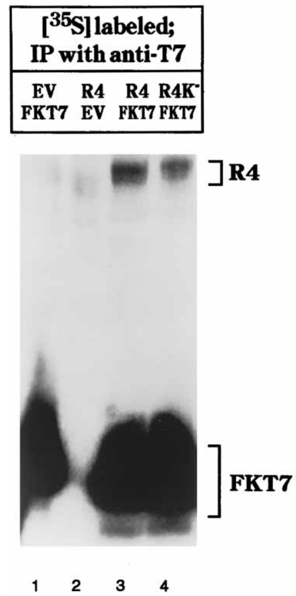

Figure 2. Interaction between FKBP12 and Ligand-Free TGFβ Type I Receptor (R4) in COS Cells

COS1 cells were transiently transfected with the indicated plasmids, metabolically labeled with [^{35}S]-methionine. Cell lysates were then immunoprecipitated with 5 μl of anti-T7 monoclonal antibody, and the immunoprecipitates were separated on 15% SDS–PAGE, which was then visualized by autoradiography. FKT7, bacteria T7 epitope is tagged at the amino terminus of FKBP12 and subcloned in pCMV8. R4, R4 pCMV6; R4K$^-$, R4 (K230R) pCMV6; EV, pCMV6. The immunoprecipitated FKT7 and the coprecipitated R4 are indicated.

To determine whether FKBP12 also interacts with other known type I receptors, five different mammalian type I receptors (R1–R4, and ALK6) (He et al., 1993; ten Dijke et al., 1993, 1994), two Drosophila type I receptors (Sax and Tkv) (Brummel et al., 1994; Xie et al., 1994), and three mammalian type II receptors (tRII, aRII, mRII, type II receptors for TGFβ, activin, and Müllerian inhibiting substance [MIS], respectively) (Mathews and Vale, 1991; Lin et al., 1992; Teixeira et al., 1996) were tested for interaction with FKBP12, in the yeast two-hybrid system (Figure 1A). All tested type I receptors exhibited strong interaction with FKBP12, while the tested type II receptors did not, which indicates that FKBP12 is a specific interactor for all type I receptors.

FKBP12 Binds to the TGFβ Type I Receptor in the Absence of TGFβ

To confirm the specific interaction between FKBP12 and the cytoplasmic domains of the type I receptors observed in yeast, we further tested the interaction between FKBP12 and the known functional TGFβ type I receptor (R4) in COS cells (Figure 2). FKBP12 was tagged at its amino-terminus with a T7 tag, and coexpressed with either the wild-type R4, or the kinase-deficient R4 mutant (R4K$^-$=R4K230R) in COS cells that were metabolically labeled with [^{35}S]methionine. Coimmunoprecipitation assays using the transfected COS cell lysates were carried out with monoclonal anti-T7 antibody. The coprecipitated type I receptor was detected by autoradiography (Figure 2). Both the wild-type and the kinase

	IP with anti-T7							
	R4 RII FKT7	R4 RIIK⁻ FKT7	R4K⁻ RII FKT7	R4K⁻ RIIK⁻ FKT7	R4V•A• RIIK⁻ FKT7	R4V•A• RII FKT7	R4K⁻ RIIK⁻ EV	R4K⁻ RIIK⁻ FKT7
T7 Peptide	-	-	-	-	-	-	-	+

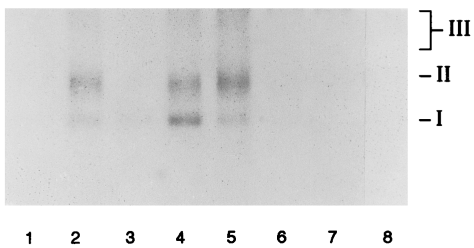

Figure 3. FKBP12 Is Released from the Ligand-Bound TGFβ Type I Receptor

COS1 cells transiently transfected with the indicated constructs or vector alone (EV) were affinity labeled two days after transfection by incubation with [^{125}I]-TGFβ followed by disuccinimidyl suberate. Cells were lysed in 500 μl of lysis buffer. Anti-T7 monoclonal antibody (5 μl) was added into cell lysates with equal counts of labeled receptors for immunoprecipitation, in the absence (lanes 1–7) or presence (lane 8) of 3 μg of T7 peptide. The immunoprecipitates were then separated on 10% SDS–polyacrylamide gel, which was then subjected to autoradiography for 6 days.

deficient mutant were coprecipitated with T7 tagged FKBP12 (Figure 2, lanes 3 and 4), indicating that the kinase activity of the type I receptor is not required for FKBP12 binding, as was also observed in yeast.

FKBP12 Is Released from the TGFβ-Bound Activated Type I Receptor

To test how ligand-binding affects R4 interaction with FKBP12, R4 was cotransfected with TGFβ type II receptor (RII) and the T7 tagged FKBP12 into COS cells, and activated by TGFβ. The ligand-bound type I and type II receptors were affinity labeled with [^{125}I]-iodinated TGFβ by chemical cross-linking (Massagué and Like, 1985). Little, if any ligand-bound wild-type R4 was coprecipitated with T7-tagged FKBP12 (Figure 3, lane 1), suggesting that FKBP12 is released from the ligand-bound type I/type II receptor complex.

The Type II Receptor Phosphorylates the Type I Receptor within an Undefined Site to Release FKBP12

The type I receptor is known to be phosphorylated by the type II receptor in the presence of TGFβ, and may thus be activated as a kinase to autophosphorylate or to phosphorylate unknown substrates (Wrana et al., 1994a). To test whether these phosphorylation events are responsible for the observed release of FKBP12 from the ligand-bound R4, we replaced the wild-type receptors with the kinase deficient mutant receptors. [^{125}I]-TGFβ-labeled receptor complexes were coprecipitated with FKBP12 only when the type II receptor kinase was deficient (Figure 3, lanes 2 and 4), but not when the type I receptor kinase was deficient (Figure 3, lane 3), suggesting that the type II receptor-mediated phosphorylation of the type I receptor, not type I receptor autophosphorylation, is necessary for the release of FKBP12.

To test whether phosphorylation of the two threonines and three serines within the core sequence of the GS domain TTSGSGSG of the TGFβ type I receptor is responsible for releasing FKBP12, the threonines and serines were mutated to valines and alanines, respectively (R4VVAAA). Like the wild-type R4, the R4VVAAA mutant failed to coprecipitate with FKBP12 in the presence of the wild-type type II receptor (Figure 3, lane 6), but coprecipitated with FKBP12 in the presence of the kinase deficient type II receptor (Figure 3, lane 5), indicating that phosphorylation of these sites is not responsible for releasing FKBP12.

Nonfunctional Derivatives of FK506 Can Compete with the Type I Receptor for FKBP12 Binding in COS Cells

Since the macrolide binding site of FKBP12 appears to be involved in binding to the type I receptors (Wang et al., 1994), we directly tested whether the interaction between R4 and FKBP12 in COS cells can be blocked by an excess amount of macrolides. Both FK506 and rapamycin each alone exhibited growth inhibition activities when added into the TGFβ responsive mink lung epithelial cell line (Mv1Lu); therefore, we turned to two nonfunctional derivatives of FK506, L685,818 and 15-*O*-desmethyl-FK520. Both bind to FKBP12 at the same site and with a similar affinity as FK506, but have minimal cellular effects even at very high doses, because of their defect in calcineurin binding (Dumont et al., 1992; Liu et al., 1992; Becker et al., 1993). Both derivatives, as illustrated by 15-*O*-desmethyl FK520 (10 nM), are able to block R4/FKBP12 coimmunoprecipitation (Figure 4A, lane 4), further confirming that the macrolide binding site of FKBP12 is involved in binding to the TGFβ type I receptor.

Release of FKBP12 from the Type I Receptors by FK506 Derivatives Enhances TGFβ and MIS-Induced Functional Responses

The ability of the nonfunctional FK506 derivatives to block FKBP12 binding to the inactive TGFβ type I receptor allows us to address the functional role of FKBP12 in type I receptor-mediated signaling events, in which FKBP12 could play either a positive, or a negative role. For example, if FKBP12 is responsible for transducing the activation signal from the receptors to downstream players through binding to the type I receptors, then blocking the binding with excess FK506 derivatives should also block the signaling pathways; alternatively, if FKBP12 is an inhibitor of the type I receptors, excess FK506 derivatives should help to activate the downstream events. In the mink lung epithelial cell line Mv1Lu, TGFβ causes transcriptional activation of the plasminogen activator inhibitor (PAI) promoter driving the luciferase reporter gene (3TP-Luc) (Wrana et al., 1992), as well as cell cycle G1 arrest, monitored by a decrease in ^{3}H-thymidine incorporation. When excess 15-*O*-desmethyl-FK520 or L685,818 (500 nM) were added to Mv1Lu cells, neither TGFβ-induced response was blocked (data not

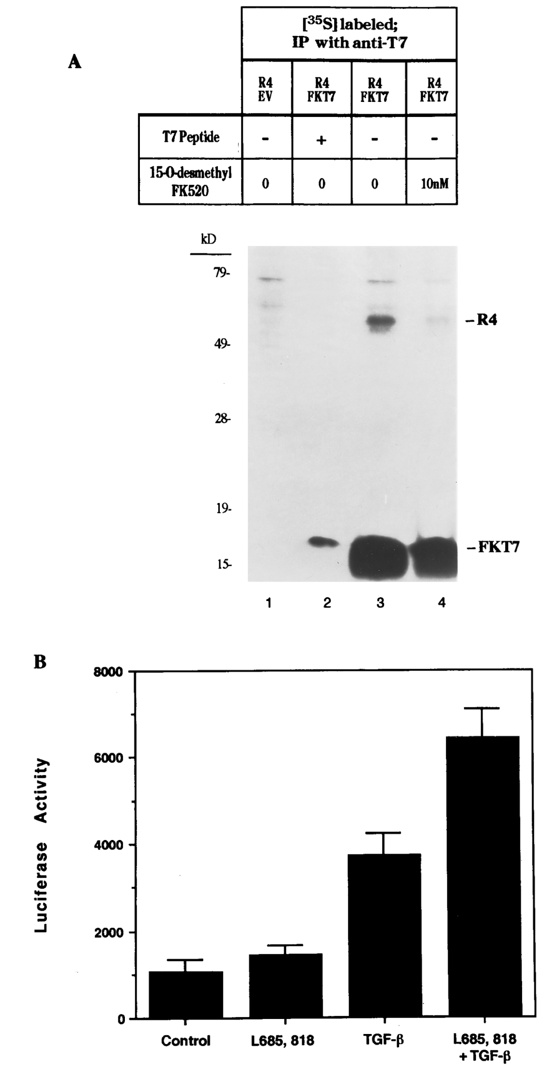

Figure 4. Nonfunctional Derivatives of FK506 Can Compete with the TGFβ Type I Receptor in Binding to FKBP12 and Thus Enhance TGFβ Effects in Mv1Lu Cells

(A) Competition of 15-O-desmethyl-FK520 with R4 in binding to FKBP12 in COS cells. COS1 cells transfected with the indicated cDNAs in either pCMV8 (FKT7) or pCMV6 (R4) or pCMV8 vector alone (EV) were treated with or without 15-*O*-desmethyl-FK520, as indicated, and metabolically labeled with [^{35}S]-methionine. Cells were then lysed, and cell lysates were subjected to immunoprecipitation using anti-T7 monoclonal antibody, either with (lane 2), or without (lanes 1, 3, and 4) preincubation with excess T7 peptide. The immunoprecipitates were separated on 15% SDS–polyacrylamide gel, which was then subjected to autoradiography.

(B) Enhancement of the TGFβ induced up-regulation of the 3TP-Luc reporter in Mv1Lu cells by L685,818. Mv1Lu cells transfected with p3TP-Lux reporter construct (control) were incubated with 200 nM L685,818 alone (L685,818), or 1 pM TGFβ alone (TGFβ), or 200 nM L685,818 together with 1 pM TGFβ (L685,818+TGFβ). Luciferase activities were measured in the cell lysates as described in Experimental Procedures, with each point determined in triplicates.

shown). Thus FKBP12 binding to the TGFβ type I receptor is not required for the activation of downstream signaling events, suggesting that FKBP12 is not likely an active signaling molecule.

To determine whether FKBP12 is instead an inhibitor of the type I receptor signaling, we first tested whether abolishing FKBP12 binding to the type I receptor with a high dose of FK506 derivatives can activate the downstream events in the absence of TGFβ in Mv1Lu cells. No significant gene activation responses were detected when the derivatives were added alone (Figure 4B, "L685, 818"), indicating that releasing FKBP12 from the type I receptor alone is not sufficient to activate downstream pathways. This is not surprising, since the derivatives only release FKBP12, but can not induce other events known to be essential for the activation of the type I receptor, such as the type II receptor mediated GS domain phosphorylation (Wrana et al., 1994a), and the release of the farnesyltransferase α subunit (FNTA) (Wang et al., 1996). Therefore, the FKBP12-releasing effect of the derivatives can only be detected in the presence of TGFβ. Since the release of FKBP12 is normally induced by the ligand/type I receptor binding (Figure 3), the specific effect of releasing FKBP12 by the FK506 derivatives can be detected only if there are threshold doses of TGFβ, which are sufficient for inducing other necessary activation events but not for releasing FKBP12 completely. By adding the derivatives together with such doses of TGFβ, the derivatives would release the retained FKBP12, which may then lead to an increase of the TGFβ specific response, if FKBP12 binding is inhibitory to the signaling activity of the type I receptor.

To find the threshold doses of TGFβ, excess FK506 derivatives were added with different doses of TGFβ to Mv1Lu cells. No effect was detected when 1 μM derivatives were added together with a maximal (250 pM) dose of TGFβ. This is as expected, since a high dose TGFβ may completely release FKBP12. When 1 pM TGFβ was added to Mv1Lu cells, it induced a 3- to 4-fold increase of the luciferase activity of the 3TP-Luc reporter (Figure 4B, "TGFβ"), which is normally induced more than 100-fold by maximal dose (250 pM) of TGFβ, thus indicating a very weak activation of the signaling pathways at this dose. When excess FK506 derivatives (1 μM of either 15-*O*-desmethyl-FK520, or L685,818) were added together with 1 pM TGFβ, a 6- to 8-fold increase of luciferase activity was detected (Figure 4B, "L685,818 + TGFβ"). Therefore, although the derivatives alone can not activate the 3TP-Luc (Figure 4B, "L685,818"), they can significantly potentiate low dose TGFβ-induced 3TP-Luc activation, by specifically releasing FKBP12 from the type I receptor, suggesting that FKBP12 binding is inhibitory to the type I receptor-mediated signaling.

Since FKBP12 binds to all known type I receptors, its inhibitory activity should also be detected in the signaling events mediated by other TGFβ family members. Müllerian inhibiting substance (MIS) is a member of the TGFβ family responsible for regression of the Müllerian duct in fetal males through an active apoptotic process (Jost, 1947; Price et al., 1977; Trelstad et al., 1982). The activity of MIS in the regression of the Müllerian ducts can be measured directly in an organ culture assay (Donahoe et al., 1977). In this assay, urogenital ridges containing both Wolffian and Müllerian duct isolated from

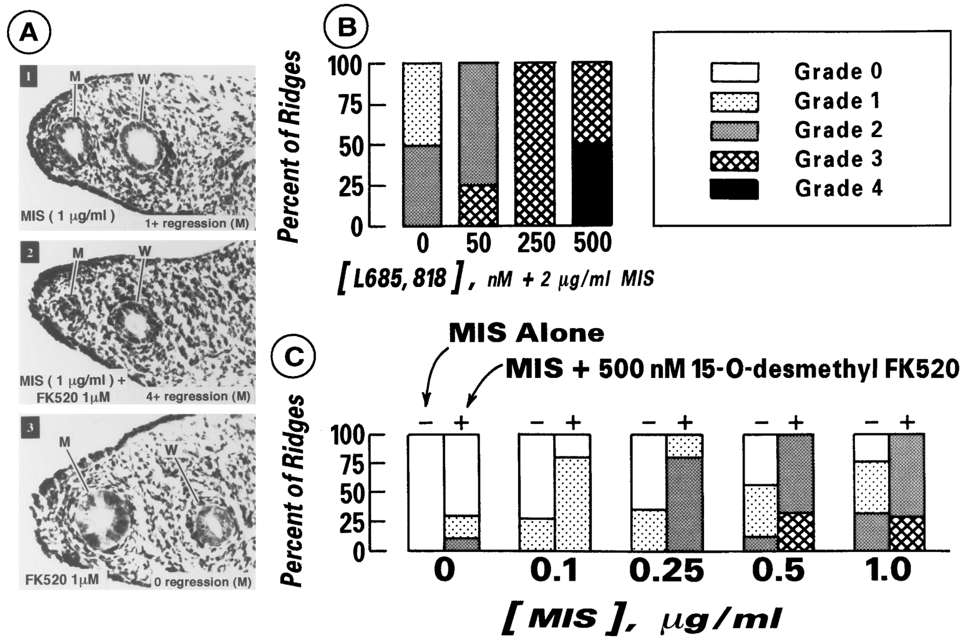

Figure 5. MIS-Mediated Müllerian Duct Regression Enhanced by FK506 Derivatives

(A) Hematoxylin- and eosin-stained paraffin sections of urogenital ridges in the organ culture. The dissected 14.5-day female rat urogenital ridges containing the Wolffian duct (W) and the Müllerian duct (M) were incubated in the presence of either 1 μg/ml MIS (top), or 1 μM 15-*O*-desmethyl-FK520 (bottom), or with 1 μg/ml MIS and 1 μM 15-*O*-desmethyl-FK520 (middle; n = 3 for each panel). The depicted Müllerian duct regression in each panel is representative of the three tested ridges, which are similar.

(B) Regression activities of 2 μg/ml MIS in the presence of different doses of L685,818. More than 5 different individual ridges were assayed at each dose and regression scored as described previously (Donahoe et al., 1977). For each dose, the percentage of ridges ranked within specific regression grades are included.

(C) Regression activities of different doses of MIS, as indicated, in the presence or absence of 500 nM 15-*O*-desmethyl-FK520. More than five ridges were assayed within each group.

14.5-day rat female fetuses, which have had no previous endogenous MIS exposure, are incubated with different doses of exogenous MIS. Regression of the Müllerian duct is then monitored histologically and the degree of the regression graded from 0 (no regression) to 5 (complete regression) as described (Donahoe et al., 1977). When 1 μg/ml MIS was added to the organ culture, grade 1 regression, characterized by condensation of the mesenchymal cells surrounding a smaller and irregularly shaped Müllerian duct, was observed (Figure 5A, top). When 1 μM 15-*O*-desmethyl-FK520 alone was added to the organ culture, no regression was detected (Figure 5A, bottom). However, when 1 μM 15-*O*-desmethyl-FK520 was added together with 1 μg/ml of MIS, the duct was replaced by a cord of pycnotic epithelial cells and connective tissue (grade 4) (Figure 5A, middle). We further tested the effect of four different doses of L685,818 on the regression activity of 2 μg/ml MIS. At least five organ cultures were carried out for each tested dose of L685,818 alone (data not shown), or L685,818 with 2 μg/ml MIS, the results of which were represented by the percentage of the Müllerian ducts ranked within each regression grade (Figure 5B). When treated with 2 μg/ml MIS alone, 50% of ridges were grade 1, and 50% were grade 2; when 50 nM L685, 818 was added together with 2 μg/ml MIS, 75% were grade 2 and 25% were grade 3; when 250 nM drug was added together with 2 μg/ml MIS, 100% were grade 3; when 500 nM drug was added together with 2 μg/ml MIS, 50% of the ridges were grade 3 and 50% were grade 4, while ridges treated with drugs alone were grade 0–1 (data not shown). Therefore, increasing concentrations of L685,818 progressively enhanced the Müllerian duct regression. Different doses of MIS were also tested with 500 nM 15-*O*-desmethyl-FK520 (Figure 5C). In the presence of the drug, 0.25 μg/ml MIS can cause grade 2 regression in more than 70% of the ridges, while 1 μg/ml MIS alone can cause grade 2 regression in only 25% of the ridges. Grade 4 regression was achieved when 2 μg/ml MIS was added together with the drug, while the same degree of regression activity normally requires more than 5 μg/ml MIS.

These data indicate that FKBP12 also inhibits the MIS-induced signaling pathway, and that Müllerian duct regression induced by MIS can be significantly enhanced by addition of the nonfunctional FK506 derivatives. Since FKBP12 interacts with all members of the TGFβ family type I receptors through two highly conserved type I receptor-specific domains (T. Wang et al., unpublished data), we can anticipate that the nonfunctional FK506 derivatives can produce similar effects on other members of the TGFβ family. These data also suggest that the nonfunctional derivatives of FK506 may have exciting therapeutic applications: one might use low dose TGFβ family ligands for targeted specificity, with addition of FK506 nonfunctional derivatives to potentiate the biological activities, thus reducing potential clinical side effects caused by high dose TGFβ family ligands.

Myristylated Wild-Type FKBP12 But Not a Calcineurin-Binding-Deficient FKBP12 Mutant Specifically Blocks TGFβ Responses

If, as our data suggests, FKBP12 functions as an inhibitor of the type I receptor, its release from the ligand-bound type I receptor should be essential for the activation of the ligand-induced downstream events, and blocking its release should also inhibit the type I receptor-mediated signaling. There are two possible ways to block the release of FKBP12: first, by abolishing the phosphorylation site on the type I receptor responsible for releasing FKBP12; second, by fusing FKBP12 with the cytoplasmic domain of the type I receptor. Since the precise phosphorylation site responsible for FKBP12 release has yet to be identified, and direct fusion of FKBP12 to the type I receptor interfered with the functional conformation of the type I receptor (data not shown), we tested whether the inhibitory activity of FKBP12 could be detected by localizing FKBP12 to the cell membrane to increase its local concentration around the membrane-bound type I receptor. A 13 amino acid *src* myristylation site was placed at the amino terminus of FKBP12 in a mammalian expression vector pBJ5 (Clipstone and Crabtree, 1992). Overexpression of this construct (mFE) in Mv1Lu cells greatly reduced the TGFβ induced 3TP-Luc luciferase activities (from 150- to 25-fold) (Figures 6A and 6B, "control" and "mFE").

To rule out nonspecific toxicity resulting from overexpressing FKBP12 at the membrane, we used mFE in another TGFβ response assay, the TGFβ-dependent suppression of a cyclin A promoter driven luciferase reporter (Figures 6C and 6D). Unlike the 3TP-Luc reporter, which is transcriptionally up-regulated by TGFβ and monitors the pathway that leads to increased production of the extracellular matrix, the cyclin A-Luc reporter is transcriptionally downregulated by TGFβ and monitors the pathway that leads to cell cycle G1 arrest (Feng et al., 1995). A specific inhibition of the TGFβ response by overexpression of myristylated FKBP12 should block TGFβ-induced down-regulation of the luciferase activity from the cyclin A-Luc reporter, which should be reflected in an increase in the luciferase activity in the mFE-transfected, TGFβ-treated Mv1Lu cells when compared with the untransfected, TGFβ-treated Mv1Lu cells. A nonspecific toxicity effect of myristylated FKBP12, however, should cause a further decrease of the luciferase activity in the TGFβ-treated, mFE-transfected cells. Overexpression of mFE almost completely blocked the TGFβ-induced repression of the cyclin A-Luc reporter, indicating that the myristylated FKBP12 can specifically inhibit two separate pathways activated by TGFβ in Mv1Lu cells (Figures 6C and 6D, "control" and "mFE"). Since FKBP12 is normally released from the type I receptor upon ligand binding (Figure 3), the myristylated FKBP12, although not free

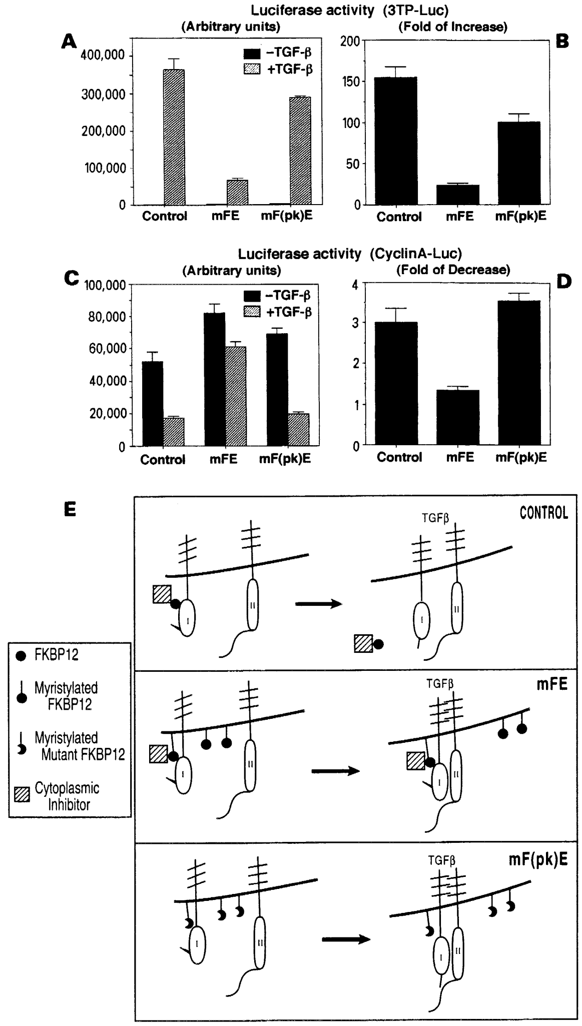

Figure 6. Myristylated Wild-Type FKBP12 But Not a Calcineurin-Binding-Deficient FKBP12 Mutant Specifically Blocks TGFβ Responses

(A) Mv1Lu cells were transfected with 3TP-Luc alone (control), or with 3TP-Luc and the myristylated FKBP12 in PBJ5 (mFE), or with 3TP-Luc and the myristylated mutant FKBP12 in PBJ5 (mFpkE). Equal amount of myristylated wild-type FKBP12 and the mutant FKBP12 are expressed, as determined by Western blot using anti-FKBP12 antiserum (data not shown). The luciferase activities were measured as described in Experimental Procedures. The arbitrary units, with error bars, are presented. The experiments were performed twice, with each point determined in triplicates.

(B) The arbitrary units in (A) from the TGFβ-treated cells were divided by those from the untreated cells to show fold of increase of the luciferase activity upon TGFβ treatment.

(C) Same as (A), except the cells were transfected with cyclin A-Luc reporter rather than 3TP-Luc.

(D) The arbitrary units in (C) from untreated cells were divided by those from the TGFβ-treated cells to show fold of decrease of the luciferase activity upon TGFβ treatment.

(E) A cartoon to depict a potential mechanistic difference between the endogenous free cytoplasmic FKBP12 (control), the membrane-bound myristoylated FKBP12 (mFE) and the membrane-bound myristylated calcineurin-binding-deficient FKBP12 (mFpkE), during ligand-induced activation of the TGFβ type I receptor. See text for details.

to travel into the cytoplasm, should also be dissociated from the ligand-bound type I receptor. Therefore, the inhibitory activity of the myristylated FKBP12 is not likely mediated by its direct interaction with the type I receptor.

An alternative mechanism for the inhibitory activity of the myristylated FKBP12 is suggested by overexpression of a mutant myristylated FKBP12 (G89P, I90K) in Mv1Lu cells. These mutations are known to be located within the 80s loop of FKBP12 and to abolish calcineurin binding without affecting either FK506 binding or FKBP12 prolyl-isomerase activity (Yang et al., 1993). The mutant (mF(pk)E) failed to inhibit either of the TGFβ-dependent pathways (Figure 6, ''mF(pk)E''). This data further indicates that FKBP12 may inhibit the type I receptor by docking a cytoplasmic inhibitor, such as calcineurin, as depicted by the cartoon (Figure 6E). Before ligand binding, FKBP12 docks the inhibitor to the type I receptor, thus preventing receptor signaling. Upon ligand binding, the type II receptor phosphorylates the type I receptor to release FKBP12 and with it, the associated inhibitor (see Figure 6E, "Control"). The overexpressed myristylated FKBP12 competes with the endogenous FKBP12 to bind to the ligand-free type I receptor, and cannot be released into the cytoplasm upon ligand-induced phosphorylation of the type I receptor, due to its anchorage to the membane, thus allowing the cytoplasmic protein to inhibit the type I receptor in the presence of ligand (see Figure 6E, "mFE"). The overexpressed mutant FKBP12, although localized to the membrane, fails to dock the inhibitor to the type I receptor, either before, or after ligand binding to the type I receptor. Therefore, normal signaling activity of the type I receptor is detected (see Figure 6E, "mF(pk)E").

Concluding Remarks

Much is known about the growth stimulatory pathways mediated by tyrosine kinase receptors, while little is known about the growth inhibitory pathways activated by the serine/threonine kinase receptors of the TGFβ family. The evolutionarily conserved, abundant cytoplasmic protein FKBP12 is known to bind exogenous macrolide molecules to mediate immunosuppression; however, its normal physiological roles remain obscure.

By using the yeast two-hybrid system, we identified FKBP12 as a common interactor of several cytoplasmic domains of the TGFβ family type I serine/threonine kinase receptors. Recent domain mapping studies revealed that two highly conserved type I receptor specific motifs are essential for the FKBP12/type I receptor binding (T. W. et al., unpublished data), which explains the molecular basis for the observed binding specificity for all known type I receptors. Using the TGFβ type I receptor as a model, we demonstrated coimmunoprecipitation of FKBP12 with the ligand-free type I receptor when both proteins were coexpressed in COS cells. In the same system, we further demonstrated that FKBP12 is released from the ligand-bound type I receptor, and that the release is dependent upon type II receptor mediated phosphorylation of the type I receptor within an undefined site. Interestingly, the receptor/FKBP12 interaction can be abolished by FK506 derivatives that have the same FKBP12 binding properties as FK506 but lack the calcineurin binding domain, and thus lack functional immunosuppressive activity (Dumont et al., 1992; Liu et al., 1992; Becker et al., 1993). Based upon this observation, we used the nonfunctional FK506 derivatives to specifically disrupt the FKBP12/type I receptor interaction in both a TGFβ-responsive cell line, Mv1Lu, and an MIS responsive organ, the fetal Müllerian duct. By finding doses of either TGFβ or MIS that were sufficiently low to retain FKBP12, yet adequate to induce other activation events, a specific enhancement of TGFβ responses was uncovered in Mv1Lu cells, and an augmentation of the degree of Müllerian duct regression was observed in a large number of organ culture assays. These data revealed, for the first time, that FKBP12 binding to the type I receptor is physiological and is inhibitory to the signaling activity of the receptors. Since detecting the FKBP12-releasing effect by the FK506 derivatives is dependent upon the dissociation of the ligand-induced FKBP12 release from other activation events by using threshold dose ligand, the relatively weaker enhancement effect of the derivatives on the TGFβ-induced gene response, in comparison to the effect on the MIS-induced Müllerian duct regression, may reflect a less successful dissociation of the ligand-induced FKBP12 release from the rest of the activation events.

The inhibitory activity of FKBP12 was further tested by overexpression of a myristylated FKBP12 in Mv1Lu cells. Strong and specific inhibition of two separate pathways mediated by TGFβ were detected. The inhibitory activity of the myristylated FKBP12 was abolished by two point mutations known to disrupt FKBP12 binding to calcineurin, but not FKBP12 prolyl-isomerase activity (Yang et al., 1993). Since ligand-induced type I receptor phosphorylation releases FKBP12, we therefore can conclude that the inhibitory activity of FKBP12 is not mediated by its prolyl-isomerase activity, nor by its direct binding to the type I receptor, but more likely by its ability to dock a cytoplasmic inhibitor of the type I receptor.

One candidate for such an inhibitor is calcineurin. FKBP12 is known to bind to calcineurin with high affinity in the presence of the macrolide FK506. In the absence of FK506, FKBP12 has a weak but specific and functional interaction with calcineurin in yeast (Cardenas et al., 1994). Recently, FKBP12 was shown to recruit calcineurin to the IP_3 receptor where calcineurin dephosphorylates specific sites on the IP_3 receptor to regulate Ca^{2+} influx (Cameron et al., 1995). Interestingly, FK506 can compete with both the IP_3 receptor and the TGFβ type I receptor to bind FKBP12, suggesting that similar motifs of the receptors are involved in binding to the same FK506 binding site of FKBP12. The known binding sites on FKBP12 for calcineurin and for FK506 are immediately adjacent to each other (Yang et al., 1993; Kissinger et al., 1995). Therefore, binding of the TGFβ family type I receptors and the huge IP_3 receptor to the FK506 binding site of FKBP12 suggests that calcineurin, if also directly bound to FKBP12 in the receptor/FKBP12/calcineurin complex, must be binding to sites removed from the FK506 binding site of FKBP12. Since calcineurin is a serine/threonine phosphatase, while type

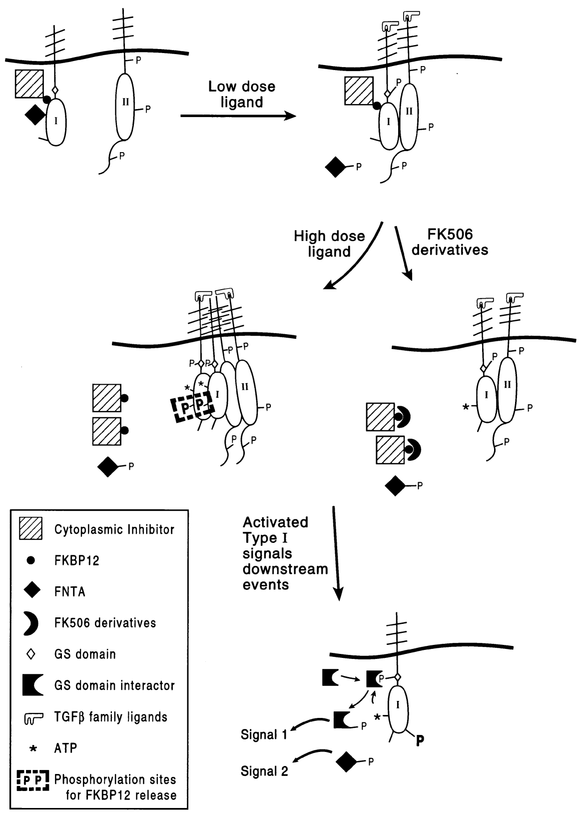

Figure 7. A Model for the Events Involved in the Activation of the TGFβ Type I Receptor.
See text for details.

I receptors are serine/threonine kinases, and phosphorylation of the type I receptor as well as its downstream substrates is essential for signaling via the type I receptors, one plausible mechanism for calcineurin to inhibit the type I receptor signaling activity is to dephosphorylate the type I receptor, or its bound substrates. It will be necessary to detect the ternary complex of calcineurin/FKBP12/type I receptor and these dephosphorylation events in vivo to provide the final proof for such a mechanism.

The model in Figure 7 summarizes our observations on the inhibitory role of FKBP12 and our hypothesis regarding its potential link to other known events involved in the activation of the TGFβ type I receptor. Before TGFβ binding, FKBP12 binds to the type I receptor, and recruits a cytoplasmic inhibitor, such as calcineurin, to keep the type I receptor inactive, possibly by keeping the type I receptor and its interactor(s), such as the α subunit of the farnesyltransferase (Wang et al., 1996), hypophosphorylated. The complete activation of the type I receptor can be accomplished in one of two ways. Naturally, high dose TGFβ induces the formation of a tetrameric active signaling complex between the type I and the type II receptors (Weis-Garcia and Massagué, 1996), in which possible sequential activation events occur, involving: (1) GS domain phosphorylation and release of the α subunit of the farnesyltransferase, and probable additional events, and (2) phosphorylation of an additional site to release FKBP12 and its docked inhibitor. Alternatively, complete activation of the type I receptor can be accomplished by using low dose TGFβ to complete (1), and then excess nonfunctional FK506 derivatives to release FKBP12 and its associated inhibitor. Additional mechanisms involved in the direct inactivation of the inhibitor docked by FKBP12, such as phosphorylation of the inhibitor, may also be required during the normal activation of the type I receptor by high dose ligand. Once activated, the type I receptor may then phosphorylate a cytoplasmic substrate, which could be bound to the phosphorylated GS domain, or to other phosphorylated domains. The phosphorylated cytoplasmic substrate(s), together with the released and phosphorylated α subunit of the farnesyltransferase (Wang et al., 1996), are then responsible for transducing the activation signals downstream. Future studies to map the phosphorylation site(s) responsible for the release of FKBP12, to identify the substrates of the type I receptor kinase, and to dissect the signaling role of the farnesyltransferase α subunit will provide important information for understanding the activation events at or downstream of the TGFβ family type I receptors.

Experimental Procedures

Strains and Cell Lines

The yeast strain EGY48 *MATa trp1 ura3 his3 LEU2::pLexAop6-LEU2*, the E. coli K-12 strain KC8 *pyrF::Tn5, hsdR, leuB600, trpC9830, lacD74, strA, galK, hisB436* are gifts from Dr. Roger Brent (Massachusetts General Hospital, Boston). The mink lung epithelial cell line Mv1Lu is a gift from the Massagué laboratory.

Plasmids and Plasmid Construction

The multiple LexA and B42 fusion constructs were made by PCR amplification of the designed cDNA sequences with two restriction sites attached at 5′ (EcoRI) and 3′ (XhoI) ends for subcloning into the respective sites within the multiple cloning sites of either pEG202 and pJG4-5 (Gyuris et al., 1993, Zervos et al., 1993). Mammalian expression constructs of TGFβ type I (He et al., 1993) and type II receptors and their kinase deficient mutants were described previously (Bassing et al., 1994). R4VVAAA was a gift from Dr. Jeffery Wrana. The T7 tagged FKBP12 was made by first subcloning FKBP12 cDNA into EcoRI/XhoI sites of plasmid PET 28a (Novogen) and then subcloned into the pCMV8 vector (from Dr. David Russel). Plasmids mFE and mFpkE in pBJ5 are generous gifts from Dr. Stuart Schreiber at Harvard. The 3TP-Luc reporter is from Dr. Jeffery Wrana. The cyclin A-Luc reporter is from Dr. Rik Derynck.

Yeast Two-Hybrid Screen

We used a modified version of the yeast two-hybrid system developed by Roger Brent and his colleagues, as previously described in detail (Gyuris et al., 1993; Zervos et al., 1993).

COS Cell Transfection, Metabolic Labeling, Receptor Cross-Linking, Immunoprecipitation, and Western Blot

For testing FKBP12 binding to ligand-free TGFβ type I receptor by coimmunoprecipitation, COS1 cells grown in P-100 dishes were transfected with 4 μg of T7 tagged FKBP12 in pCMV8 and 6 μg of R4 (or mutant R4) in pCMV6, using the DEAE-dextran method. In the case of using metabolic labeling to detect coprecipitation of FKBP12 and R4, 40–44 hr after transfection COS1 cells were incubated for 4 hr with 50 μCi/ml [^{35}S]-methionine in methionine-free media and lysed in lysis buffer (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.5% Triton X-100, 1 mM EDTA) in the presence of a mixture of protease inhibitors. Anti-T7 monoclonal antibody (5 μl) was added into 200 μl of cell lysates, incubated at 4°C for 2 hr, absorbed with protein A sepharose beads, and washed three times with wash buffer (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.1% Triton X-100, 1 mM EDTA). Proteins were eluted from the sepharose beads using 2× SDS–polyacrylamide gel electrophoresis (SDS–PAGE) denaturing sample buffer, separated on 15% SDS–polyacrylamide gel.

For testing the effect of nonfunctional FK506 derivatives (L685,818 from Merck Laboratories and 15-*O*-desmethyl-FK520 from Sandoz) on FKBP12 binding to R4, 2 hr after transfection COS1 cells were treated with the derivatives for 40–44 hr and then labeled for 4 hr with 50 μCi/ml [^{35}S]-methionine in methionine-free. Cells were then lysed and immunoprecipitation was carried out as described above.

For testing FKBP12 binding to the ligand-bound type I receptor, COS cells were transfected with 4 μg of T7-FKBP12 pCMV8, 6 μg of R4 (or R4K$^-$)pCMV6 and 5 μg of RII (or RIIK$^-$)pcDNA1. Forty-eight hours after transfection, cells were incubated for 3 hr at 4°C with [^{125}I]-labeled TGFβ at 250 pM. Cross-linking of receptor with the ligand was carried out as described previously (Massagué and Like, 1985). Cells were lysed in lysis buffer as above followed by immunoprecipitation, SDS–PAGE, and autoradiography.

Urogenital Ridge Organ Culture Assay

The organ culture assay was carried out as described previously (Donahoe et al., 1977). To test the effect of the nonfunctional derivatives of FK506 (L685,818 or 15-*O*-desmethyl FK520) on Müllerian duct regression, the specimens were treated with the indicated doses of MIS in the presence or absence of the nonfunctional derivatives of FK506. For each dose of MIS or MIS plus the FK506 derivatives, more than five individual urogenital ridges were assayed and graded, and the ordinal results represented by the percentage of the ridges that ranked within a specific regression grade.

Transcriptional Response Assay

The TGFβ responsive mink lung epithelial cell lines MvlLu were used in transcriptional response assays employing a DEAE-dextran transfection method (Bassing et al., 1994; Wrana, 1994a). In brief, cells grown to 50% confluency in 6-well plates were transiently transfected with 1 μg of either the transcriptional response reporter construct p3TP-lux, or cyclin A-Luc, alone, or together with 1 μg of mFE or mFpkE in PBJ5. Sixteen to twenty hours after transfection, cells were treated with TGFβ in 0.2% serum medium for 24–28 hr. For testing the effect of FK506 derivatives, cells were treated with the drug beginning 2 hr prior to the addition of TGFβ. Cell lysates were made and luciferase activity was measured in a luminometer. Each assay was carried out in triplicates.

Acknowledgments

We thank R. Brent, A. Zervos, A. Roberts, S. Schreiber, B. Stockwell, J. Massagué, H. Lodish, W. W. He, and X.-F. Wang, who generously provided valuable probes and Y.-M. Wang for technical assistance. We thank B. Stockwell for valuable suggestions and L. Perkins for critical reading of the manuscript. We also thank Merck laboratories and Sandoz for providing us with the nonfunctional FK506 derivatives. This work is supported by a National Institute of Child Health and Human Development (NICHD) Reproductive Sciences Training Grant (P-30 HD07396) and by NIH R29 grant GM53710 (T. W.), by fellowships from the Gynecologic Oncology in the Department of Obstetrics and Gynecology at Massachusetts General Hospital (B.-Y. L.), the Department of Surgery at MGH (P. C. S.), the Department of Surgery, University of Rochester School of Medicine (P. D. D.), and the Surdna Foundation (P. C. S. and P. D. D.), and by NIH grants HD 32112 and CA 17393 (P. K. D.), and a P30 HD 28138 Reproductive Sciences Center grant (P. K. D.).

Received December 11, 1995; revised June 21, 1996.

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