Farnesylthiosalicylic Acid Inhibits Mammalian Target of Rapamycin (mTOR) Activity Both in Cells and *in Vitro* by Promoting Dissociation of the mTOR-Raptor Complex

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The mammalian target of rapamycin (mTOR) functions with raptor and mLST8 in a signaling complex that controls rates of cell growth and proliferation. Recent results indicate that an inhibitor of the Ras signaling pathway, farnesylthiosalicylic acid (FTS), decreased phosphorylation of the mTOR effectors, PHAS-I and S6K1, in breast cancer cells. Here we show that incubating 293T cells with FTS produced a stable change in mTOR activity that could be measured in immune complex kinase assays using purified PHAS-I as substrate. Similarly, FTS decreased the PHAS-I kinase activity of mTOR when added to cell extracts or to immune complexes containing mTOR. Incubating either cells or extracts with FTS also decreased the amount of raptor that coimmunoprecipitated with mTOR, although having relatively little effect on the amount of mLST8 that coimmunoprecipitated. The concentration effect curves of FTS for inhibition of mTOR activity and for dissociation of the raptor-mTOR complex were almost identical. Caffeine, wortmannin, LY294002, and rapamycin-FKB12 also markedly inhibited mTOR activity *in vitro*, but unlike FTS, none of the other mTOR inhibitors appreciably changed the amount of raptor associated with mTOR. Thus, our findings indicate that FTS represents a new type of mTOR inhibitor, which acts by dissociating the functional mTOR-raptor signaling complex. *(Molecular Endocrinology 19: 175–183, 2005)*

The mammalian target of rapamycin (mTOR), is a Ser/Thr protein kinase involved in the control of cell growth and proliferation (1, 2). One of the best-characterized substrates of mTOR is PHAS-I (also known as 4E-BP1) (3–6). PHAS-I binds to eukaryotic initiation factor (eIF) 4E and represses cap-dependent translation by preventing eIF4E from binding to eIF4G (7, 8). When phosphorylated by mTOR, PHAS-I dissociates from eIF4E, allowing eIF4E to engage eIF4G, thus increasing the formation of the eIF4F complex needed for the proper positioning of the 40S ribosomal subunit and for efficient scanning of the 5’-untranslated region (UTR). In cells, mTOR is found in mTORC1, a complex also containing raptor and mLST8 (5, 9–11). Raptor (also known as mKOG1) is a newly discovered Mr = 150,000 protein, which possesses a unique NH2 terminal region followed by three HEAT motifs and seven WD-40 domains (5, 9, 11). mLST8 (also known as Gβl) is homologous to members of the family of β-subunits of heterotrimeric G proteins, and it consists almost entirely of seven WD-40 repeats (10, 11). The roles of the two mTOR-associated proteins are still not fully defined, but both appear necessary for optimal mTOR function because depleting cells of either raptor or mLST8 with small interfering RNA decreases mTOR activity (5, 9, 10). Raptor binds directly to PHAS-I and mutations in PHAS-I that decrease raptor binding also inhibit phosphorylation of PHAS-I by mTOR *in vitro* (12, 13). It has been proposed that raptor functions in TORC1 as a substrate-binding subunit that presents PHAS-I to mTOR for phosphorylation (5).

Rapamycin is the prototypic inhibitor of mTOR function (14). Determining the sensitivity to rapamycin has been an invaluable approach for identifying processes in cells controlled by mTOR. In addition to its experimental use, rapamycin and/or the related drug, CCI-779, are used clinically to inhibit host rejection of transplanted organs, the occlusion of coronary arteries after angioplasty, and the growth of tumor cells (15–17). Rapamycin action is complicated in that to bind mTOR with high affinity, the drug must first form a complex with the peptidylprolyl isomerase, FKBP12 (14). Rapamycin-FKB12 binds upstream of the kinase domain in a region of mTOR referred to as the FRB. Binding of the complex markedly attenuates, but does not fully inhibit, mTOR activity *in vitro* (4, 6). The incomplete inhibition raises the possibility that there are rapamycin-insensitive functions of mTOR in cells.
Thus, agents that interfere with mTOR by mechanisms different from that of rapamycin may prove to be useful experimental and/or clinical tools.

It has been demonstrated that farnesylthiosalicylic acid (FTS) inhibits phosphorylation of the mTOR effectors, PHAS-I and S6K1, in response to estrogen stimulation of breast cancer cells (Yue, W., J. Wang, Y. Li, and R. J. Santen, manuscript submitted). FTS is best known for its effects on the Ras signaling pathway, which it inhibits by disrupting the association of Ras with the plasma membrane, a localization essential for both the action and stability of Ras (18, 19). In this report, we present evidence that FTS inhibits mTOR activity through a novel mechanism involving dissociation of raptor from TORC1.

RESULTS

To investigate the effects of FTS on mTOR function in 293T cells, we monitored changes in the phosphorylation of PHAS-I, a well-characterized target of mTOR (1, 8). Phosphorylation of Ser64 and Thr69 in PHAS-I causes a dramatic decrease in the mobility of the protein in SDS-PAGE (8), so that changes in the mobility provide an index of changes in phosphorylation state. Incubating cells with increasing concentrations of FTS decreased the phosphorylation of PHAS-I, as evidenced by an increase in the electrophoretic mobility (Fig. 1A). To determine whether FTS also promoted dephosphorylation of Thr36 and Thr45, the preferred sites for phosphorylation by mTOR (20, 21), an immunoblot was prepared with PThr36/45 antibodies (Fig. 1A). Increasing FTS markedly decreased the reactivity of PHAS-I with the phosphospecific antibodies. Although the change in the intensity of the immunoblot does not provide an exact measure of the change in phosphorylation because the antibodies react with PHAS-I phosphorylated in either Thr36 or Thr45 (22), it is clear that the drug promotes the dephosphorylation of these sites.

To investigate further the inhibitory effects of FTS on mTOR signaling, we determined the effect of the drug on the association of mTOR, raptor, and mLST8. AU1-mTOR and hemagglutinin (HA)-tagged forms of raptor and mLST8 were overexpressed in 293T cells, which were then incubated with increasing concentrations of FTS before AU1-mTOR was immunoprecipitated with anti-AU1 antibodies. Immunoblots were prepared with anti-HA antibodies to assess the relative amounts of HA-raptor and HA-mLST8 that coimmunoprecipitated with AU1-mTOR (Fig. 1A). Both HA-tagged proteins were readily detectable in immune complexes from cells incubated in the absence of FTS, indicating that mTOR, raptor, and mLST8 form a complex in 293T cells. FTS did not change the amount AU1-mTOR that coimmunoprecipitated; however, increasing concentrations of FTS produced a progressive decrease in the amount of HA-raptor that coimmunoprecipitated (Fig. 1A). When results from three experiments were analyzed, the half-maximal effect on raptor dissociation from mTOR was observed at approximately 30 μM FTS (Fig. 2B). FTS did not appear to change the amount of HA-mLST8 associated with AU1-mTOR (Figs. 1A and 2B).

Results obtained with overexpressed proteins are not necessarily representative of responses of endogenous proteins. Therefore, experiments were conducted to investigate the effect of FTS on the endogenous TORC1 in nontransfected cells. The 293T cells were incubated with increasing concentrations of FTS before mTOR was immunoprecipitated with the mTOR antibody, mTAb1 (Fig. 1B). Immunoblots were then prepared with antibodies to mTOR, mLST8, and raptor. FTS markedly decreased the amount of raptor that coimmunoprecipitated with mTOR. Thus, FTS had comparable effects on the association of endogenous and overexpressed mTOR and raptor proteins. FTS also decreased the amount of mLST8 that coimmuno-
immunoprecipitated. AU1-mTOR and HA-raptor (Fig. 2B) were very similar, with half-maximal effects occurring between 20 and 30 μM. These results indicate that FTS inhibits mTOR in cells by promoting dissociation of raptor from mTORC1.

We also investigated the effects of incubating cells with increasing concentrations of 5-geranyltiosalicylate (GTS) on mTOR activity and the association of AU1-mTOR and HA-raptor (Fig. 2). GTS is identical with FTS except that it contains the 10-carbon geranyl group instead of the 15-carbon farnesyl group. GTS is much less effective than FTS in down-regulating the Ras signaling pathway (23), and it serves as a control for nonspecific detergent-like actions that occur with high concentrations of isoprenoid derivatives. Incubating cells with increasing concentrations of GTS slightly decreased mTOR activity (Fig. 2A); however, GTS was clearly less effective than FTS. GTS had relatively little effect on the association of AU1-mTOR with either HA-raptor or HA-mLST8 (Fig. 2B). Incubating cells with 200 μM sodium salicylate was also without effect on either mTOR activity or the association of mTOR and raptor (McMahon, L. P., K. M. Choi, and J. C. Lawrence, Jr., unpublished observations).

The findings with FTS in intact cells would be consistent with either an action of FTS on TORC1 or an action on a signaling pathway controlling the association of mTOR and raptor. Because the integrity of most signaling pathways is disrupted when cells are homogenized, we investigated the effects of FTS in extracts of cells in which AU1-mTOR, HA-raptor, and HA-mLST9 had been overexpressed. Incubating extracts with increasing concentrations of FTS progressively decreased the PHAS-I kinase activity of AU1-mTOR, assessed both by 32P incorporation from [γ-32P]ATP and by immunoblotting with PThr36/45 antibodies (Fig. 3A). Approximately four times lower concentrations of FTS were needed to inhibit mTOR activity in vitro than in intact cells. Presumably factors related to protein binding and membrane permeability account for the difference in concentrations of FTS needed in cells and extracts. Incubating extracts with increasing concentrations of FTS also decreased the amount of HA-raptor that coimmunoprecipitated with AU1-mTOR. The dose-response curves of kinase inhibition (Fig. 4A) and dissociation of AU1-mTOR and HA-raptor (Fig. 4B) were almost identical, indicating that loss of raptor from mTORC1 accounted for the inhibition of mTOR activity by FTS under these in vitro conditions. Again, the effects of FTS did not depend on overexpression of the mTORC1 components. Incubating extracts of nontransfected cells with FTS decreased the amount of endogenous raptor that coimmunoprecipitated with mTOR (Fig. 3B). The effects occurred at the same concentrations that promoted dissociation of the complex between the transfected proteins (Fig. 3A). The inhibitory effects of FTS on mTOR activity were apparent either when FTS was added directly to immune complexes just before the protein kinase assay, or when FTS was added to extracts before the immunoprecipitation of mTOR and HA-raptor (Fig. 2B).
Thus, if FTS action depends on factors other than the known components of mTORC1, such factors must coimmunoprecipitate with the complex. FTS also modestly decreased the amounts of both transfected and endogenous mLST8 proteins that coimmunoprecipitated with mTOR proteins (Fig. 3, A and B), but the effects were observed only at the highest concentrations of FTS investigated (Fig. 4B).

Because a portion of mTORC1 may be associated with membranous structures, we determined whether the effect of FTS depended on the presence of membranes. A fraction containing soluble mTORC1 was generated by centrifuging detergent-free extracts of HEK293 cells for 40 min at 200,000 × g. Adding 100 μM FTS to the supernatant fraction completely dissociated the complex between mTOR and raptor (McMahon, L. P., K. M. Choi, and J. C. Lawrence, Jr., unpublished observations).

Incubating extracts of transfected cells with increasing concentrations of GTS also led to progressive decreases both in AU1-mTOR activity (Fig. 4A) and in the association of AU1-mTOR and HA-raptor (Fig. 4B). These effects of GTS occurred at approximately 10 times higher concentrations than those of FTS. Thus, a degree of selectivity is conferred by the isoprenyl component of the drug.
We next compared the effects of FTS to those of rapamycin and several other inhibitors of mTOR that have been described previously (6, 24, 25). Incubating extracts from transfected cells with FTS decreased both mTOR activity (Fig. 5, A and B) and the association of AU1-mTOR and HA-raptor (Fig. 5, A and C). In contrast, incubating these complexes with concentrations of caffeine, rapamycin-FKBP12, LY294002, or wortmannin that decreased mTOR activity by more than 80% had little, if any, effect on decreasing the association of AU1-mTOR and HA-raptor.

DISCUSSION

The results of this study provide direct evidence that FTS inhibits mTOR activity. The finding that the inhibition of mTOR activity by increasing concentrations of FTS correlated closely with the dissociation of the mTOR-raptor complex, both in cells (Fig. 2) and in vitro (Fig. 3), supports the conclusion that FTS acts by promoting dissociation of raptor from mTORC1.

The peptidomimetic farnesyltransferase inhibitor, L-744,832, has also been shown to inhibit mTOR signaling (26, 27). By analogy to the Ras signaling pathway, it is logical to suspect that FTS and farnesyltransferase inhibitors might act at the same target in the mTOR signaling pathway. Both farnesyltransferase inhibitors and FTS disrupt the plasma membrane localization of Ras, one by blocking in the isoprenylation of Ras necessary for its membrane localization, the other by displacing Ras from its membrane binding sites. Farnesyltransferases prenylate the Cys found in a carboxy-terminal motif, sometimes referred to as the CAAX box (where C is Cys, A is an aliphatic amino acid, and X is any amino acid) (28). The COOH terminal sequence in mTOR is CysProPheTrp, which has some features of a CAAX box. However, based on studies with model peptides, the Phe in the mTOR sequence represents a strong negative determinant (28). Indeed, peptides with Phe in this position served as the basis for the design of L-744,832, and other potent competitive inhibitors of farnesyltransferase.

Although none of the proteins in mTORC1 are known to be prenylated, there are potential targets for FTS upstream in the mTOR signaling pathway. One example is the farnesylated GTP-binding protein Rheb (Ras homolog enriched in brain) (29). Rheb is activated in response to growth factors that inhibit TSC1/TSC2, which functions as the Rheb GTPase-activating protein (30–32). Although the mechanism is still unclear, activation of Rheb increases mTOR signaling. Mutating the Cys in the CAAX box of Rheb abolished the ability of overexpressed Rheb to increase S6K activity immunoprecipitated and are expressed as percentages of the respective controls. Means ± 1/2 the range from two experiments are presented.
Thus, Rheb is a potential target for farnesyltransferase inhibitors, and it is feasible that an action of FTS to displace Rheb from intracellular binding sites contributes to the inhibitory effects of FTS on mTOR signaling in intact cells. However, FTS does not appear to coimmunoprecipitate with mTOR (31). Thus, it is not clear that Rheb was involved in the inhibitory effects of FTS on mTOR activity and the association of mTOR and raptor in vitro. Interestingly, the inhibition of mTOR signaling by L-744,832 in cells seems to occur too rapidly (within 1.5 h) to be explained by inhibition of protein farnesylation (27, 34). Moreover, incubating 293T cells for 18 h with 60 μM L-744,832 did not promote dissociation of endogenous or overexpressed mTORC1 (McMahon, L. P., K. M. Choi, and J. C. Lawrence, Jr., unpublished observations). Additional studies will be required to determine the actual sites of action of both FTS and L-744,832.

Consistent with its action to inhibit Ras signaling, FTS blocks the activation of MAPK (19), and it inhibits the proliferation of several types of tumor cells, both in vitro and in vivo (35–38; and Yue, W., J. Wang, Y. Li, and R. J. Santen, manuscript submitted). In view of the number of different proteins that are farnesylated, the actions of FTS would be expected to involve more than inhibition of Ras signaling. As evidence of the complexity of FTS action, recent results indicate that inhibition by FTS of the effect of estrogen on stimulating the proliferation of breast cancer cells correlate much better with dephosphorylation of PHAS-I and S6K-1, two downstream elements of the mTOR signaling pathway, than with the inhibition of MAPK (Yue, W., J. Wang, Y. Li, and R. J. Santen, manuscript submitted).

Inhibition of mTOR with rapamycin has been shown to inhibit translation of capped mRNAs (39) and messages having a TOP (tract of pyrimidines) motif adjacent to the cap site (40). There are also reasons to suspect that the inhibition of mTORC1, with the decrease in PHAS-I phosphorylation and the resulting decrease in the availability of eIF4E for translation, contributes to the antiproliferative effect of FTS. Increasing eIF4E may result not only in an increase in cap-dependent translation, but also in an increase in cell proliferation. eIF4E levels are elevated in most breast cancer cells (41). Overexpressing eIF4E in 3T3 fibroblasts not only increased the rate of growth and caused an aberrant morphology of HeLa cells (42). Stable overexpression of eIF4E in 3T3 fibroblasts not only increased the rate of proliferation but actually caused malignant transformation, as evidenced by anchorage-independent growth and formation of tumors when implanted in nude mice (43).

It has been suggested that eIF4E stimulates proliferation by preferentially increasing translation of proteins that facilitate mitogenesis (44). The 5′-UTRs of mRNAs encoding many oncogenes, growth factors, and signal transduction proteins are predicted to contain regions of relatively stable secondary structure (45). These structured regions have been shown to interfere with binding and/or scanning by the 40S ribosomal subunit (46). Translation of such messages appears to be more dependent on eIF4E availability than translation of mRNAs having unstructured 5′-UTRs, a characteristic of many messages encoding house-keeping proteins. The dependency on eIF4E is believed to be explained by the requirement of eIF4E for formation of eIF4F, which melts secondary structure in the 5′-UTR via the helicase activity of the eIF4A subunit (44). As predicted from this mechanism, overexpressing PHAS-I, which decreases eIF4E availability, caused reversion of cells overexpressing eIF4E (47). Interestingly, overexpressing a constitutively active PHAS-I protein was recently shown to decrease the proliferation of MCF7 breast cancer cells (48).

By inhibiting mTOR activity and decreasing PHAS-I phosphorylation, FTS should decrease the contribution of eIF4E to proliferative responses. Other mTOR-dependent processes that are independent of changes in eIF4E availability are surely involved in the control of cell proliferation. Although they are not investigated in the present study, it can be predicted that FTS will be found to inhibit those processes requiring the raptor-mTOR interaction.

**MATERIALS AND METHODS**

**Antibodies**

Antibodies recognizing endogenous mTOR (mTAb1 and mTAb2) (49), PHAS-I (50), and raptor (12) were generated by immunizing rabbits with peptides having sequences corresponding to regions in the respective proteins. The phosphospecific antibodies, P-Thr36/45 and P-Thr69, that recognize phosphorylated sites in PHAS-I were generated as described previously (22). P-Thr36/45 antibodies bind to PHAS-I phosphorylated in either Thr36 or Thr45, as the sequences surrounding these sites are almost identical (51). Ascs fluid containing monoclonal antibody to the AU1 epitope tag was from Covance Research Products (Denver, PA) 9E10, which recognizes the myc epitope tag, and 12CA5, which recognizes the HA epitope tag, were purified from hybridoma culture medium by the University of Virginia Lymphocyte Culture Center.

To generate antibodies to mLST8, a synthetic peptide (CVETGEIKREYGGHQK) having a sequence identical with sequences around these sites are almost identical (51). Ascs fluid containing monoclonal antibody to the AU1 epitope tag was from Covance Research Products (Denver, PA) 9E10, which recognizes the myc epitope tag, and 12CA5, which recognizes the HA epitope tag, were purified from hybridoma culture medium by the University of Virginia Lymphocyte Culture Center.

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**cDNA Constructs**

The pcDNA3<sup>AU1</sup>-mTOR (3), pcDNA3<sup>SHA</sup>-Raptor (12), and pCMV-Tag 3<sup>A</sup><sub>PHAS-I</sub> (53) constructs for overexpressing AU1-mTOR, HA-Raptor, and myc-PHAS-I were described previously (3, 12, 53). pcDNA3<sup>SHA</sup>-mLST8 encodes mLST8 having an NH<sub>2</sub>-terminal triple HA epitope tag (HA-mLST8). To generate pcDNA3<sup>SHA</sup>-mLST8 a 5<sup>′</sup>-EcoRI site and a 3<sup>′</sup>-NotI site were introduced into mLST8 cDNA by PCR using l.MAGE clone 3910883 as template, and GAGTCGAATTCATGAACACCTCGCACACTGTC as forward and reverse primers, respectively.
After digesting the product with EcoRI and NotI, the mLSH8 cDNA was inserted in pcDNA33HA-Raptor in place of raptor insert, which had been removed with EcoRI and NotI. The coding region of the resulting pcDNA33HA-mLST8 was sequenced and found to be free of errors.

Overexpression of AU1-mTOR, HA-Raptor, HA-mLSH8, and Myc-PHAS-I

The 293T cells were cultured for 24 h in growth medium composed of 10% (vol/vol) fetal bovine serum in DMEM (6). AU1-mTOR, HA-raptor, and HA-mLSH8 were coexpressed by transfecting 293T cells (100 mm diameter dish) with 4 μg each of pcDNA3AU1-mTOR, pcDNA3SHA-Raptor, and pcDNA3SHA-mLSH8 by using TransIT-LT2 (Mirus Corp., Madison, WI) as described previously (3). Other cells were transfected with pcDNA3 vector alone. Where indicated, cells were transfected with pCMV-Tag 3APHAS-I to coexpress Myc-PHAS-I. Cells were used in experiments 18–20 h after transfection.

Immune Complex Assay of mTOR Activity

AU1-mTOR was immunoprecipitated by using anti-AU1 antibody to protein G-agarose beads as described previously (6). Endogenous mTOR was immunoprecipitated in the same manner, except that mTab1was used instead of anti-AU1 antibody. To measure kinase activity, exhaustively washed immune complexes were suspended in 20 μl of Buffer A (50 mM NaCl, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 mM microcystin LR, 10 mM Na-HEPES, and 50 mM β-glycerophosphate (pH 7.4)). The kinase reactions were initiated by adding 20 μl of buffer A supplemented with 2 μm [γ-32P]ATP, 20 mM MnCl2, and 40 μg/ml of [Hist]PHAS-I. In experiments in which the effects of wortmannin were investigated, dithiothreitol was omitted from the reactions, and [Hist]PHAS-I that had been reduced and alkylated with N-ethylmaleimide was used as substrate. Reactions were terminated after 10 min at 30 C by adding sodium dodecyl sulfate sample buffer. Samples were subjected to SDS-PAGE and relative amounts of 32P incorporated into [Hist]PHAS-I were determined.

Electrophoretic Analyses

SDS-PAGE was performed by using the method of Laemmli (54). Immunobots were prepared after electrophoretically transferring protein to Immobilon (Millipore, Billerica, MA) membranes. The relative amounts of 32P incorporated into [Hist]PHAS-I were determined by phosphorimaging. Signal intensities of bands in immunoblots were determined by scanning laser densitometry.

Other Materials

FTS and S-geranyltiosalicylic acid (GTS) were kindly provided by Dr. Yoel Klooq (Tel-Aviv University, Tel-Aviv, Israel) and Wayne Bardin (Thyreos, New York, NY). Rapamycin and LY294002 were from Calbiochem-Novabiochem International (San Diego, CA). Caffeine was from Sigma Chemical Co. (St. Louis, MO). Glutathione-S-transferase-FKBPI2 (55) and [Hist]PHAS-I (56) were expressed in bacteria and purified as described previously (55, 56). [γ-32P]ATP was from NEN Life Science Products (Beverly, MA).

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