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Communication

Interaction of Transforming Growth Factor-β Receptor I with Farnesyl-protein Transferase-α in Yeast and Mammalian Cells*

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Transforming growth factor β (TGF-β) signals through two transmembrane serine/threonine kinases, known as TβR-I and TβR-II. Several lines of evidence suggest that TβR-II acts as a primary receptor, binding TGF-β and phosphorylating TβR-I whose kinase activity then propagates the signal to unknown substrates. We report an interaction between TβR-I and the farnesyl-protein transferase-α subunit (FT-α) both in a yeast two-hybrid system and in mammalian cells. These findings raise the possibility that TGF-β might regulate cellular functions by altering the ability of FT-α to catalyze isoprenylation of targets such as G proteins, lamins, or cytoskeletal components. However, we provide evidence that TGF-β action does not alter the overall protein isoprenyl transferase activity in Mv1Lu mink lung epithelial cells. In fact, the β subunits of farnesyl transferase and geranylgeranyl transferase, which are necessary for the activity of FT-α, prevent the association of FT-α with TβR-I. Furthermore, farnesyl transferase activity is shown to be dispensable for TGF-β signaling of growth inhibitory and transcriptional responses in these cells. These results suggest that the interaction between TβR-I and FT-α does not affect the known functions of these two proteins.

TGF-β[1] is a multifunctional cytokine that controls proliferation, differentiation, and many other functions in the cell (1–4). The anti-mitogenic effects of TGF-β in particular have attracted much attention. By inhibiting cyclin-dependent kinases, TGF-β can override the action of mitogens without directly blocking their signal transduction pathways (5–8). TGF-β initiates signaling at the membrane by contacting two types of transmembrane serine/threonine kinase receptors, the type I and II receptors (TβR-I and TβR-II) (9–12). These receptors belong to a family that also includes receptors for other TGF-β-related factors such as activins and bone morphogenic proteins (BMPs) (4). Type II receptors bind ligand present in the medium, and this complex associates with and phosphorylates type I receptors (12). Phosphorylation is at serine and threonine residues clustered in the GS domain, a region just upstream of the kinase domain and conserved in all type I receptors, and mutation of these sites blocks TGF-β signaling (13). Certain mutations in the GS domain generate a constitutively active TβR-I that does not require the presence of ligand or TβR-II for signaling (14). These observations suggest that TβR-I, acting downstream of TβR-II, is directly involved in transducing TGF-β signals to downstream substrates.

In order to search for proteins that interact with the cytoplasmic domain of TβR-I, we used protein interaction cDNA cloning in yeast. This led to the identification of farnesyl transferase-α (FT-α) as a TβR-I interacting protein. FT-α is a shared subunit of heteromeric transferases that attach farnesyl or geranylgeranyl moieties to a variety of proteins that play key roles in signal transduction, protein secretion, and cytoskeleton assembly (15, 16). We provide evidence that the interaction between TβR-I and FT-α can also take place in mammalian cells. Similar observations were reported by two other groups (17, 18) while this paper was in preparation. Additionally, we have analyzed the physiological relevance of this interaction.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screening and Interaction Assay—A HeLa cDNA library was screened by the two-hybrid system essentially as described (19, 20) using as bait the wild type cytoplasmic domain of TβR-I (amino acids 148–503) (10) or this domain containing the T204D mutation (14). For interaction assays, the cytoplasmic domains of TβR-II (190–576) (9), TSR-I (amino acids 144–503) (11), ActR-I (amino acids 147–509) (11), ActR-IB (amino acids 150–505) (22), TβR-I wild type or mutated versions were fused in frame to the LexA DNA binding domain in the vector pEG202 (19). Yeast transformation and β-galactosidase assays were done as described (20, 21).

Mammalian Expression Vectors and Transfections—The human FT-α cDNA (15) was modified by polymerase chain reaction to encode this protein with the Flag epitope sequence at the C terminus and subcloned into the mammalian expression vector pCMV5. Human FT-β and GGT-β cDNAs in the pcDNA3 vector were generous gifts of Drs. Joseph Goldstein and Michael Brown. The generation of mutant as well as chimeric receptors and reporter constructs had been previously described (14, 22). The cell lines COS-1, Mv1Lu, and R-1B/L-17 were cultured and transiently transfected with the indicated vectors (11, 22).

Immunoprecipitation and Western Blotting—Precipitation with anti-Flag antibodies (IBI-Kodak) or Ni^{2+}-NTA-agarose (Qiagen) was done as described (20). Western immunoblotting with anti-Flag or anti-TβR-I antibodies was done using a 1:2000 dilution of these antibodies, a 1:5000 dilution of secondary antibodies, and the ECL detection system (Amersham Corp.). Monoclonal antibodies against TβR-I were raised using a juxtamembrane peptide sequence as described previously (10).

Metabolic Labeling—Cell labeling with [^{3}H]mevalonolactone (23) and with [^{35}S]methionine or [^{32}P]phosphate (13, 24) was done as described.

In Vitro Ras Farnesylation Assay—Mv1Lu cells were incubated for 1 h with the indicated concentrations of TGF-β and subsequently lysed in 20 mM Tris, 10 mM $MgCl_2$, 5 μM $ZnCl_2$, and 5 mM dithiothreitol, pH 7.4. After a brief sonication, 1 μM [^{3}H]farnesyl pyrophosphate (DuPont

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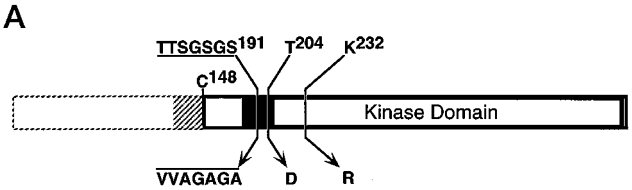
[1] The abbreviations used are: TGF-β, transforming growth factor-β; BMP, bone morphogenic protein; FT-α and -β, farnesyl-protein transferase α and β subunits, respectively; GGT-β, geranylgeranyl transferase β subunit; PAGE, polyacrylamide gel electrophoresis; NTA, nitrilotriacetate.



B

Bait	Description	Relative activity
Lex A	(Empty vector)	1
TβR-I	TGFß type I receptor	20
ActR-IB	Activin type I receptor	146
ActR-I	Activin type I receptor	10
TSR-I / ALK1	TGFß/Act. type I receptor	6
TβR-II	TGFß type II receptor	2
TβR-I(K232R)	Kinase-defective TβR-I	3
TβR-I(T204D)	Constitutively active TβR-I	1296
TβR-I(TS/VA)	Phosph'n-defective TβR-I	155
TβR-I(IIGS)	Altered GS domain TβR-I	1330

FIG. 1. **Analysis of the interaction between FT-α and various baits in yeast.** *A*, schematic representation of TβR-I with the extracellular and transmembrane domains (*dashed box*) and the cytoplasmic region (*solid box*). The position of the GS domain (*black box*), the kinase domain, and various mutations used in this study are indicated. *B*, *Saccharomyces cerevisiae* EGY48 was transformed with the *lacZ* reporter gene pSH18–34 bearing eight LexA binding sites, a vector encoding the indicated receptor cytoplasmic domains as LexA fusion proteins, and a full-length FT-α vector. β-Galactosidase activity was determined from four colonies of each strain. Values were normalized relative to the activity of cells transformed with empty vector. The experiment was repeated twice with the same results.

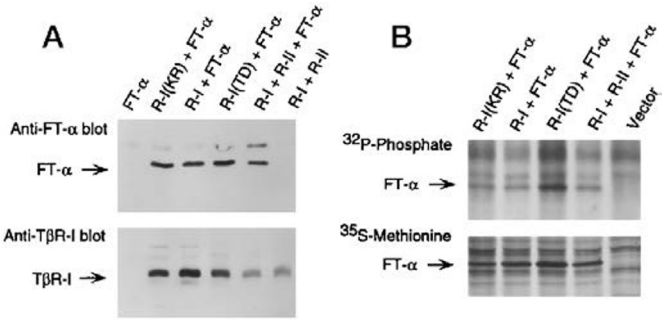


FIG. 2. **FT-α interaction with TβR-I and phosphorylation in mammalian cells.** COS-1 cells were transfected with FT-α tagged with the Flag epitope and different TβR-I constructs tagged with a hexahistidine sequence. Assays were done 2 days after transfections. *A*, histidine-tagged TβR-I receptors were isolated from cell lysates using Ni^{2+}-NTA-agarose, separated by SDS-PAGE, and then immunoblotted with anti-Flag antibody to detect FT-α (*upper panel*) or anti-TβR-I as a control (*lower panel*). *B*, cells transfected with the indicated constructs were metabolically labeled with [^{35}S]methionine (*lower panel*) or [^{32}P]phosphate (*upper panel*). FT-α was immunoprecipitated with anti-Flag antibody and resolved on SDS-PAGE.

NEN) and 100 mM bacterially expressed human Ha-*ras* was added to cell lysates and incubated for 40 min at 30 °C. Samples were analyzed by SDS-PAGE and autoradiography or by precipitation with trichloroacetic acid and counted. The farnesyl transferase inhibitor L-744-832 was a generous gift of Neal Rosen, MSKCC, and Allan Oliff, Merck (25).

RESULTS

We generated bait constructs for a yeast two-hybrid system (19) using cDNAs encoding the human TβR-I cytoplasmic domain, either wild type or containing the T204D mutation (Fig. 1*A*). This mutation elevates the kinase activity of TβR-I *in vitro* and endows this receptor with the ability to signal in the absence of TGF-β or TβR-II (14). Screening of a HeLa cell cDNA library with either bait yielded three major classes of cDNAs. Two of these encoded, respectively, the FK506/rapamycin binding protein FKBP12 and the BMP type II receptor BMPR-II, both of which have been previously described as TβR-I interacting proteins in the yeast two-hybrid system (20, 26, 27). The third class of cDNAs isolated in these screenings encodes FT-α. This class accounted for 25% of all clones isolated with TβR-I as bait, and 40% of those isolated with TβR-I(T204D) as bait (of over 100 clones analyzed in each case). The isolated FT-α clones encoded the full-length protein or lacked no more than 79 amino acids at the N terminus, suggesting that the interaction with TβR-I requires most of the FT-α protein.

FT-α interacted weakly or not at all with the cytoplasmic domains of TβR-II (9), the activin type I receptor ActR-I (11), the mixed specificity type I receptor TSR-I (11), or with empty vector (Fig. 1*B*). However, FT-α interacted strongly with the cytoplasmic domain of the activin type I receptor ActR-IB (Fig. 1*B*), which has high sequence similarity (90% identity) to that of TβR-I (22). To analyze the structural requirements of TβR-I for its interaction with FT-α, we used baits containing mutations that are known to alter the kinase activity of TβR-I and its signaling ability in mammalian cells. The (K232R) bait, which contains a mutation that eliminates kinase activity in TβR-I (13), interacted very weakly with FT-α, whereas the (T204D) bait interacted with FT-α more strongly than the wild type bait (Fig. 1*B*). Curiously, mutations that eliminate TβR-II phosphorylation sites in the TβR-I GS domain and prevent signaling in mammalian cells (13, 14) increased the interaction of the TβR-I bait with FT-α (Fig. 1*B*). A similar result was obtained with a bait containing a full replacement of the GS domain with an unrelated juxtamembrane sequence of TβR-II (construct TβR-I (IIGS)) (Fig. 1*B*), indicating that the GS domain is not directly involved in the interaction.

In order to investigate these interactions in mammalian cells, FT-α was tagged with the Flag epitope sequence at its N terminus and transfected into COS-1 cells alone or in combination with full-length TβR-I constructs. The latter were tagged at the C terminus with a hexahistidine sequence that binds to Ni^{2+}-NTA-agarose. An interaction between the transfected TβR-I and FT-α was demonstrated by incubating the cell lysates with Ni^{2+}-NTA-agarose beads followed by immunoblotting of the bound material using Flag antibody (Fig. 2*A*). In contrast to the results in yeast, the interaction with FT-α was not detectably affected by the mutations K232R or T204D in TβR-I. Cotransfection of TβR-II and addition of TGF-β were also without effect on the association between TβR-I and FT-α, as judged from the amount of coprecipitated FT-α (Fig. 2*A*). Metabolic labeling of these transfectants with [^{35}S]methionine and [^{32}P]phosphate followed by precipitation of total FT-α with anti-Flag antibody indicated that FT-α is a phosphoprotein in the absence of TGF-β or cotransfected receptors (Fig. 2*B*). No change in the ratio of ^{32}P/^{35}S labeling of FT-α was observed in cells cotransfected with the wild type, kinase-defective, or constitutively active TβR-I constructs or with both TβR-I and TβR-II in the presence of TGF-β (Fig. 2*B*). Similar experiments using an untagged FT-α construct and precipitation with a FT-α monoclonal antibody (28) also yielded no evidence of TGF-β-induced FT-α phosphorylation (data not shown).

The question was raised as to whether TβR-I recognized FT-α as part of a holoenzyme with FT-β or GGT-β. In experiments designed to test this possibility, we observed that cotransfection of the β subunits inhibited the interaction of FT-α with TβR-I (Fig. 3). Transfection of FT-β or GGT-β generated holoenzyme complexes with cotransfected FT-α while it inhibited the association of FT-α with TβR-I. Controls showed that this effect was not due to a decreased expression of FT-α or TβR-I (Fig. 3), suggesting that TβR-I does not recognize the isoprenyl transferase holoenzymes.

In order to determine the functional consequence of the in-

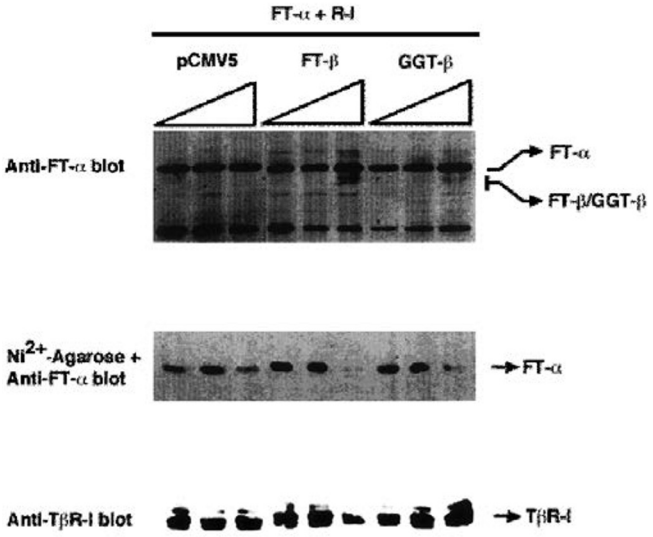


FIG. 3. **Effect of expression of isoprenyl transferase β subunits on the receptor-FT-α interaction.** COS-1 cells were transfected with 0.5 μg of plasmids encoding Flag-tagged FT-α and hexahistidine-tagged TβR-I, respectively, and increasing amounts (0.025, 0.15, and 1 μg) of plasmids encoding FT-β or GGT-β or empty vector. After 2 days, cells were metabolically labeled with [^{35}S]methionine for 2 h. FT-α was precipitated with anti-Flag antibody and subjected to SDS-PAGE and autoradiography (*upper panel*). TβR-I was retrieved with Ni^{2+}-NTA-agarose, separated by SDS-PAGE, and immunoblotted either with anti-Flag antibody to detect FT-α (*middle panel*) or TβR-I antibody (*bottom panel*).

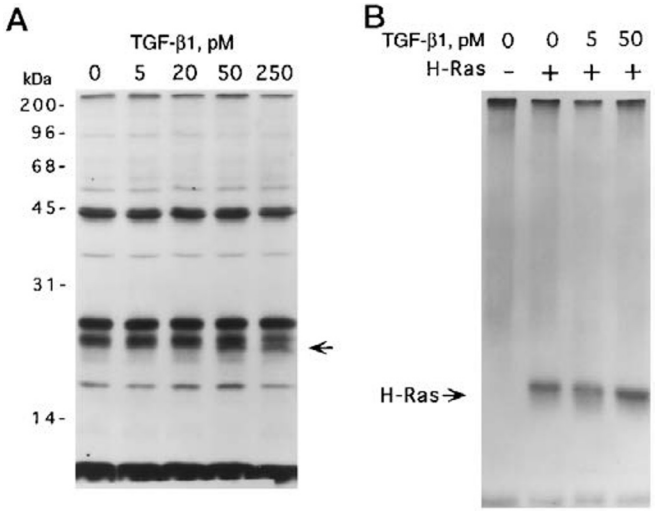


FIG. 4. **TGF-β effects on protein isoprenylation activity.** *A*, Mv1Lu cells were metabolically labeled with [^{3}H]mevalonolactone and treated with different TGF-β concentrations for 8 h. Triton-soluble proteins were resolved by SDS-PAGE and visualized by autoradiography. *B*, extracts of cells treated with increasing TGF-β concentrations were assayed for farnesyl transferase activity using H-ras and [^{3}H]farnesyl pyrophosphate as substrates. Labeled Ha-*ras* was visualized by SDS-PAGE and autoradiography.

teraction between FT-α and TβR-I, we tested the effect of TGF-β on protein isoprenylation. Mv1Lu epithelial cells were used since they are highly responsive to TGF-β. Cells were metabolically labeled with [^{3}H]mevalonolactone, a biosynthetic precursor of both farnesyl and geranylgeranyl pyrophosphate. Incubation for 8 h with physiological concentrations of TGF-β did not modify the level of ^{3}H incorporation into Triton-soluble proteins detectable under these conditions (Fig. 3*A*). A labeled protein of 20 kDa (presumably a small G protein; Ref. 23) migrated slightly faster after cell treatment with TGF-β (Fig. 4*A*). TβR-I has no consensus isoprenylation motif and did not become labeled in [^{3}H]mevalonolactone-labeled cells (data not shown). Farnesyl transferase activity assayed in cell lysates with recombinant Ha-*ras* as a substrate was similar in control and TGF-β-treated cells (Fig. 4*B*). Therefore, TGF-β action does not significantly alter the overall protein isoprenyl transferase activity in the cell.

Finally, we determined the effect of farnesyl transferase inhibitor L-744-832 (25) on TGF-β responsiveness in Mv1Lu cells. This agent blocks farnesyl transferase activity at 20 μM or lower concentrations in diverse cell types (25) including Mv1Lu cells.[2] Mv1Lu cell proliferation was inhibited half-maximally by ~20 μM L-744-832 and maximally by 40 μM L-744-832 (Fig. 5*A*). Mv1Lu cells respond to TGF-β with increased transcription of plasminogen activator inhibitor-1 (22). Using a luciferase reporter construct (p3TP-lux) that contains the TGF-β response region of the plasminogen activator inhibitor-1 promoter (22), the luciferase response to TGF-β was not affected by addition of 20 μM L-744-832 and was decreased by 40 μM L-744-832 but only at the lower (<10 pM) TGF-β concentration range (Fig. 5*B*). In experiments designed to determine the effect of L-744-832 on the growth inhibitory response to TGF-β, a partial inhibition of ^{125}I-deoxyuridine incorporation into DNA observed with 20 μM L-744-832 was simply additive to the inhibitory effect of TGF-β (Fig. 5*C*). These results suggest that farnesyl transferase activity is not essential for TGF-β signaling in Mv1Lu cells and has little or no participation in the two TGF-β responses analyzed here.

DISCUSSION

We have used a yeast two-hybrid cloning system to search for proteins that interact with the cytoplasmic domain of TβR-I since this is a downstream component of the TGF-β receptor system. Separate screenings of a HeLa cell cDNA library with either the wild type TβR-I cytoplasmic domain or a constitutively active mutant version yielded multiple isolates of three different classes of clones. One class corresponds to the BMP type II receptor BMPR-II previously shown to interact with TβR-I in yeast (20, 27). Another class corresponds to the FK506- and rapamycin-binding protein FKBP-12 whose interaction with TβR-I has also been previously documented and remains of unknown significance (26). The third class, as reported here, are clones encoding FT-α. While this report was in preparation, both Kawabata *et al.* (17) and Wang *et al.* (18) using the same approach reported an interaction of TβR-I with FT-α and proposed that this interaction might regulate the activity of the enzyme and explain the antiproliferative effects of TGF-β. Here we provide evidence for this interaction in mammalian cells and address the question of its physiological significance.

TβR-I has no consensus isoprenylation motif and was not isoprenylated in our assays. Therefore, it is unlikely that TβR-I is a substrate of FT-α. On the other hand, the kinase activity of certain TβR-I constructs correlates with their ability to interact with FT-α in yeast, and similar findings have been made by Kawabata *et al.* (17). Recombinant TβR-I kinase is able to phosphorylate FT-α *in vitro* (17).[3] However, some of our evidence challenges the notion that TβR-I association with FT-α simply reflects a kinase-substrate recognition event. Mutations that eliminate ligand-dependent phosphorylation sites in the GS domain actually increase the interaction of TβR-I with FT-α in yeast even though they decrease the kinase activity of TβR-I and block TβR-I signaling activity (14). Furthermore, in contrast to their effect on the receptor-FTα interaction in yeast,

[2] L. Sepp-Lorenzini and N. Rosen, personal communication.

[3] M. Kretzschmar and J. Massagué, unpublished work.

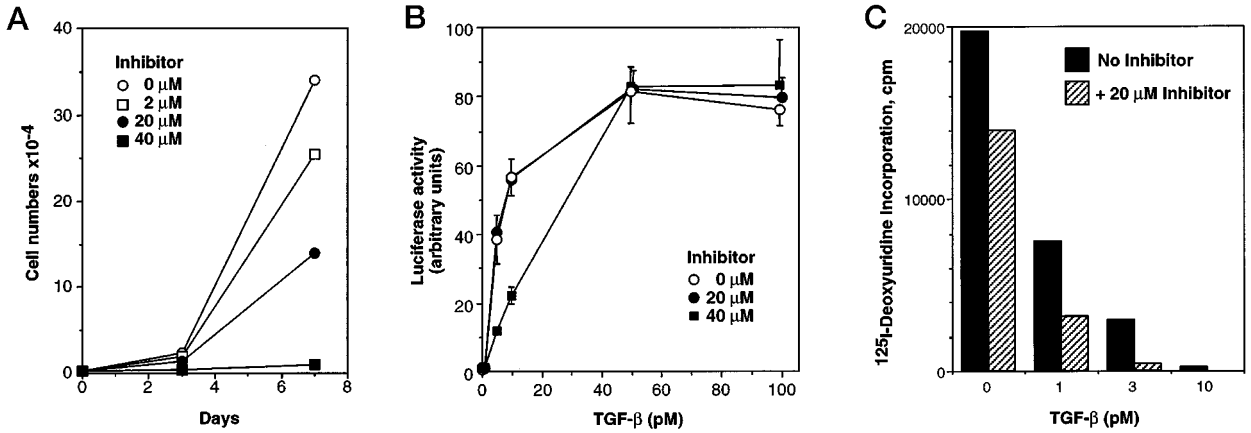


FIG. 5. **Effect of farnesyl transferase inhibitor on TGF-β action.** *A*, effect of various concentrations of farnesyl transferase inhibitor L-744-832 on Mv1Lu cell proliferation. *B* and *C*, Mv1Lu cells were treated with the indicated concentrations of TGF-β and L-744-832 for 15 h and assayed for luciferase activity from a transfected p3TP-lux reporter construct (22) (*B*) or [^{125}I]deoxyuridine incorporation (22) (*C*). Data are the average of triplicate determinations.

the K232R and T204D mutations had no detectable effect on this interaction in COS-1 cells. We observed no change in the phosphorylation level of FT-α by cotransfection of receptors and TGF-β addition to the cells, although this result stands in contrast to those of Wang *et al.* (18) who reported ligand-induced phosphorylation of FT-α. Further investigation is required to determine the reason for this discrepancy and the basis for the interaction between TβR-I and FT-α.

The overall level of protein isoprenylation in intact cells or the Ras farnesylation activity in cell extracts is not affected by TGF-β addition to Mv1Lu cells, a cell line that is strongly growth-inhibited by this factor. TGF-β could have altered isoprenyl transferase activity in a transient manner that escaped detection in our experiments. However, this seems unlikely since protein isoprenylation has a relatively long half-life in the cell and is not known to undergo a highly dynamic regulation (16). We also considered the possibility that farnesyl transferase activity might be required for TGF-β signaling. This question was investigated with the use of the farnesyl transferase inhibitor L-744-832. This agent inhibits cell proliferation by inhibiting protein farnesylation (25). Yet, at a concentration that blocks cell proliferation, L-744-832 has little effect on the basal or TGF-β-activated expression of a reported gene in these cells. On the other hand, the growth inhibitory effects of L-744-832 and TGF-β in Mv1Lu cells are additive with no evidence of synergy. These results suggest that farnesyl transferase activity does not participate in these TGF-β responses.

Significantly, in addition to forming complexes with FT-α, the cotransfected FT-β or GGT-β subunits inhibit the interaction of FT-α with TβR-I. Thus, the receptor can recognize isolated FT-α but not the holoenzymes and, therefore, may not be associated with isoprenyl transferase activity. It is possible that the receptor acts as a negative regulator of isoprenyl transferases by sequestering FT-α. However, overexpression of TβR-I alone does not cause the alterations in proliferation and other cellular functions that might be expected from an efficient sequestration of endogenous FT-α. Alternatively, FT-α might have a high tendency to associate with β subunits and, in their absence, with other proteins that for unknown reasons include TβR-I. In any case, we find no evidence that TβR-I or FT-α affects the known functions of each other. The hypothesis that Ras activity and cell proliferation are regulated through a direct interaction of the TGF-β receptor with farnesyl transferase is not supported by the evidence to date.

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