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Ras *CAAX* Peptidomimetic FTI-277 Selectively Blocks Oncogenic Ras Signaling by Inducing Cytoplasmic Accumulation of Inactive Ras-Raf Complexes*

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Ras-induced malignant transformation requires Ras farnesylation, a lipid posttranslational modification catalyzed by farnesyltransferase (FTase). Inhibitors of this enzyme have been shown to block Ras-dependent transformation, but the mechanism by which this occurs remains largely unknown. We have designed FTI-276, a peptide mimetic of the COOH-terminal Cys-Val-Ile-Met of K-Ras4B that inhibited potently FTase *in vitro* (IC_{50} = 500 pM) and was highly selective for FTase over geranylgeranyltransferase I (GGTase I) (IC_{50} = 50 nM). FTI-277, the methyl ester derivative of FTI-276, was extremely potent (IC_{50} = 100 nM) at inhibiting H-Ras, but not the geranylgeranylated Rap1A processing in whole cells. Treatment of H-Ras oncogene-transformed NIH 3T3 cells with FTI-277 blocked recruitment to the plasma membrane and subsequent activation of the serine/threonine kinase c-Raf-1 in cells transformed by farnesylated Ras (H-RasF), but not geranylgeranylated, Ras (H-RasGG). FTI-277 induced accumulation of cytoplasmic non-farnesylated H-Ras that was able to bind Raf and form cytoplasmic Ras/Raf complexes in which Raf kinase was not activated. Furthermore, FTI-277 blocked constitutive activation of mitogen-activated protein kinase (MAPK) in H-RasF, but not H-RasGG, or Raf-transformed cells. FTI-277 also inhibited oncogenic K-Ras4B processing and constitutive activation of MAPK, but the concentrations required were 100-fold higher than those needed for H-Ras inhibition. The results demonstrate that FTI-277 blocks Ras oncogenic signaling by accumulating inactive Ras/Raf complexes in the cytoplasm, hence preventing constitutive activation of the MAPK cascade.

Ras proteins are plasma membrane-associated GTPases that function as relay switches transducing biological information from extracellular signals to the nucleus (for review, see Refs. 1–3). In normal cells, Ras proteins cycle between the GDP (inactive)- and GTP (active)-bound forms to regulate proliferation and differentiation. The mechanism by which extracellular signals, such as epidermal and platelet-derived growth factor, transduce their biological information to the nucleus via Ras proteins has recently been unraveled. Binding of these growth factors to tyrosine kinase receptors results in autophosphorylation of various tyrosines which bind *src*-homology 2 (SH2) domains of several signaling proteins. One of these, a cytoplasmic complex of GRB-2 and a Ras exchanger (SOS-1), is recruited by the tyrosine-phosphorylated receptor, where SOS-1 catalyzes the exchange of GDP for GTP on Ras, hence activating it. GTP-bound Ras recruits c-Raf-1, a serine/threonine kinase, to the plasma membrane where its kinase activity is activated by as yet undetermined membrane-associated events. Raf triggers a kinase cascade by phosphorylating MAP kinase kinase (MEK) which, in turn, phosphorylates MAPK[1] on threonine and tyrosine residues. Activated MAPK translocates to the nucleus where it phosphorylates transcription factors. In a large number of human cancers, Ras is locked in its GTP-bound form due to mutations in amino acids 12, 13, or 61 (4, 5). As a result, the Ras pathway no longer requires an upstream growth signal, is uninterrupted and the enzymes in this pathway such as Raf, MEK, and MAPK are constitutively activated (1–3).

In addition to its inability to hydrolyze GTP, oncogenic Ras must associate with the plasma membrane to cause malignant transformation (6–8). Ras membrane association is mediated through a series of posttranslational modifications (9–12). The first step is catalyzed by a cytosolic heterodimer farnesyltransferase (FTase), which attaches farnesyl to the thiol group of cysteine of the carboxyl-terminal tetrapeptide C*AAX*, where *A* is isoleucine or valine and *X* is serine or methionine (13–16). Because farnesylation is required and sufficient for Ras transformation (8, 17), FTase is an attractive target for the development of a potential new class of anti-cancer agents (18, 19). Although C*AAX* peptides are potent competitive inhibitors of FTase, rapid degradation and low cellular uptake limit their use as therapeutic agents. Over the last 3 years, we (20–22) and others (23–26) have designed C*AAX* peptidomimetics that potently inhibit FTase *in vitro* and Ras processing *in vivo* but that retain several peptidic features. More recently, we have

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[1] The abbreviations used are: MAPK, mitogen-activated protein kinase; C*AAX*, tetrapeptide where C = cysteine, *A* = aliphatic amino acid, and *X* = serine or methionine; FTase, farnesyltransferase; GGTase I, geranylgeranyltransferase I; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; FTI, farnesyl transferase inhibitor; Boc, *t*-butoxycarbonyl; HPLC, high performance liquid chromatography.

designed non-peptide C*AAX* mimetics that have several desirable features for further development as anti-cancer agents (27). Although these non-peptide mimics and C*AAX* peptidomimetics inhibit FTase potently (nM), their ability to disrupt Ras processing in whole cells occurs at micromolar concentrations that would not be easily achievable in *in vivo* settings. Therefore, there is a need for improved FTase inhibitors with more potent activity in whole cells and *in vivo.*

Ras C*AAX* peptidomimetics have been shown to reverse oncogenic H-Ras transformation, inhibit the growth of H-Ras-transformed, but not normal cells in culture, and slow the growth of Ras but not Raf-transformed cells in nude mice (23, 24, 28). Recently, FTase inhibitors have also been shown to inhibit oncogenic Ras activation of MAPK in H-Ras-transformed cells (29, 30). Whether FTase inhibitors also inhibit oncogenic K-Ras signaling is not yet known. This is an important question, since K-Ras is a more efficient substrate for FTase, rendering it more difficult to block by FTase inhibitors, and since K-Ras mutations are most prevalent in human tumors where Ras is mutated. Furthermore, the mechanism by which FTase inhibitors suppress MAPK activation has not been investigated. Specifically, the effects of FTase inhibitors on the interactions between Ras and its downstream effectors such as Raf have not been studied. The present work describes the design of a highly potent (pM/nM) Ras C*AAX* peptidomimetic which antagonizes both H- and K-Ras oncogenic signaling. The results demonstrate that the mechanism by which this inhibitor blocks Ras-dependent signaling involves sequestering Raf in the cytoplasm away from the plasma membrane where it would be activated.

EXPERIMENTAL PROCEDURES

Synthesis of CAAX Analogues—The peptidomimetic FTI-276 was synthesized as follows: 2-bromo-4-nitrotoluene was coupled with phenylboronic acid. The product was oxidized with $KMnO_4$ to 2-phenyl-4-nitrobenzoic acid which was coupled with L-methionine methyl ester followed by reduction with stannous chloride. The resulting 4-amino-2-phenylbenzoyl methionine methyl ester was reacted with *N*-Boc-*S*-trityl-cysteinal to give *N*-Boc-*S*-trityl methyl ester of FTI-276. This methyl ester was hydrolyzed by LiOH and then deprotected by trifluoroacetic acid. The pure FTI-276 was obtained through preparative HPLC. The peptidomimetic FTI-277 was made from the *N*-Boc-S-trityl methyl ester of FTI-276 by first treatment with mercuric chloride followed by hydrogen sulfide in methanol. The final product, FTI-277, was obtained as its hydrochloride salt. Spectroscopic data of both FTI-276 and FTI-277 were consistent with their assigned structures. HPLC analysis showed purity over 99.9%.

FTase and GGTase I Activity Assays—FTase and GGTase I activities from 60,000 × *g* supernatants of human Burkitt lymphoma (Daudi) cells (ATCC, Rockville, MD) were assayed exactly as described previously (27). Inhibition studies were performed by determining the ability of Ras C*AAX* peptidomimetics to inhibit the transfer of[^{3}H]farnesyl and [^{3}H]geranylgeranyl from [^{3}H]farnesyl pyrophosphate and [^{3}H]geranylgeranyl pyrophosphate to H-Ras-CVLS and H-Ras-CVLL, respectively (27).

Ras and Rap1A Processing Assay—H-RasF, H-RasGG, pZIPneo, Raf, S186 (31), and K-Ras4B cells (17) were seeded on day 0 in 100-mm dishes in Dulbecco's modified Eagle's medium supplemented with 10% calf serum and 1% penicillin/streptomycin. On days 1 and 2, cells were refed with medium containing various concentrations of FTI-277 or vehicle (10 mM DTT in Me_2SO). On day 3, cells were lysed in buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM EGTA, 25 μg/ml leupeptin, 1 mM PMSF, 2 mM Na_3VO_4, 1 mg/ml soybean trypsin inhibitor, and 6.4 mg/ml Sigma-104® phosphatase substrate. Lysates were then immunoblotted using an anti-Ras antibody (Y13-238, ATCC) or an anti-Rap1A antibody (SC-65, Santa Cruz Biotechnology, Santa Cruz, CA) as described previously (27).

Co-immunoprecipitation of Raf and Ras—Cells were seeded on day 0 in 100 mM dishes, and on days 1 and 2 cells were treated with FTI-277 (10 μM) or vehicle. On day 3, cells were collected and pellets were resuspended in ice-cold hypotonic buffer (10 mM Tris, pH 7.5, 5 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF) and sonicated to break up the cell pellet. The cell suspension was then centrifuged at 2,000 rpm for 10 min to clear debris after which the supernatant was spun for 30 min at 100,000 × *g* to separate membrane and cytosol fractions. The cytosol and membrane fractions were then lysed as described previously (32). Equal amounts of cytosol and membrane fractions were immunoprecipitated using 50 μl of a 25% protein A-Sepharose CL-4B suspension (Sigma) with 1 μg/ml anti-c-Raf-1 (SC133, Santa Cruz Biotechnology, Santa Cruz, CA). The samples were washed five times in 50 mM HEPES, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 0.1% Triton X-100, 10% glycerol, 20 mM NaF). The final pellets were run on 12.5% SDS-PAGE, transferred to nitrocellulose, and immunoblotted for the presence of Ras using anti-Ras antibody (Y13-238) and anti-Raf antibody (SC133, Santa Cruz Biotechnology, Santa Cruz, CA). Detection was the same as above for Ras and Rap1A processing.

Detection of GTP and GDP Bound to Ras—H-RasF cells were seeded and treated as above for Ras/Raf interaction studies. On day 2, however, cells were labeled overnight with [^{32}P]orthophosphate as described previously (32). On day 3, the cytosol and membrane fractions were separated, lysed, and equal amounts of lysate were immunoprecipitated using anti-Ras antibody (Y13-259) along with 30 μl of protein A-agarose goat anti-rat IgG complex (Oncogene Science). Bound nucleotide was eluted and separated by TLC as described previously (32).

Raf-I Kinase Activity Assay—Raf-I kinase activity was assayed by determining the ability of Raf to transfer phosphate from [γ-^{32}P]ATP to a 19-mer peptide containing a Raf autophosphorylation site. Membrane and cytosol fraction isolation and Raf immunoprecipitation were described above. Immunoprecipitates were washed with kinase buffer (50 mM Tris, pH 7.3, 150 mM NaCl, 12 mM $MnCl_2$, 1 mM DTT, 0.2% Tween 20). Immune complex kinase assays were performed by incubating immunoprecipitates from membrane and cytosol fractions in 96 μl of kinase buffer with 20 μCi of [γ-^{32}P]ATP (10 mCi/ml, Amersham Corp.) and 2 μl of the Raf-1 substrate peptide IVQQFGFQRRASDDGKLTD (1 mg/ml, Promega, Madison, WI) for 30 min at 25 °C. The phosphorylation reaction was terminated by spotting a 50-μl aliquot onto Whatman P81 phosphocellulose filters. The filters were washed in 0.5% orthophosphoric acid and air-dried. The amount of ^{32}P incorporated was determined by liquid scintillation counting.

MAP Kinase Immunoblotting—Cells were treated with FTI-277 and lysed as described above for Ras and Rap1A processing. Equal amounts of lysate were separated on a 15% SDS-PAGE, transferred to nitrocellulose, and immunoblotted using an anti-MAPK antibody (erk2, monoclonal, Upstate Biotechnology, Inc., Lake Placid, NY). Antibody reactions were visualized using peroxidase-conjugated goat anti-mouse IgG and enhanced chemiluminescence detection (Amersham).

RESULTS AND DISCUSSION

Recently, we (20–22, 27) and others (23–26) have designed Ras C*AAX* peptidomimetics that inhibit FTase potently with concentrations in the nM range. However, these agents inhibited Ras processing in whole cells only at μM levels (29, 30). In order to investigate the mechanism of action of FTase inhibitors, we sought to first improve the potency and selectivity of our first generation of C*AAX* peptidomimetics. Structure activity relationship studies with C*AAX* peptides and peptidomimetics predict a hydrophobic region in the active site of FTase that interacts with the central portion of the C*AAX* tetrapeptide. In our original designs (20–22), we have replaced the central aliphatic dipeptide "VI" in CVIM by aromatic spacers of the aminobenzoic acid family (Fig. 1*A*). Structural comparison of CVIM with the peptidomimetic FTI-249 (Fig. 1*A*) suggests that increased binding energy could be gained by increasing the size and hydrophobicity of the aminobenzoic acid spacer to fully occupy the FTase substrate binding pocket. In the present work, we have designed FTI-276 and its methyl ester FTI-277 (Fig. 1*A*), where reduced cysteine and methionine are linked by 2-phenyl-4-aminobenzoic acid, hence increasing the hydrophobic character of the central portion of the peptidomimetic. FTI-276 and FTI-277 were synthesized as described under "Experimental Procedures." Fig. 1*B* shows that FTI-276 inhibited FTase with an IC_{50} of 500 pM, whereas FTI-249, the unsubstituted precursor to FTI-276, had an IC_{50} of 200,000 pM (27). Thus, a phenyl ring at the 2 position of the aminobenzoic acid spacer increased inhibition potency of FTase by 400-fold, indi-

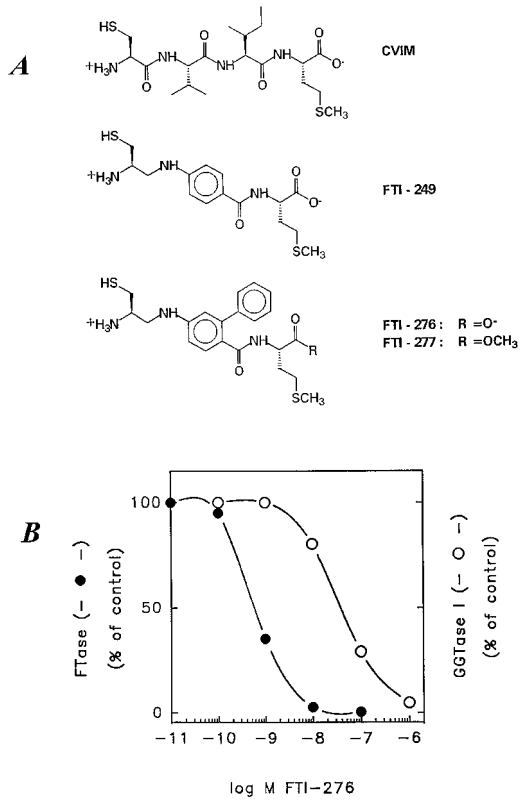

FIG. 1. **Ras C*AAX* peptidomimetics and FTase/GGTase I inhibition.** *A*, structures of CVIM, FTI-249, FTI-276, and FTI-277. *B*, FTase and GGTase I inhibition assays were carried out by determining the ability of FTI-276 to inhibit the transfer of farnesyl and geranylgeranyl to recombinant H-Ras-CVLS and H-Ras-CVLL, respectively. The data are representative of at least three independent experiments.

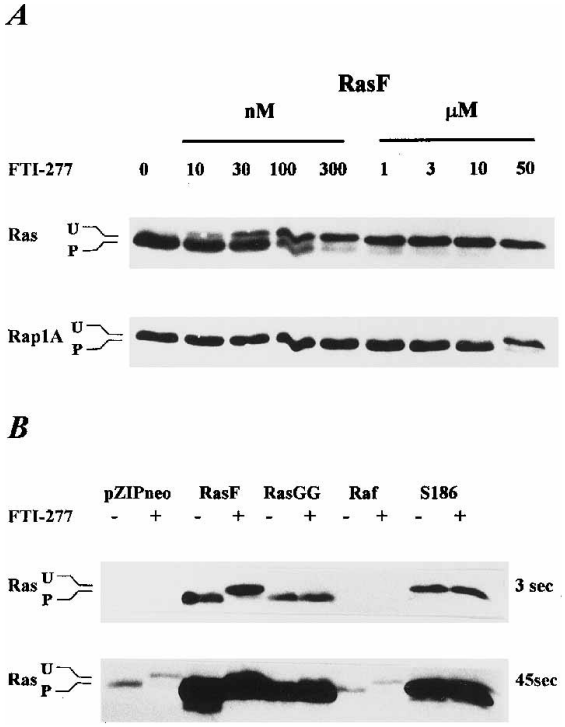

FIG. 2. **Effects of FTI-277 on Ras and Rap1A processing.** *A*, H-RasF cells were treated with various concentrations of FTI-277, lysed, and the lysates were immunoblotted with anti-Ras or anti-Rap1A antibodies as described under "Experimental Procedures." *B*, pZIPneo, H-RasF, H-RasGG, Raf, and S186 cells were treated with vehicle or FTI-277 (10 μM), lysed, and lysates were immunoblotted by anti-Ras antibody. Data are representative of five independent experiments.

cating a significant role for the hydrophobic pocket within the C*AAX* binding site of FTase. This extremely potent inhibitor was also highly selective (100-fold) for FTase over the closely related GGTase I. FTI-276 inhibited GGTase I with an IC_{50} of 50 nM (Fig. 1*B*). This 100-fold selectivity is superior to our previously reported 15-fold selectivity of the parent compound FTI-249 (27). We next determined the ability of FTI-276 to inhibit Ras processing. To facilitate cellular uptake, we have used the corresponding methyl ester, FTI-277 (Fig. 1*A*). H-RasF cells (NIH 3T3 cells transformed with oncogenic (leucine 61) H-Ras-CVLS (31)) were treated with FTI-277 (0–50 μM), and the lysates were blotted with anti-Ras or anti-Rap1A antibodies as described under "Experimental Procedures." Fig. 2*A* shows that concentrations as low as 10 nM inhibited Ras processing but concentrations as high as 10 μM did not inhibit processing of the geranylgeranylated Rap1A (Fig. 2*A*). FTI-277 inhibited Ras processing with an IC_{50} of 100 nM (Fig. 2*A*), whereas the IC_{50} of FTI-249 was 100 μM. Furthermore, the most potent C*AAX* peptidomimetics previously reported inhibited Ras processing in whole cells at micromolar concentrations (28–30). The selectivity of FTI-277 for farnesylation over geranylgeranylation processing in whole cells was further investigated by treating H-RasGG cells (NIH 3T3 cells transformed with oncogenic (leucine 61) H-Ras-CVLL (31)) with FTI-277. Fig. 2*B* shows that the processing of H-RasGG was not affected, whereas that of H-RasF was completely blocked. Furthermore, the processing of endogenous Ras was also blocked in pZIPneo cells (NIH 3T3 cells transfected with empty vector) and Raf cells (NIH 3T3 cells transfected with a transforming mutant of human Raf-1 (Raf22W) (33)). Thus, FTI-277 is a farnesylation-specific inhibitor which blocks the processing of both oncogenic and normal Ras.

In order to determine the mechanism by which FTI-277 disrupts Ras oncogenic signaling, we transfected NIH 3T3 cells with activated (GTP-locked) Ras and first investigated the effects of FTI-277 on the interaction of Ras with its immediate effector c-Raf-1 (1–3, 32). Various NIH 3T3 cell transfectants (pZIPneo, H-RasF, H-RasGG) were treated with vehicle or FTI-277, membrane and cytosolic fractions were isolated and immunoprecipitated with anti-Raf antibody, and the resulting immunoprecipitates were blotted with anti-Ras antibody as described under "Experimental Procedures." Fig. 3 shows that Raf did not associate with Ras in pZIPneo cells which do not contain GTP-locked Ras. In contrast, H-RasF and H-RasGG cells contain Ras-Raf complexes in the membrane but not in the cytosolic fractions of untreated cells (Fig. 3). Treatment with FTI-277 resulted in the accumulation of Ras-Raf complexes in the cytoplasmic but not membrane fractions of H-RasF cells (Fig. 3). The lack of Ras-Raf interaction at the cell membrane and accumulation of these complexes in the cytoplasm occurred only in Ras-F but not Ras-GG cells in agreement with the Ras processing selectivity results of Fig. 2. Thus, our results demonstrate that inhibition with FTI-277 results in the accumulation of non-farnesylated cytosolic Ras that is capable of binding to Raf. The fact that non-processed Ras can associate with Raf in a non-membranous, cytoplasmic environment was confirmed by transfecting NIH 3T3 cells with a GTP-locked Ras that lacks a farnesylation site (Ras mutant with a leucine 61 oncogenic mutation and a serine 186 mutation (34)) and, therefore, remains in the cytoplasm. These cells were shown to contain only cytoplasmic Ras-Raf complexes when immunoprecipitated with Raf and blotted with anti-Ras antibodies (Fig. 3, *S186*). Thus, farnesylation is not required for Ras to bind to Raf. Furthermore, the fact that non-farnesylated Ras binds Raf in the cytoplasm gives support to an earlier suggestion that unprocessed GTP-locked Ras is a dominant negative form of Ras (35).

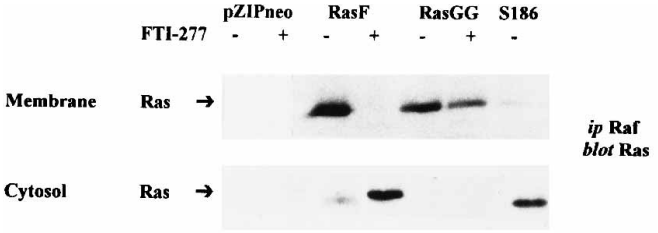

FIG. 3. **Effects of FTI-277 on Ras/Raf association.** pZIPneo, H-RasF, H-RasGG, and S186 cells were treated with vehicle or FTI-277 (10 μM), homogenized, and the membrane and cytosolic fractions were separated and immunoprecipitated by an anti-Raf antibody as described under "Experimental Procedures." The immunoprecipitates were then resolved by SDS-PAGE and immunoblotted with anti-Ras antibody. Data are representative of three independent experiments.

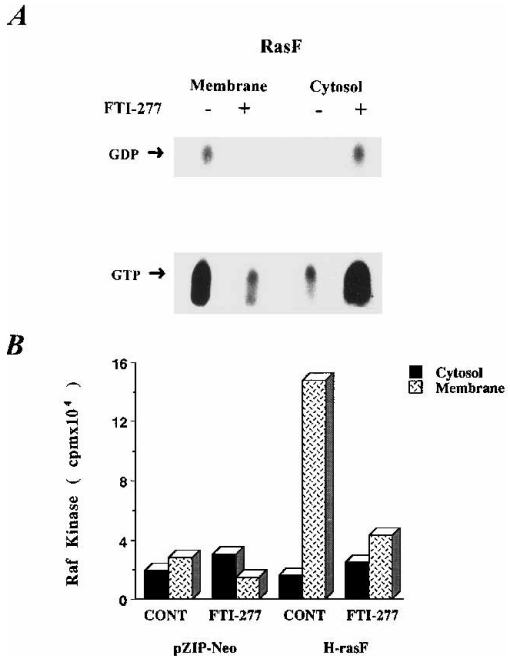

FIG. 4. **Effects of FTI-277 on Ras nucleotide binding and Raf kinase activity.** *A*, H-RasF cells were treated with vehicle or FTI-277, lysed, and the lysates were immunoprecipitated with anti-Ras antibody. GTP and GDP were then released from Ras and separated by TLC as described under "Experimental Procedures." *B*, pZIPneo and H-RasF cells were treated with vehicle or FTI-277, lysed, and cell lysates were immunoprecipitated with an anti-Raf antibody. Raf kinase was assayed as described under "Experimental Procedures." Data are representative of three independent experiments.

Since Raf binds Ras-GTP with much higher affinity than Ras-GDP (1–3), we determined the nucleotide state of Ras in the cytoplasmic Ras-Raf complexes as described under "Experimental Procedures." Fig. 4*A* shows that in H-RasF cells only membrane fractions contained GTP-locked Ras. Upon treatment with FTI-277, however, GTP-locked H-Ras was found primarily in the cytosol (Fig. 4*A*). Thus, the cytoplasmic form of H-Ras(61L) is still GTP-bound and can, therefore, still interact with Raf. We next determined the Ser/Thr kinase activity of Raf in Ras/Raf complexes by immunoprecipitating Raf and assaying for its ability to phosphorylate a 19-mer autophosphorylated peptide. Fig. 4*B* shows that oncogenic H-RasF induced activation of Raf at the plasma membrane and that treatment with FTI-277 suppressed this activation. More importantly, the cytoplasmic Ras/Raf complexes (Fig. 3) had basal levels of Raf kinase activity that were comparable with those of the parental NIH 3T3 cell line pZIPneo (Fig. 4*B*). Taken together, Figs. 3 and 4 demonstrate that oncogenic transformation with GTP-locked H-Ras results in the constitutive recruitment of Raf to

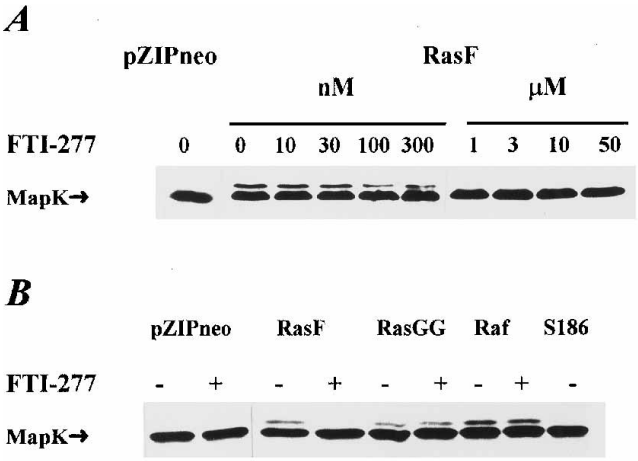

FIG. 5. **Effect of FTI-277 on oncogenic activation of MAPK.** *A*, H-RasF cells were treated with various concentrations of FTI-277, cells lysed, and lysates run on SDS-PAGE and immunoblotted with anti-MAPK antibody as described under "Experimental Procedures." *B*, pZIPneo, H-RasF, H-RasGG, Raf, and S186 cells were treated with vehicle or FTI-277 (10 μM), lysed, and cells lysates processed as for *A*. Data are representative of two independent experiments.

the plasma membrane and its subsequent activation. Furthermore, FTase inhibition by FTI-277 suppresses this activation by inducing the accumulation of Ras-Raf complexes in the cytoplasm where Ras is GTP-bound, but Raf kinase is not activated. The fact that Raf kinase is not activated when bound to Ras in a non-membranous environment is consistent with recent reports that indicate that Raf activation requires an as yet unidentified activating factor at the plasma membrane (36).

We then investigated the effects of FTI-277 on oncogenic Ras activation of MAPK, a Raf downstream signaling event (1–3). Oncogenic activation of MAPK can be easily detected, since the phosphorylated activated MAPK migrates slower in SDS-PAGE (29). Fig. 5*A* shows that NIH 3T3 cells transfected with pZIPneo contain only inactive MAPK but that upon transformation with oncogenic H-Ras, MAPK is activated (Fig. 5*A*). Pretreatment with FTI-277 results in a concentration-dependent inhibition of the constitutive activation of MAPK by oncogenic H-Ras. Concentrations as low as 300 nM were effective, and the inhibition was complete at 1 μM. Taken together, Figs. 2 and 5 demonstrate that at least 50% but less than 100% inhibition of H-Ras processing is required for inhibition of MAPK activation. To determine whether the inhibition of MAPK activation is due to selectively antagonizing H-Ras function we have used a series of NIH 3T3 cells lines transformed with various oncogenes. Fig. 5*B* shows that FTI-277 was able to block H-RasF but not H-RasGG activation of MAPK, and this is consistent with its ability to inhibit H-RasF but not H-RasGG processing. Selectivity of FTI-277 toward inhibition of Ras-dependent activation of MAPK was substantiated by using NIH 3T3 cells, where MAPK is constitutively activated by transformation with the Raf oncogene (33). Fig. 5*B* shows that oncogenic Raf activation of MAPK is not blocked by FTI-277, even though processing of endogenous Ras was inhibited in these cells (Fig. 2*B*). Taken together these results clearly demonstrate that FTI-277 is highly effective and selective in disrupting constitutive H-Ras-specific activation of MAPK.

Since K-Ras4B, the predominant form of Ras mutated in human tumors, is a much more efficient substrate (C*AAX* = CVIM) for FTase than is H-Ras (C*AAX* = CVLS) (13, 37), its processing has been difficult to disrupt. To determine whether or not FTI-277 inhibits K-Ras processing, we have treated K-Ras4B cells (NIH 3T3 cells transformed with oncogenic (valine 12) K-Ras4B-CVIM (17)) with FTI-277 (0–30 μM). Fig. 6

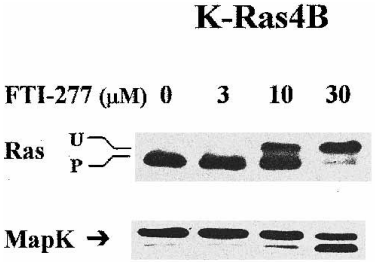

FIG. 6. **FTI-277 inhibits oncogenic K-Ras4B processing and activation of MAPK.** NIH 3T3 cells that overexpress oncogenic K-Ras4B were treated with FTI-277 (0, 3, 10, and 30 μM), and the cell lysates were immunoblotted with anti-Ras or anti-MAPK antibody as described under "Experimental Procedures." Data are representative of three and two independent experiments, respectively.

shows that FTI-277 inhibited K-Ras4B processing with an IC_{50} of 10 μM. Thus, inhibiting K-Ras4B processing (Fig. 6) requires 100-fold higher concentration than that needed for inhibition of H-Ras processing (Fig. 2*A*). This lower sensitivity to FTI-277 could be because K-Ras4B-CVIM is a much better substrate than H-Ras-CVLS (13, 37). An alternative explanation is that K-Ras4B-CVIM could be geranylgeranylated (37), especially when cellular FTase is inhibited. The fact that inhibition of K-Ras4B processing occurs only at concentrations that inhibit the processing of the geranylgeranylated Rap1A (Fig. 2*A*) is consistent with this latter possibility. We next determined whether the inhibition of K-Ras processing results in disruption of oncogenic K-Ras4B constitutive activation of MAPK. The same cell lysates that were blotted with anti-Ras antibody (Fig. 6) were reblotted with anti-MAPK antibody as described under "Experimental Procedures." Fig. 6 shows that NIH 3T3 cells that overexpress oncogenic K-Ras4B (17) contain mainly hyperphosphorylated (activated) MAPK. Treatment of these cells with FTI-277 (30 μM) inhibited oncogenic K-Ras4B constitutive activation of MAPK (Fig. 6). Furthermore, consistent with inhibition of Ras processing data (Fig. 6), higher concentrations were required to inhibit MAPK activation by K-Ras4B as compared with H-Ras. Nevertheless, the data clearly demonstrate for the first time that an FTase inhibitor disrupts both H- and K-Ras processing and oncogenic signaling.

Thus, we have designed an extremely potent and highly selective FTase inhibitor. FTI-277 inhibited H-Ras processing with concentrations as low as 10 nM, and processing was blocked by more than 95% at 3 μM. The most potent inhibitors previously reported blocked H-Ras processing completely only at 100 μM (28–30). The tremendous increase of potency in intact cells is due to increased hydrophobicity of the central portion of the peptidomimetic. FTI-277 inhibition of FTase resulted in the accumulation of non-farnesylated, GTP-locked H-Ras in the cytoplasm, where it was capable of binding Raf. This sequestration of Raf in the cytoplasm prevented its recruitment to the plasma membrane and subsequent activation. Thus, non-farnesylated cytoplasmic H-Ras could act as a dominant inhibitor by sequestering its downstream effector. Furthermore, FTI-277 was very selective in antagonizing H-Ras-specific signaling. The fact that FTI-277 suppressed only H-RasF but not H-RasGG or Raf oncogenic signaling demonstrates that the suppression is due to inhibition of H-Ras function and not the function of other farnesylated proteins that may be required for H-Ras transformation. Finally, we demonstrated for the first time that an FTase inhibitor can inhibit K-Ras processing and signaling but at much higher doses than required for H-Ras. Furthermore, we have recently demonstrated that FTI-276 and FTI-277 inhibit tumor growth in nude mice of a human lung carcinoma that has a K-Ras mutation and a p53 deletion (38). Since the great majority of human tumors with Ras mutations are of the K-type rather than the H-type, this finding is critical to further development of these agents as anti-cancer drugs.

Note Added in Proof—We have recently demonstrated that oncogenic K-Ras4B processing and signaling are inhibited potently with a GGTase I-specific inhibitor (39).

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