Inhibition of DNA Synthesis by a Farnesyltransferase Inhibitor Involves Inhibition of the p70s6k Pathway*

Brian K. Law‡§§, Peter Nørgaard†, Luigi Gnudi**, Barbara B. Kahn**, Hans S. Poulsen‡, and Harold L. Moses‡‡‡

From the §Vanderbilt Cancer Center and §Department of Cell Biology, Vanderbilt University, Nashville, Tennessee 37232, the **Section for Radiation Biology, The Finsen Center Righospitalet, Copenhagen DK-2100, Denmark, and the **Diabetes Unit, Department of Medicine, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, Massachusetts 02215.

Previously, the protein farnesyltransferase inhibitor (FTI), L-744,832, has been shown to inhibit the proliferation of a number of tumor cell lines in vitro in a manner that correlated with the inhibition of the mitogen-activated protein kinase cascade. Here we show that FTI inhibits p70s6k phosphorylation in mammary tumors in vivo in transgenic mice. Furthermore, in a mouse keratinocyte cell line, FTI inhibits p70s6k phosphorylation and activity and inhibits PHAS-1 phosphorylation in vitro in both rapidly growing cells and in growth factor-stimulated quiescent cells. Dominant-negative Ras expression inhibits p70s6k stimulation by epidermal growth factor, demonstrating a requirement for Ras activity during p70s6k activation. FTI does not inhibit protein kinase B phosphorylation on Ser473, indicating that FTI does not act by inhibiting phosphatidylinositol 3-kinase. FTI also inhibits DNA synthesis in keratinocytes, and inhibition of DNA synthesis correlates closely with p70s6k inhibition. Rapamycin, an inhibitor of p70s6k and PHAS-1 phosphorylation, causes a 30–45% reduction in DNA synthesis in keratinocytes, while FTI induces an 80–90% reduction in DNA synthesis. These observations suggest that alteration of p70s6k and PHAS-1 function by FTI are responsible for a substantial portion of the growth-inhibitory properties of FTI. Together, these data demonstrate that p70s6k and PHAS-1 are novel downstream targets of FTI and suggest that the antitumor properties of FTI are probably due to the inhibition of multiple mitogenic pathways.

Ras is mutationally activated in approximately 30% of human tumors (1). Since Ras proteins, with the exception of Ki-Ras, must be farnesylated to be functionally active, protein farnesyltransferase inhibitors are potentially useful as anti-cancer drugs. FTIs have proven quite effective in the treatment of cancers in animal models (2–5), and they appear to induce tumor regression by increasing apoptosis and by inducing a G1 cell cycle arrest (4). The signaling pathways inhibited by FTIs to produce these anti-tumor effects, with the exception of the MAPK pathway (6), are not well defined. Identifying these pathways is an important step in elucidating the mechanism of action of FTI and in identifying novel targets for new anti-cancer drugs.

The efficacy of FTI as an anti-tumor agent likely stems from the central role of Ras in mitogenic signaling. Ras interacts with Raf, mitogen-activated protein kinase kinase, and other downstream signaling proteins in a GTP-dependent manner (reviewed in Refs. 7 and 8). Most of the mechanistic studies involving FTIs, however, have focused on the MAPK signal transduction pathway and have not explored the effects of FTIs on other Ras-dependent mitogenic pathways. Ras has recently been shown to support cell survival through the sequential activation of PI 3-kinase and the serine/threonine kinase protein kinase B (PKB) (9, 10). Other studies have indicated that PI 3-kinase can mediate mitogenic signaling (11–14). These studies raise the possibility that FTIs may induce tumor regression by increasing apoptosis and decreasing mitogenesis through the inhibition of Ras-dependent PI 3-kinase activation. One PI 3-kinase-dependent pathway required for mitogenic signaling involves p70s6k (15, 16). This enzyme was initially identified as a kinase capable of phosphorylating the ribosomal S6 protein in a growth factor-sensitive manner (17, 18). Phosphorylation of S6 is thought to regulate the synthesis of proteins whose transcripts contain 5′-oligopyrimidine tracts. More recently, in studies using embryonic stem cells containing targeted disruptions of both copies of the p70s6k gene, p70s6k was shown definitively to be the kinase responsible for S6 phosphorylation and for the growth factor-dependent stimulation of the translation of transcripts containing 5′-oligopyrimidine tracts (19). Another protein regulated in a PI 3-kinase-dependent manner is the translation initiation inhibitor PHAS-1 (properties of heat and sodium stability). PHAS-1 was identified independently as a protein that becomes phosphorylated in response to insulin and that interacts directly with the mRNA cap binding protein elongation initiation factor 4B (reviewed in Ref. 20). Specifically, PHAS-1 regulates cap-dependent translation by binding to elongation initiation factor 4B in a phosphorylation-dependent manner. Interestingly, the phosphorylation of both p70s6k and PHAS-1 are inhibited by the macroline rapamycin (21–23). Rapamycin acts by binding to its intracellular receptor FKBP12. The rapamycin-FKBP12 complex then binds to the mammalian target of rapamycin (mTOR) and forming growth factor, MOPS, 4-morpholinopropanesulfonic acid.

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‡‡ To whom correspondence should be addressed: Vanderbilt Cancer Center, 649 Medical Research Bldg. II, Nashville, TN 37232-6838.

Tel.: 615-936-1782; Fax: 615-936-1790; E-mail: hal.moses@mcmail.vanderbilt.edu.

† The abbreviations used are: FTI, farnesyltransferase inhibitor; EGF, epidermal growth factor; MAPK, mitogen-activated protein kinase; PI, phosphatidylinositol; PKB, protein kinase B; FKBP12, FK506-binding protein 12; mTOR, mammalian target of rapamycin; Erk1 and -2, extracellular regulated kinase 1 and 2, respectively; TGF, transforming growth factor; MOPS, 4-morpholinopropanesulfonic acid.

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blocks its function. FK506, a macrolide structurally related to rapamycin, competes for the same binding site on FKBP12 and blocks the effect of rapamycin on TOR-dependent pathways. The TOR genes were initially identified in yeast genetic screens as genes involved in rapamycin sensitivity (24) and subsequently shown to be involved in the control of G1 progression and protein synthesis (25). Later, mTOR was purified from mammalian cell extracts as a protein that specifically binds to FKBP12 in a rapamycin-dependent manner (26). The mechanisms by which mTOR regulates p70^euk and PHAS-1 function are unclear but may involve direct phosphorylation of these proteins by mTOR (27, 28).

The fact that rapamycin induces a G1 cell cycle arrest in many cell lines and regulates p70^euk and PHAS-1 function underscores the potential role of these proteins in mitogenic signaling. In this report, we investigate the effect of FTI on p70^euk and PHAS-1 and find that FTI mimics the ability of rapamycin to inhibit p70^euk phosphorylation and activation, PHAS-1 phosphorylation, and DNA synthesis. Taken together, these studies demonstrate that inhibition of the p70^euk pathway plays an important role in the growth-inhibitory effects of FTI.

EXPERIMENTAL PROCEDURES

Tumor Biopsies—The TGFα/11g double transgenic mice have been described previously (29). Excision biopsies from spontaneously arising tumors in murine mammary tumor virus-TGFα/11g double transgenic mice were collected when tumors reached approximately 300 mm^3. The mice were then treated for 3 weeks with 40 mg/kg of FTI given daily as a solution in 10% dextrose. After treatment, the mice were killed by cervical dislocation, and the tumors were resected and minced. The minced tumor tissue was homogenized in Laemmli sample buffer, and 20 μg of protein was resolved on 7.5, 10, or 15% SDS-containing polyacrylamide gels for the analysis of p70s6k, MAPK, and PKB, or PHAS-1, respectively. Proteins were transferred to nitrocellulose membranes, blocked with 5% nonfat dry milk, probed with antibodies directed against p70s6k (sc-230; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), Erk1 (sc-93; Santa Cruz Biotechnology), the dually phosphorylated serine residue at amino acid 17 is mutated to an asparagine residue (N17Ras) or β-galactosidase at a multiplicity of infection of 700 and 1900, respectively. This dose of N17Ras adenovirus was the lowest multiplicity of infection capable of completely inhibiting MAPK activation in response to EGF. The N17Ras-encoding adenovirus was generated as described (31). The β-galactosidase-encoding adenovirus was kindly provided by Dr. Christopher Newgard (32, 33) (University of Texas Southwestern Medical Center). Plates treated with MeSO or wortmannin were pretreated for 2 h in EGF(−) medium. To stimulate the cells, all plates except the EGF(−) control were refed with complete medium containing 4 ng/ml EGF. For the FTI-, MeSO-, and wortmannin-treated plates, these compounds were present in the EGF(−)containing medium. After a 15-min EGF stimulation, cell lysates were prepared and handled as described earlier.

Kinase Assays—Three hundred micrograms of protein were immunoprecipitated with 1 μg of rabbit polyclonal antibody specific for either p70^euk or Erk1, and the immune complexes were precipitated with 20 μl of packed protein A-Sepharose beads (Amersham Pharmacia Biotech). Immunoprecipitates were washed three times in lysis buffer and two times in 50 mM MOPS, pH 7.0, 5 mM MgCl2, 1 mM diithiothreitol, 10 mM paramethoxyphosphophosphate, and 10 μM microcystin (kinase buffer). Five micrograms of S6 substrate peptide (Santa Cruz Biotechnology) or myelin basic protein (Boehringer Mannheim) dissolved in kinase buffer were added to the washed beads in a volume of 10 μl. Kinase assays were set up with 10 μl of 333 μM ATP containing 10 μCi of [γ-32P]ATP. Reactions were allowed to proceed at 37 °C for 30 min and stopped with the addition of 10 μl of 4× Laemmli sample buffer followed by boiling for 5 min. Twenty microliters of each reaction mixture were resolved on 20% SDS-polyacrylamide gels, and bands were visualized using a PhosphorImager and ImageQuant software (Molecular Dynamics). Since comparable results were obtained using phospho-MAPK antibodies and immune complex kinase assays, these MAPK assays were used interchangeably.

DNA Synthesis Assays—Cells were plated at 20,000 cells/well in 24-well plates in complete growth medium and incubated for 24 h. The medium was removed, and the cells were cultured for an additional 22 h with 1 ml/well of the indicated treatments. Forty microliters of 10 μl of 3Hthymidine were added to each well, and the cells were incubated for an additional 2 h. The cells were washed, and incorporation of [3H]thymidine into DNA was quantitated as described previously (30).

RESULTS AND DISCUSSION

Since Ras is able to initiate multiple signaling cascades, we investigated the effect of FTI on several Ras-dependent signaling pathways in tumor-bearing transgenic mice. Protein extracts of tumor biopsies from TGFα/11g double transgenic mice taken before and after FTI treatment were analyzed by immunoblot using a variety of antibodies. Immunoblots of the tumor extracts using antibodies directed against p70^euk (Fig. 1) revealed a shift of the bulk of the p70^euk after FTI treatment to less phosphorylated forms that migrate more rapidly during SDS-polyacrylamide gel electrophoresis. Importantly, the mobility of p70^euk closely correlates with kinase activity, with the more slowly migrating forms representing catalytically active enzymes and the faster migrating forms representing inactive enzymes (34). The observed mobility shift indicates a decrease in p70^euk phosphorylation and probably correlates with a decrease in p70^euk activity following FTI treatment.

FTI Inhibits p70^euk in Mouse Keratinocytes—In order to more directly determine the effect of FTI treatment on p70^euk activity, we examined the ability of FTI to inhibit the activation of
p70s6k in Balb-MK cells. Balb-MK cells require EGF in order to proliferate and if deprived of EGF for 72 h exhibit very low levels of p70s6k activity and MAPK activity (Fig. 2). Balb-MK cells are therefore similar to mammary tumors in the MMTV-TGFα/neu transgenic mice in terms of their epithelial origin and presumably a dependence upon EGF signaling for proliferation. Treatment of the quiescent cells with EGF for 15 min resulted in a marked activation of both p70s6k activity and MAPK activity (Fig. 2). Pretreatment of the cells with 20 μM FTI for 72 h resulted in a 42% reduction in the activation of p70s6k by EGF. Inclusion of FTI in the p70s6k assays had no effect on enzyme activity, indicating that the inhibition of p70s6k by FTI is not direct but that FTI acts upstream of p70s6k (data not shown). This observation is consistent with the changes in p70s6k phosphorylation induced by FTI. Interestingly, FTI treatment did not cause a significant reduction in MAPK activation under these conditions. The PI 3-kinase inhibitor wortmannin also blocked p70s6k activation but did not affect MAPK activation. PKB activation is dependent upon PI 3-kinase activity, and expression of activated forms of PKB results in p70s6k activation (35–37). PKB activation by PI 3-kinase is mediated by the phosphorylation of Thr308 and Ser473 (38). In order to examine the effect of FTI on PKB phosphorylation, immunoblots were probed with an antiserum specific for PKB phosphorylated at Ser473. FTI had no significant effect on PKB phosphorylation at Ser473. Since phosphorylation of this site is inhibited by wortmannin, but not by FTI, this demonstrates that FTI does not act by inhibiting PI 3-kinase activity. Immunoblots performed using antibodies reactive against all forms of PKB demonstrated that the effect of wortmannin and lack of any FTI effect are not due to changes in the level of PKB protein. The possibility does exist, however, that FTI could inhibit PKB activity either directly or indirectly by inhibiting Thr308 phosphorylation.

**Dominant Negative Ras Abrogates p70s6k Activation**—Since FTIs were originally designed to inhibit Ras function but could also alter the function of other farnesylated proteins, we used the expression of dominant-negative Ras to distinguish Ras-dependent pathways from Ras-independent ones. Pretreatment of the cells for 48 h with an adenovirus encoding N17Ras, but not an adenovirus encoding β-galactosidase, strongly inhibited the activation of both p70s6k and MAPK by EGF. This result demonstrates that endogenous Ras is required for the activation of both p70s6k and MAPK and that a 72-h pretreatment with 20 μM FTI is insufficient to completely block Ras activity, since MAPK is still activated by EGF under these conditions. Treatment with 0.5 μM FTI was sufficient to block the farnesylation of adenosvirally expressed N17Ras although this treatment has no effect on either p70s6k or MAPK activities (Fig. 2, lower panel). Adenosvirally expressed Ha-Ras was used to monitor farnesyltransferase inhibition, because endogenous Ha-Ras was undetectable in the Balb-MK cells by immunoblot. The observation that low concentrations of FTI block farnesylation of adenosvirally expressed Ras suggests that the concentrations of FTI employed are sufficient to block farnesyltransferase activity but that there is residual Ras activity after a 72-h treatment with 20 μM FTI. Since farnesylation is an irreversible modification, Ras activity would not be expected to be abrogated until the endogenous Ras is completely turned over. Alternatively, the residual Ras activity might be due to K-Ras, which can be geranylgeranylated rather than farnesylated in the presence of FTIs (39). The differential effect of FTI on p70s6k and MAPK might then be explained by either a difference in the relative sensitivity of these pathways to the level of Ras activity or to different Ras isoforms. The latter hypothesis is supported by recent studies (40) that show that K-Ras is more effective at activating MAPK than Ha-Ras, while Ha-Ras is more effective at activating PI 3-kinase than K-Ras. It is possible that in Balb-MK cells FTI is preferentially inhibiting PI 3-kinase activation by abrogating Ha-Ras farnesylation but having a weak effect on MAPK activation because K-Ras is being geranylgeranylated and is thus still functional.

The role of Ras in p70s6k activation is unclear. In one report, Ras was found to be unnecessary for the activation of p70s6k (41). In another report, V12Ras, a constitutively active form of Ras, was shown to be sufficient for p70s6k activation (42). Similar observations have been made regarding the PI 3-kinase-dependent activation of PKB, which was found to be abrogated by V12Ras (43) but not inhibited by N17Ras (35). Our results show that N17Ras inhibits PKB phosphorylation on Ser473, suggesting that Ras is important for PKB activation in Balb-MK cells. These discrepancies might be explained by the fact that PI 3-kinase can be activated by the binding of its p85 subunit to tyrosine-phosphorylated proteins and also by directly binding the catalytic subunit to activated Ras. Whether the
activation of a particular PI 3-kinase-dependent pathway is Ras-dependent will probably vary with the mechanisms of PI 3-kinase activation in a cell type- and growth factor-specific manner (44). Our results demonstrate that in Balb-MK cells, Ras activity is necessary for p70s6k activation.

**FTI Inhibits DNA Synthesis in Balb-MK Cells and in LNCaP and MDA-MB-468 Tumor Cell Lines**—Since FTI induces a G1 cell cycle arrest in mouse mammary tumors and p70\textsuperscript{euk} is required for the initiation of DNA synthesis (15, 16), we examined whether FTI inhibits the proliferation of Balb-MK cells. To measure growth inhibition by FTI, \[^{3}H\]thymidine incorporation into DNA in rapidly growing Balb-MK cells was measured under various conditions. As shown in Fig. 3A, FTI caused a dose-dependent inhibition of EGF-induced DNA synthesis in Balb-MK cells. In order to determine whether FTI also inhibits DNA synthesis in tumor cells, thymidine incorporation experiments were performed using the LNCaP prostatic adenocarcinoma and MDA-MB-468 human breast adenocarcinoma cell lines (Fig. 3B). FTI also dramatically inhibited DNA synthesis in these two tumor cell lines. Thus, FTI inhibits DNA synthesis not only in the nontransformed Balb-MK cell line but also in two different tumor cell lines of epithelial origin. If p70\textsuperscript{euk} activity plays a role in stimulating mitogenesis in Balb-MK cells, then inhibition of p70\textsuperscript{euk} by rapamycin should also inhibit DNA synthesis. As shown in Fig. 3, A and B, rapamycin inhibited DNA synthesis to differing degrees in all three cell lines. The effect of rapamycin is specific, since in MDA-MB-468 cells, which are not growth-inhibited by FK506, FK506 blocks growth inhibition by rapamycin. The inhibition of thymidine incorporation by FTI, however, is greater than the inhibition by rapamycin, suggesting that FTI inhibits additional mitogenic pathways.

**FTI Mimics the Ability of Rapamycin to Induce p70\textsuperscript{euk} and PHAS-1 Dephosphorylation**—In order to determine which signaling elements might be involved in the growth inhibition observed in Fig. 3, A and B, cell extracts from FTI- or rapamycin-treated Balb-MK cells were prepared and analyzed by assays for p70\textsuperscript{euk} activity and PHAS-1 phosphorylation. Keratinocytes were treated for 24 h in the presence of 4 ng/ml EGF and incubated for 2 h with \[^{3}H\]thymidine, and the incorporation into DNA was quantitated. Data represent the average of quadruplicate determinations ± S.D. B, LNCaP human prostatic adenocarcinoma cells (left) or MDA-MB-468 human breast adenocarcinoma cells (right) were treated as in Fig. 3A, except the mitogenic stimulus used was 10% fetal bovine serum rather than EGF. Data represent the average of quadruplicate determinations ± S.D. C, FTI and rapamycin inhibit p70\textsuperscript{euk} activity and inhibit p70\textsuperscript{euk} and PHAS-1 phosphorylation. Keratinocytes were treated for 24 h and a, and extracts were prepared as in Fig. 2. Equal amounts of protein were subjected to p70\textsuperscript{euk} assay as in Fig. 2 (upper part) or subjected to immunoblotting with antibodies specific for p70\textsuperscript{euk}, PHAS-1, the dually phosphorylated active forms of Erk1 and Erk2 (P-Erk1,2), or antibodies recognizing both phosphorylated and unphosphorylated Erk1 and Erk2 (Erk1,2) (lower parts).
to its sensitivity to PI 3-kinase inhibitors and rapamycin (reviewed in Ref. 20), we investigated whether FTI can induce changes in PHAS-1 phosphorylation similar to those seen with rapamycin treatment. Rapamycin induced the formation of a faster migrating, less phosphorylated band of PHAS-1α (Fig. 3C). FTI treatment also caused the appearance of PHAS-1α. These data suggest that FTI acts at a site upstream of both p70s6k and PHAS-1. Since the regulation of these two proteins bifurcates at mTOR or immediately upstream of p70s6k (45), these results are consistent with the hypothesis that FTI acts at the level of mTOR or upstream of mTOR.

Since FTI treatment inhibits MAPK activity and colony formation in tumor cell lines (6), immunoblots of Balb-MK extracts were performed with antibodies specific for the dually phosphorylated active form of MAPK. These experiments showed a slight reduction in the levels of active MAPK at 60 μM FTI. The inhibition of MAPK, however, was weak, and in some experiments not apparent at all (see Fig. 2). Out of a total of nine experiments where the effect of FTI on MAPK was examined, FTI had no effect on MAPK activity in seven experiments and had a weak effect on MAPK activity in two experiments. In all nine experiments, FTI had a strong inhibitory effect on p70s6k. Moreover, the same level of inhibition of DNA synthesis by FTI was observed in experiments where no inhibition of MAPK was apparent, suggesting that the inhibition of MAPK by FTI in Balb-MK cells is not critical for the growth-inhibitory properties of FTI. This observation differs from a previous report (6) in which growth inhibition of various tumor cell lines by FTI correlated with MAPK inhibition. Further studies will be required to determine whether FTI inhibition of cell growth correlates more closely with MAPK inhibition or p70s6k inhibition or whether each effect is important in a subset of cell lines. The inhibition of DNA synthesis by rapamycin, along with the inhibition of p70s6k activity and p70s6k, and PHAS-1 phosphorylation by FTI, suggests that these effects are involved in the inhibition of DNA synthesis by FTI.

**FTI Rapidly Inhibits p70s6k.** Since farnesylation is an irreversible modification, the period of FTI treatment required to inhibit p70s6k activation should provide an estimate of the half-life of the farnesylated protein(s) involved. In order to determine the time course of FTI inhibition of p70s6k, rapidly growing Balb-MK cells were treated for various intervals with FTI and cell extracts prepared. Analysis of the extracts for p70s6k activity (Fig. 4) showed that p70s6k activity was inhibited relative to the untreated control by 50% as early as 0.5 h. p70s6k immunoblots revealed four distinct phosphoforms of the enzyme. The control sample consisted primarily of the three most slowly migrating bands. FTI induced a partial loss of the most slowly migrating band as early as 0.5 h. p70s6k was most highly dephosphorylated at the 6- and 24-h time points. At 24 h, p70s6k activity was inhibited by 73% and consisted primarily of the two most rapidly migrating bands of p70s6k.

FTI also induced a partial dephosphorylation of PHAS-1 indicated by the appearance of a third more rapidly migrating phosphoform (α). PHAS-1 dephosphorylation was reproducible but was modest relative to the dephosphorylation of p70s6k. The time course of PHAS-1 dephosphorylation was slower than the p70s6k dephosphorylation, with the α-band becoming apparent 2 h after FTI treatment. This difference between the time courses of p70s6k and PHAS-1 dephosphorylation suggests the possibility that FTI inhibits p70s6k and PHAS-1 dephosphorylation by acting on distinct upstream targets. The differences, however, might be due to differences in the relative kinetics of the kinases and phosphatases acting on these two proteins. The level of phosphorylation of MAPK was unaltered relative to the untreated control at all time points examined. The relatively rapid effect of FTI suggests that the relevant farnesylated protein(s) have a very short half-life or, alternatively, that the effect of FTI on p70s6k and PHAS-1 is elicited by some mechanism other than through the inhibition of farnesyltransferase activity.

An FTI structurally similar to the one used in this report was shown to revert the phenotype of Ras-transformed cells, including the loss of membrane ruffles (46). Since Rac is required for the formation of membrane ruffles in response to growth factors (47), the loss of membrane ruffles observed suggests that FTI may be acting by altering Rac function. We see similar FTI-induced morphological changes in Balb-MK cells (data not shown). Interestingly, these morphological changes, like the effects on p70s6k, occur more rapidly than would be expected if the FTI is working by blocking Ras prenylation. These observations, along with the fact that Rac is essential for Ras transformation (48) and G1 progression (49) and has been implicated in p70s6k activation (50), make it tempting to speculate that the FTI effects on cell morphology, cell proliferation, and p70s6k activity may be mediated by altering Rac function. This possibility is currently being investigated.

These studies demonstrate that FTI inhibits p70s6k phosphorylation and activation and PHAS-1 phosphorylation. The observation that FTI inhibits the phosphorylation of p70s6k in vivo in tumors from TGFα/neu transgenic mice under conditions, where FTI induces tumor regression accompanied by a G1 arrest and increased apoptosis, suggests that the modulation of p70s6k and PHAS-1 function may be partially responsible for the anti-tumor effects of FTI. In Balb-MK cells, FTI inhibits p70s6k and PHAS-1 phosphorylation rapidly, and inhibition is observed in both rapidly growing cells and quiescent cells stimulated with EGF. We further show that Ras activity is required for p70s6k activation in response to EGF. The fact that FTI mimics the effects of rapamycin on p70s6k and PHAS-1 demonstrates that the effects of FTI on these pathways are involved in the inhibition of DNA synthesis by FTI. It should be noted that since FTI and rapamycin both inhibit p70s6k and PHAS-1 phosphorylation, the growth-inhibitory effects of these drugs could be mediated by p70s6k, PHAS-1, or some other effector in the same signaling pathway. These data lead us to speculate that an upstream element of the p70s6k signaling pathway may be one of the much sought after targets of FTI responsible for its anti-tumor effects. Future studies directed toward determining the pathways inhibited by FTIs during tumor regression may lead to the identification of new molec-
FTI Inhibits the p70^s6k Pathway

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