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High Affinity for Farnesyltransferase and Alternative Prenylation Contribute Individually to K-Ras4B Resistance to Farnesyltransferase Inhibitors*

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Farnesyltransferase inhibitors (FTIs) block Ras farnesylation, subcellular localization and activity, and inhibit the growth of Ras-transformed cells. Although FTIs are ineffective against K-Ras4B, the Ras isoform most commonly mutated in human cancers, they can inhibit the growth of tumors containing oncogenic K-Ras4B, implicating other farnesylated proteins or suggesting distinct functions for farnesylated and for geranylgeranylated K-Ras, which is generated when farnesyltransferase is inhibited. In addition to bypassing FTI blockade through geranylgeranylation, K-Ras4B resistance to FTIs may also result from its higher affinity for farnesyltransferase. Using chimeric Ras proteins containing all combinations of Ras background, C*AAX* motif, and K-Ras polybasic domain, we show that either a polybasic domain or an alternatively prenylated C*AAX* renders Ras prenylation, Ras-induced Elk-1 activation, and anchorage-independent cell growth FTI-resistant. The polybasic domain alone increases the affinity of Ras for farnesyltransferase, implying independent roles for each K-Ras4B sequence element in FTI resistance. Using microarray analysis and colony formation assays, we confirm that K-Ras function is independent of the identity of the prenyl group and, therefore, that FTI inhibition of K-Ras transformed cells is likely to be independent of K-Ras inhibition. Our results imply that relevant FTI targets will lack both polybasic and potentially geranylgeranylated methionine-C*AAX* motifs.

The four human Ras proteins (H-Ras, N-Ras, K-Ras4A, and K-Ras4B) are small GTP-binding proteins that are found at the inner leaflet of the plasma membrane, have intrinsic GTPase activity, and play a central role in signaling such diverse cellular responses as proliferation, differentiation, motility, and apoptosis. Ras mutations that reduce or eliminate its inherent GTPase function (typically at position 12, 13, or 61) lead to constitutive GTP binding and activation and are associated with ~30% of human tumors (1). Not surprisingly, inhibition of Ras function has become the focus of numerous anti-cancer drug development efforts. One such effort has been the development of pharmacological inhibitors of farnesyltransferase (FTase),[1] the enzyme that catalyzes the obligate first step in a series of post-translational modifications of Ras that are collectively required for Ras to localize to the plasma membrane, where it is biologically active (2). The COOH-terminal four amino acids, collectively called the "C*AAX*" motif, are sufficient to initiate the modification of Ras by FTase. The C*AAX* motif includes a conserved cysteine (C), two aliphatic residues (*AA*) and any other amino acid (*X*). FTase catalyzes the covalent addition of a 15-carbon farnesyl isoprenoid to the cysteine residue of the C*AAX* motif. This isoprenylation step is followed by proteolytic removal of the -*AAX* sequence and carboxymethylation of the resulting carboxyl terminus. Finally, in the case of H-Ras, N-Ras, and K-Ras4A, one (N-Ras) or two (H-Ras and K-Ras4A) cysteine residues just upstream from the C*AAX* motif become palmitoylated. K-Ras4B is not palmitoylated and instead contains a stretch of six consecutive lysine residues (the polybasic domain, "K_6") in place of the palmitoylatable cysteines and surrounding residues. Similar post-translational modifications occur on a relatively small set of other farnesylated cellular proteins, not all of which have been identified or characterized.

FTase inhibitors (FTIs) have now been studied extensively (3, 4). In preclinical studies (5, 6), FTIs inhibited farnesylation of H-Ras *in vitro* and *in vivo*, blocked H-Ras signaling and transformation, inhibited cell cycle progression in human tumor cell lines, and induced regression of mammary and salivary tumors in H-Ras transgenic mice and lymphoid tumors in N-Ras transgenic mice. Moreover, FTIs preferentially induce apoptosis and increase radiosensitivity in transformed cells and show additive or synergistic effects with standard cancer chemotherapies, especially taxanes. Although Ras is required for normal cell cycle progression, FTIs selectively block transformed cell growth compared with normal cells and interfere with signaling from oncogenic Ras but not from growth factors that require endogenous Ras function. This selectivity, although surprising, allayed initial fears that the clinical utility of FTIs might be limited by grossly toxic effects on nontransformed cells. Several FTIs are currently in clinical trials as cancer treatments.

Although they were originally designed to be anti-Ras drugs, and although they effectively inhibit H-Ras signaling and transformation, FTIs do not block either the processing or the

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[1] The abbreviations used are: FTase, farnesyltransferase; FTI, farnesyltransferase inhibitor; GGTase I, geranylgeranyltransferase I; HA, hemagglutinin.

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function of K-Ras4B (7), the isoform of Ras most frequently mutated in human tumors. Instead, in the presence of FTIs, K-Ras can become prenylated by the FTase-related enzyme geranylgeranyltransferase I (GGTase I) (8, 9) by virtue of a C*AAX* motif in which *X* is methionine (an M-C*AAX*). Nevertheless, FTI treatment can significantly inhibit the growth of tumors containing mutated K-Ras4B (10–12). This indicates either that farnesylated proteins other than Ras must also play a role in the biological consequences of FTI treatment (13) or that farnesylated and geranylgeranylated K-Ras function differently. Other farnesylated proteins that may be important FTI targets include the Ras-related protein RhoB (14, 15), the centromere-binding proteins CenP-E and CenP-F (16, 17), the phosphatases PRL1, -2, and -3 (18), and nuclear lamins (19), but no protein yet identified can explain all of the actions of FTIs.

As it becomes increasingly clear that farnesylated proteins other than Ras are important FTI targets, it also becomes important to define clearly the mechanisms by which K-Ras FTI resistance is conferred and to identify unambiguously the K-Ras sequence elements that are involved. K-Ras resistance to FTI is the result of two distinct mechanisms. The first is alternative prenylation by GGTase which allows K-Ras to bypass the FTI block. The second results from K-Ras having a higher affinity for FTase than does H-Ras or N-Ras (20, 21), making it more difficult for peptidomimetic FTIs to compete with K-Ras for FTase binding. That only K-Ras has both increased FTase affinity and a polybasic domain suggests that the polybasic domain, but not the M-C*AAX* common to N- and K-Ras, contributes significantly to the Ras/FTase interaction. However, James *et al.* (20) observed that neither an M-C*AAX* nor a polybasic domain was able by itself to increase Ras FTase affinity or the resistance of H-Ras to a benzodiazepine FTI.

In order to resolve this discrepancy, further define the relative contributions of each Ras sequence element to Ras/FTase association and K-Ras FTI resistance, and develop more stringent criteria for identifying other farnesylated protein targets of FTI, we generated a comprehensive set of activated Ras mutants, consisting of all combinations of Ras background (H-, N-, and K-Ras4B), C*AAX* motif (-CVLS, -CVVM, and -CVIM), and polybasic domain (with or without). We then assessed their sensitivity to FTI with respect to alterations in Ras prenylation status, Ras-induced Elk-1 activation, and Ras-induced anchorage-independent cell growth. We show that the polybasic domain and the C*AAX* motif contribute individually to the resistance of K-Ras4B to the FTI L-744,832. We propose, in contrast to previous reports, that the polybasic domain alone confers partial FTI resistance by increasing the affinity of K-Ras4B for FTase, thereby rendering FTIs less effective as competitive inhibitors, whereas the M-C*AAX* motif acts primarily by promoting alternative prenylation in the presence of FTI. Finally, we also provide confirmation of the notion that FTI inhibition of the Ras-mediated transformed phenotype results in part from functional inhibition of farnesylated proteins other than Ras. These data have implications for understanding the mechanism of FTI inhibition of tumor growth.

EXPERIMENTAL PROCEDURES

Generation of Ras Mutants—Ras constructs were generated by PCR using primers (University of North Carolina Lineberger Comprehensive Cancer Center Nucleic Acids Core Facility) coding for the desired mutant COOH-terminal sequence (Fig. 1). Parental oncogenic H-, N-, and K-Ras4B coding sequences (H-Ras (61L), N-Ras (12D), and K-Ras (12V)) were used as PCR templates in order to generate constitutively active Ras proteins as described (22). Primers were also designed to generate restriction sites (*Sal*I at the 5′-end of the coding sequence, *Bam*HI at the 3′-end) for directional insertion into the mammalian expression vector, pDCR, in frame with the coding sequence for the hemagglutinin (HA) tag. After amplification, PCR products were purified and digested with *Sal*I and *Bam*HI, extracted with phenol/chloroform, and ethanol-precipitated. pDCR was treated similarly. Insert was ligated into vector and transformed into competent *Escherichia coli* strain DH5α. Plasmid sequences were confirmed prior to use by automated sequencing (University of North Carolina Automated DNA Sequencing Facility). Protein expression was driven by the cytomegalovirus promoter of pDCR and confirmed by Western analysis using anti-HA antibody MonoHA (BAbCo, Richmond, CA). Some mutants were also inserted into the mammalian expression vector pZIPneoSV(X)1 (22).

Cell Culture and Generation of Cell Lines Stably Expressing Ras Mutant Constructs—NIH 3T3 fibroblasts were maintained in a humidified atmosphere at 37 °C in DMEM-H (Invitrogen) with 10% Colorado Calf Serum (Colorado Serum Company, Denver, CO) and antibiotics. Cells (plated at 5×10^5/60-mm dish on the day before transfection) were transfected with 50–100 ng of pDCR-Ras by calcium phosphate precipitation as described previously (23). Briefly, plasmid DNA was precipitated with high molecular weight carrier DNA (calf thymus DNA, Roche Applied Science) in 125 mM calcium phosphate and layered onto cells for 3–5 h. Cells were washed, shocked in 15% glycerol for 3 min, and returned to complete medium without selection for 48 h. Cells were then passaged into selective antibiotic (750 μg of active G418 per ml) and fed regularly with fresh G418. Colonies appeared after 2 weeks, and 40–70 individual colonies were pooled for use. Protein expression was confirmed by SDS-PAGE and Western analysis on cell lysates using antibodies directed to the HA epitope tag (BAbCo) or to Ras (pan-Ras antibody (Ras11), catalog no. OP-40; Calbiochem).

Gel Shift Assays—The sensitivity of mutant Ras proteins to FTI-induced inhibition of prenylation was evaluated by gel shift assay as described (24), based on the observation that prenylated Ras proteins migrate in SDS-PAGE more rapidly than their unprenylated counterparts. NIH 3T3 fibroblasts (1×10^5 cells/35-mm dish) were transiently transfected with H-, N-, or K-Ras4B mutant constructs as described above. Immediately after glycerol shock, cells were returned to complete medium for 48 h in the presence of a 3 or 10 μM concentration of the FTI L-744,832 (Biomol; a kind gift from Jay Gibbs and Allen Oliff (Merck)) or FTI-277 (Calbiochem; a kind gift from Saïd Sebti (University of South Florida) and Andy Hamilton (Yale)) or of vehicle (Me_2SO or Me_2SO plus 10 μM dithiothreitol, respectively). These two FTIs are structurally distinct C*AAX* peptidomimetics that are competitive with the Ras protein. Both were used throughout the study with equivalent results. We have also performed some of these studies with a variety of other C*AAX*-competitive FTI structures (including SCH 66336 (25) a kind gift from Robert Bishop and Paul Kirschmeier, Schering-Plough), again with similar results. Cells were washed with PBS and lysed in 500 μl of 1× SDS-PAGE sample buffer per dish. Thirty microliters of each lysate were separated by SDS-PAGE, transferred to Immobilon polyvinylidene difluoride membranes (Fisher) and subjected to Western analysis using anti-HA antibodies to identify only the exogenously expressed mutant Ras proteins.

Luciferase Assays—The FTI sensitivity of mutant Ras signaling through the transcription factor Elk-1 was assessed by luciferase reporter assay. NIH 3T3 cells (2×10^5 cells/35-mm dish) were transiently transfected by calcium phosphate precipitation in duplicate with 100 ng of the appropriate Ras mutant construct, 250 ng of a Gal-Elk-1 reporter plasmid, and 2.5 μg of 5×Gal-Luciferase plasmid as described (26). Immediately after glycerol shock, cells were returned to complete medium containing various concentrations of FTI. After 48 h, cells were washed twice with PBS, pH 7.2, lysed, and assayed for luciferase activity using reagents from the enhanced luciferase assay kit (PharMingen, San Diego, CA) according to the manufacturer's protocols. Raw data from duplicate samples were averaged and expressed as percentage of luciferase activity compared with vehicle-treated cells. All assays were performed at least three times, and data are displayed ± S.D.

Soft Agar Assays—The ability of cells to grow in the absence of attachment to substrate is one of the hallmarks of a transformed phenotype and can be induced by oncogenically mutated H-, N-, or K-Ras. To determine the sensitivity of anchorage-independent growth to FTI treatment, NIH 3T3 fibroblasts stably transfected with constructs encoding H-, N-, or K-Ras4B mutants were removed from dishes by trypsinization and triturated to ensure a single-cell suspension. After counting, cells were plated in soft agar as described previously (23) in the presence or absence of 3 or 10 μM FTI or vehicle. Briefly, 1×10^4 cells/35-mm dish were plated in 0.4% agar over a bottom layer of 0.6% agar. Both layers contained 1× DMEM-H, 10% serum, antibiotics, and FTI at the indicated concentrations. Plates were incubated at 37 °C in 10% CO_2 for up to 2 weeks. Cell groups containing more than four cells were

Construct	Sequence (175–189; CAAX Motif at 186–189)	Domains
H-Ras	-D E S G P G C M S C K C V L S	H, C_2, CVLS
N-Ras	-D D G T Q G C M G L P C V V M	N, C, CVVM
K-Ras	-G K K K K K K S K T K C V I M (Polybasic domain: K K K K K K)	K, K_6, CVIM
H-CVVM	-D E S G P G C M S C K C V V M	H, C_2, CVVM
H-CVIM	-D E S G P G C M S C K C V I M	H, C_2, CVIM
H-(K_6)	-D K K K K K K M S S K C V L S	H, K_6, CVLS
H-(K_6)-CVVM	-D K K K K K K S K T K C V V M	H, K_6, CVVM
H-(K_6)-CVIM	-D K K K K K K M S S K C V I M	H, K_6, CVIM
N-CVLS	-D D G T Q G C M G L P C V L S	N, C, CVLS
N-CVIM	-D D G T Q G C M G L P C V I M	N, C, CVIM
N-(K_6)	-D K K K K K K S K T K C V V M	N, K_6, CVVM
N-(K_6)-CVLS	-D K K K K K K M G L P C V L S	N, K_6, CVLS
N-(K_6)-CVIM	-D K K K K K K S K T K C V I M	N, K_6, CVIM
K-CVLS	-G K K K K K K S K T K C V L S	K, K_6, CVLS
K-CVVM	-G K K K K K K S K T K C V V M	K, K_6, CVVM
K-(Q_5)	-G Q Q Q Q Q C M S C K C V I M	K, Q5, CVIM
K-(Q_5)-CVLS	-G Q Q Q Q Q C M S C K C V L S	K, Q5, CVLS
K-(Q_5)-CVVM	-G Q Q Q Q Q C M S C K C V V M	K, Q5, CVVM

FIG. 1. **Generation of mutant Ras sequences.** The COOH-terminal sequences of the parental Ras proteins and Ras mutants are shown. All constructs contain activating mutations and an NH_2-terminal HA tag but are otherwise identical to the corresponding wild-type Ras proteins. Mutations were designed by homologous exchange between corresponding sequences in the parental constructs and generated by PCR using mutagenic 3′ primers. The C*AAX* motif, polybasic domain, and palmitoylated cysteines are indicated by *boxes*. The *boxes* in H-(K_6) and H-(K_6)-CVIM indicate additional cysteine-to-serine mutations made to prevent palmitoylation of those constructs.

considered colonies. Colony formation in FTI-treated samples was expressed as percentage of colony formation in vehicle-treated samples.

Ras/FTase Affinity Assays—To determine the affinity of Ras mutant proteins for FTase, *in vitro* prenylation assays were performed. Bacterially expressed, unprenylated Ras mutant proteins (1 μM) were incubated with [^{3}H]farnesyl pyrophosphate (1 μM final concentration; specific activity of 8–10 Ci/mmol; American Radiolabeled Chemicals, St. Louis, MO) and recombinant FTase (50 ng) in a reaction containing 50 mM Tris, pH 7.7, 20 mM KCl, 10 mM $MgCl_2$, 2 mM dithiothreitol, and 5 μM $ZnCl_2$. Reaction volume was 50 μl. Reactions were allowed to proceed for 10 min at 30 °C and were stopped by the addition of 0.5 ml of 4% SDS. Total protein was precipitated by the addition of 0.5 ml of 30% trichloroacetic acid. After 20 min, samples were filtered through 25-mm glass fiber filters (Schleicher & Schuell), which bind the protein. Reaction tubes were washed with 2 × 2 ml of 4% SDS plus 6% trichloroacetic acid, and the washes were added to the filters. Bound protein was washed with a further 4 ml of 4% SDS/6% trichloroacetic acid and then 3 × 2 ml 6% trichloroacetic acid. After drying, radioactivity bound to the filters was quantitated in a scintillation counter. Negative controls were also performed without protein substrate or using FTase that was previously inactivated by incubation for 5 min at 90 °C. Each substrate protein was reacted under standard conditions with vehicle or a range of FTI concentrations (5–625 nM) to block the farnesylation reaction. The level of farnesylation in FTI-treated samples was expressed as a percentage of maximum incorporation of [^{3}H]farnesyl for each substrate, as determined by allowing the uninhibited reaction to go to completion.

Growth Inhibition Assays—NIH 3T3 cells were transfected as described above with 50 ng of plasmid containing wild-type H-, N-, or K-Ras4B mutated to be substrates for geranylgeranylation (H-Ras(CVLL), N-Ras(CVVL), or K-Ras4B(CVIL) or empty vector (pZIPneo). After 48 h, cells were passaged into G418 selection until colonies appeared. Plates were washed twice in PBS, and the colonies were fixed in methanol/acetic acid and stained with 0.4% crystal violet before photography.

RESULTS

Design and Construction of Mutant Ras Proteins—In order to evaluate the individual and combined contributions of either the methionine-containing C*AAX* (M-C*AAX*) motif characteristic of N-Ras and K-Ras4B or the polybasic domain (K_6) unique to K-Ras4B, we generated a comprehensive set of chimeric Ras mutants (Fig. 1). We then evaluated the FTI sensitivity of their prenylation status, ability to induce anchorage-independent growth, and signaling through the transcription factor Elk-1. In addition, the affinity of several mutants for FTase was assessed directly. Since all mutations were near the COOH terminus, constructs were produced by PCR using mutagenic COOH-terminal primers. The COOH-terminal region of the parental Ras sequences were aligned as shown in Fig. 1, and the mutants were generated by homologous substitution between corresponding regions of each sequence. Two of the constructs (H-(K_6) and H-(K_6)-CVIM) were further modified to remove a palmitoylatable cysteine residue at position 184. This was done because Ras proteins normally contain, in addition to the C*AAX* motif, either a palmitoylated cysteine(s) or a polybasic domain but not both. Without this substitution, these two constructs would contain three potential membrane-targeting sequences, making it difficult to determine unambiguously the relative contributions of the C*AAX* motif and the polybasic domain to FTI resistance.

The Polybasic Domain Confers FTI Resistance in the Absence of Alternative Prenylation—The observed FTI resistance of N- and K-Ras4B as compared with H-Ras has been attributed to two possible mechanisms that may function *in vivo* separately

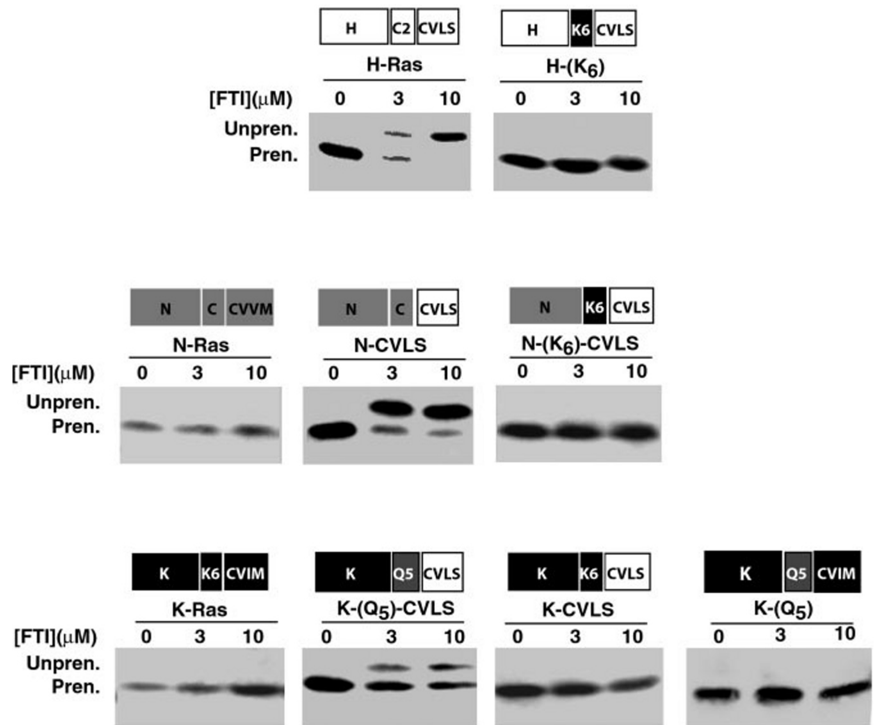

FIG. 2. **The polybasic domain confers FTI resistance in the absence of alternative prenylation.** FTI-induced inhibition of Ras prenylation was evaluated by a gel mobility shift assay. NIH 3T3 cells were transiently transfected with the indicated Ras constructs and treated with vehicle or FTI. Cell lysates were separated by SDS-PAGE and subjected to Western blot analysis using anti-HA antibody. Unprenylated Ras proteins have decreased mobility compared with their farnesylated counterparts. *Pren.*, prenylated Ras; *Unpren.*, unprenylated Ras. Representative data from at least two independent assays are shown.

or together. First, higher affinity between K-Ras4B and FTase may render FTIs less effective as competitive inhibitors against this isoform (20, 21). Second, the now well established potential for N- and K-Ras4B to become alternatively prenylated by GGTase upon FTI-induced FTase inactivation (8, 9) presumably allows these isoforms to bypass the FTI block. However, assays designed to assess Ras function *in vivo* (such as soft agar assays, focus formation assays, and transcriptional reporter assays) cannot by themselves distinguish between these two possibilities. Nor can the use of wild-type Ras proteins in such studies unambiguously identify the Ras sequence elements responsible for each mechanism. With the panel of Ras mutants we constructed, we were able to address these points.

We first assessed the ability of FTI to block Ras prenylation by showing a shift in gel mobility characteristic of unprenylated Ras proteins compared with the prenylated forms (Fig. 2). NIH 3T3 fibroblasts were transiently transfected with each HA-tagged Ras construct and treated for 48 h with various concentrations of FTI or vehicle. To ensure that effects were not due to a particular FTI structure, we used both L-744,832 and FTI-277 throughout most of these studies with equivalent results. Cell lysates were separated by SDS-PAGE and subjected to Western analysis using anti-HA antibody. At steady state, ~50% of parental H-Ras showed decreased gel mobility in response to 2.5 μM FTI, whereas 10 μM FTI produced a complete mobility shift after 48 h of treatment. These results indicate that, as expected, H-Ras became unprocessed in a dose-dependent manner in the presence of FTI. In contrast, an H-Ras construct containing a polybasic domain (H-(K_6)) showed no mobility shift at either 3 μM or 10 μM FTI. This increase in FTI resistance was unlikely to result from alternative prenylation, since proteins containing a CVLS C*AAX* sequence have been shown to be poor substrates for GGTase (8, 21) and since it was resistant to the geranylgeranyltransferase inhibitor GGTI-298 (data not shown). In one study (20), the introduction of a polybasic domain into H-Ras (H-(K_6)) was shown to permit limited geranylgeranylation *in vitro* using purified GGTase. But even with this Ras construct, farnesylation was the preferred reaction, indicating that in the presence of both enzymes, -CVLS-containing Ras constructs are preferentially farnesylated.

Similar results were obtained using N-Ras and K-Ras4B constructs. Neither parental N-Ras nor K-Ras4B undergoes an FTI-induced gel mobility shift even at 10 μM FTI, since alternative geranylgeranylation results in proteins that migrate with similar mobility as farnesylated Ras. Replacement of the native N-Ras M-C*AAX* (-CVVM) with the H-Ras C*AAX* motif (to generate N-CVLS) makes N-Ras susceptible to FTI-induced gel mobility shift (80% block at 3 μM FTI) (Fig. 2). Similarly, replacement of the native K-Ras M-C*AAX* (-CVIM) with -CVLS, along with replacement of the polybasic domain with a neutral stretch of glutamines (5Q), renders K-(Q_5)-CVLS susceptible to FTI-induced inhibition of prenylation (40% block at 3 μM FTI). The subsequent addition of the polybasic domain into these FTI-sensitive N-Ras or K-Ras constructs reduced sensitivity to FTI-induced gel mobility shift to undetectable levels. Similarly, introduction into FTI-sensitive K-(Q_5)-CVLS of a CVIM motif to generate K-(Q_5) also resulted, as expected, in FTI resistance, presumably through induction of alternative prenylation. The sensitivity of K-(Q_5)-CVLS to FTI and its insensitivity to GGTI (data not shown) confirmed that this construct was not normally geranylgeranylated. Together, these data demonstrate that the polybasic domain alone renders farnesylation of Ras proteins partially resistant to FTI even in the absence of alternative prenylation. Further, that parental H-, N-, and K-Ras proteins are not equally susceptible to FTI (50, 80, and 40% shifts in gel mobility, respectively) suggests that sequences outside of the C*AAX* motif and the polybasic domain are also involved in FTI resistance.

The Polybasic Domain Confers FTI Resistance on H-Ras-induced Elk-1 Activation—Unlike more complex phenotypes such as transformation, transcriptional transactivation of a Gal-Elk-1-luciferase reporter represents a simple system for studying Ras-driven signaling. Further, this pathway is important for the transformation and anchorage-independent growth of Ras-transformed NIH 3T3 fibroblasts. Therefore, we assessed the effect of the polybasic domain and M-C*AAX* motifs alone and in combination on FTI resistance in H-, N-, or

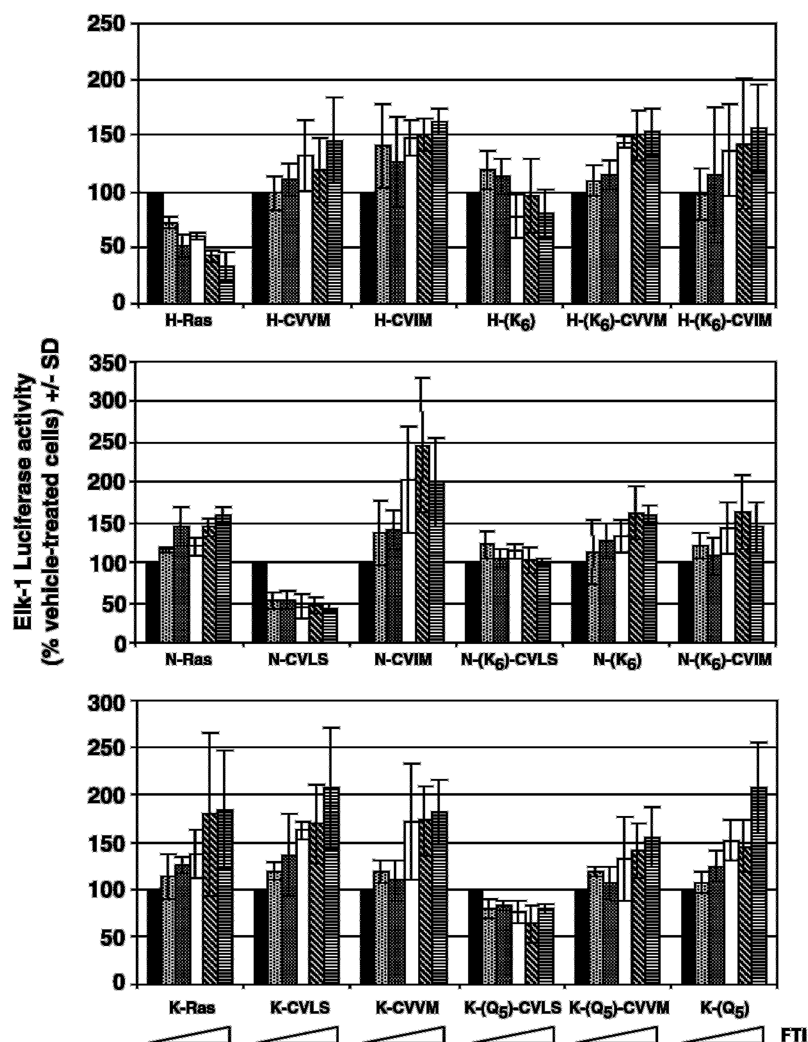

FIG. 3. **The polybasic domain confers FTI resistance on H-Ras-induced Elk-1 activation.** To determine the sensitivity of mutant Ras signaling through Elk-1, NIH 3T3 fibroblasts were transiently co-transfected with Ras mutant constructs and an Elk-1-driven luciferase plasmid and treated with various concentrations of FTI or vehicle. *Solid bars*, vehicle; *light gray bars*, 0.5 μM FTI; *dark gray bars*, 1 μM FTI; *open bars*, 2 μM FTI; *hatched bars*, 4 μM FTI; *horizontally striped bars*, 8 μM FTI. Luciferase activity is shown as percentage of activity compared with vehicle-treated cells. Samples were evaluated in duplicate in each assay. Data from at least three independent assays were averaged and are shown ± S.D.

K-Ras4B backgrounds with respect to signaling through the transcription factor Elk-1. Each of the Ras constructs was transiently transfected along with Elk-1-luciferase reporter plasmids into NIH 3T3 cells that were then treated for 48 h with various concentrations of FTI. Luciferase activity in cell lysates reflects the ability of the transfected Ras construct to activate Elk-1 and induce transcription from the Elk-1-dependent luciferase promoter. The data presented in Fig. 3 show that either the K-Ras4B polybasic domain or a methionine-containing C*AAX* motif (-CVVM or CVIM) alone can render Ras proteins resistant to FTIs regardless of Ras background. Oncogenic H-Ras with a wild-type COOH-terminal sequence is inhibited by FTI up to 70% in a dose-dependent manner. Replacement of the native H-Ras C*AAX* motif (-CVLS) with that of either N-Ras (-CVVM) or K-Ras4B (-CVIM) abrogates this effect and makes H-Ras resistant to FTI at concentrations up to 8 μM, presumably by permitting alternative prenylation. More importantly, in the absence of an M-C*AAX* motif, the presence of the polybasic domain also renders H-Ras resistant to FTI (H-(K_6)). This is consistent with our prenylation data (Fig. 2), which showed increased FTI resistance of H-Ras farnesylation when a polybasic domain is present.

The same conclusion can be drawn from the N-Ras mutants tested (Fig. 3*B*). Parental N-Ras, which contains an M-C*AAX*, is resistant to FTI, and this resistance is maintained even when the native N-Ras C*AAX* motif (-CVVM) is replaced by the K-Ras4B C*AAX* motif (-CVIM). When neither motif is present (N-CVLS), this resistance is eliminated, and N-Ras becomes susceptible to FTI (55% block at 8 μM FTI). However, introduction of the polybasic domain into the FTI-sensitive N-Ras restores FTI resistance (N-(K_6)-CVLS; 0% block at 8 μM). This observation is generally consistent with the reduced ability of FTI to prevent the prenylation of N-(K_6)-CVLS (Fig. 2). Together, these observations demonstrate that either motif alone can result in the FTI resistance of Ras-dependent Elk-1 activation.

Since parental K-Ras4B contains both motifs, we predicted that replacement of both would be necessary to render this protein FTI-sensitive. Thus, a K-Ras4B construct in which the polybasic domain was replaced with a series of neutral glutamine residues (Q_5) and containing the H-Ras C*AAX* motif (-CVLS) was generated. As predicted, this construct (K-(Q_5)-CVLS) was significantly sensitive to FTI (20% block at 8 μM FTI; Fig. 3*C*), and this sensitivity was reversed by reintroducing either an M-C*AAX* (K-(Q_5)-CVVM and K-(Q_5)) or a polybasic domain (K-CVLS). However, the FTI sensitivity of K-(Q_5)-CVLS was not nearly as pronounced as that of H-Ras or N-CVLS (60 and 55% at 8 μM FTI, respectively) although all three lack both an M-C*AAX* and polybasic domain. This suggests that K-Ras4B also has sequence elements outside of the COOH-terminal region (amino acids 176–188) that contribute to FTI resistance, probably by increasing FTase affinity. Since such elements would have to be found only in K-Ras4B, it is likely that they would be located NH_2-terminal of the polybasic domain but still within the region least conserved among Ras proteins (*i.e.* amino acids 165–175). The fact that N-CVLS was

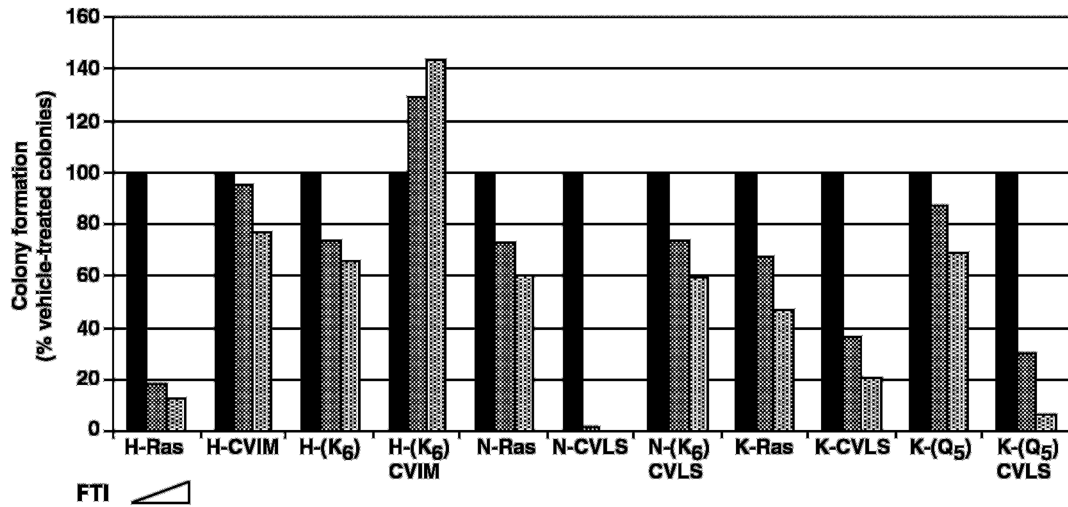

FIG. 4. **The polybasic domain confers FTI resistance on Ras-induced anchorage-independent growth.** NIH 3T3 fibroblasts stably transfected with active Ras mutant constructs were plated in 0.4% agar in the presence of FTI L-744,832 and maintained for 14 days. The percentage of cells forming colonies in soft agar in the presence of FTI is expressed in comparison with the percentage of vehicle-treated cells forming colonies. *Solid bars*, vehicle (Me_2SO); *dark gray bars*, 3 μM FTI; *light gray bars*, 10 μM FTI.

inhibited in Elk-1 signaling by FTI to a similar extent as was H-Ras despite an incomplete shift in gel mobility (Fig. 2) suggests that the unprocessed population of this construct might be acting as a dominant negative inhibitor for signaling through the remaining prenylated protein. This dominant negative effect would result from the sequestration of effector proteins such as Raf that bind unprenylated Ras, which itself remains cytosolic, and suggests that complete inactivation of FTase by FTIs may not be necessary for effective cancer treatment (27).

All alternatively geranylgeranylated M-C*AAX*-containing constructs evaluated showed a characteristic increase in Ras signaling through Elk-1 with increasing FTI concentration. A similar phenomenon has been reported previously with signaling to Elk-1 and c-Jun (28). In our assay, FTI-induced increases in Elk-1 activation ranges from 20 to 100% over untreated control cells and occurs even when nonnative M-C*AAX* motifs replace native versions. In contrast, two constructs that lack an M-C*AAX* but contain a polybasic domain (H-(K_6) and N-(K_6)-CVLS) show a flattened FTI response, indicating neither a decrease nor an increase in signaling in response to FTI. This suggests that geranylgeranylation of Ras proteins increases their signaling capabilities. Although it is not clear why this might be the case, it is possible that geranylgeranylation targets Ras to different membrane subdomains than does farnesylation, bringing it into closer proximity with effector proteins. The demonstration that the Ras prenyl group is involved in effector association and activation (29) supports the possibility that geranylgeranylated Ras more effectively activates effectors through enhanced association. An FTI-induced increase in Ras signaling via Elk-1 cannot be the result of inhibition of other farnesylated proteins that might normally inhibit Ras function or else all Ras constructs would be similarly affected, but H-Ras, N-(K_6)-CVLS, and K-(Q_5)-CVLS are not. Interestingly, this phenomenon also occurs with K-CVLS although this protein is not geranylgeranylated. When the polybasic domain is then removed (K-(Q_5)-CVLS), this increase is reversed (albeit to a lesser extent that that seen with H-Ras and N-CVLS), suggesting once again that the polybasic domain confers FTI resistance. That this resistance is greater in K-Ras4B than in H-Ras or N-Ras may result from the contribution of other sequence elements unique to K-Ras4B. However, this FTI-induced increase in Elk-1 reporter activation does not appear to correlate with transforming activity, which is independent of the specific isoprenoid modification of Ras (30).

The Polybasic Domain Confers FTI Resistance on Ras-induced Anchorage-independent Growth—Although we had demonstrated the independent roles of the polybasic domain and the C*AAX* motif in functional FTI resistance with respect to Ras prenylation and Ras signaling, we could not conclude that these data would reflect Ras function *in vivo* with respect to a more complex aspect of the transformed phenotype such as anchorage-independent growth. In order to address the functional significance of the polybasic domain and/or the C*AAX* motif in transformation, we stably transfected NIH 3T3 fibroblasts with several activated Ras mutants and assessed their ability to form colonies in soft agar in the presence or absence of FTI.

As shown in Fig. 4, NIH 3T3 cells expressing all of the activated Ras constructs were able to generate colonies in soft agar in the absence of FTI. In the three activated Ras constructs that lacked both the polybasic domain and an M-C*AAX* motif (H-Ras, N-CVLS, K-(Q_5)-CVLS), colony growth was inhibited by 10 μM FTI to approximately the same degree (87, 100, and 94%, respectively), suggesting that at least one of these motifs is necessary for functional Ras FTI resistance.

The H- and N-Ras constructs assayed consistently support the assertion that either a polybasic domain or an M-C*AAX* motif confers some functional FTI resistance on Ras proteins. Both H-CVIM and H-(K_6) (24 and 34% block at 10 μM FTI, respectively), each of which contains one of the motifs, show approximately the same increase in FTI resistance as compared with H-Ras (87% block). When both motifs are simultaneously placed into H-Ras, functional FTI sensitivity is eliminated, suggesting that the individual contributions of each motif to FTI resistance are additive. Similarly, whereas N-CVLS (which contains neither motif) is highly sensitive to FTI (100% block at 10 μM FTI), N-Ras constructs containing either motif (N-Ras or N-(K_6)-CVLS) are significantly less sensitive to FTI treatment (40 and 40% blocks at 10 μM FTI). Also, the addition of an M-C*AAX* motif to the FTI-sensitive K-(Q_5)-CVLS construct (94% block at 10 μM FTI) reduces the effect of FTI to approximately the same degree (K-Ras6KCVLS; 31% block at 10 μM FTI) as it does in the corresponding H- and N-Ras constructs (H-(K_6), 34% block at 10 μM FTI; N-(K_6)-CVLS, 40% block at 10 μM FTI). Together, these data indicate that either a polybasic domain or an M-C*AAX* motif can render Ras proteins less sensitive to FTI-induced functional inhibition but that

both may be necessary for maximum functional resistance.

However, the anchorage-independent growth data for K-Ras and K-CVLS are not consistent with this model. Although K-Ras contains both motifs, it shows significant FTI sensitivity (53% block at 10 μM FTI), which contrasts sharply with H-(K_6)-CVIM (0% block at 10 μM FTI), which also contains both motifs. And although K-CVLS (79% block at 10 μM FTI) predictably shows greater FTI sensitivity than K-Ras (53% block at 10 μM FTI), it unexpectedly shows considerably greater FTI sensitivity than the corresponding H-(K_6) (34% block at 10 μM FTI). These differences may result from functional differences between H-Ras and K-Ras4B that are unrelated to prenylation. Several lines of evidence have suggested that H-Ras and K-Ras4B are functionally distinct, possibly related to their different microlocalization (31–34). If this is the case, K-Ras4B-induced soft agar colony growth may depend more strongly on the function of other farnesylated proteins (35, 36) that are themselves sensitive to FTI, whereas H-Ras-induced colony growth may rely less on other farnesylated proteins. The observations that the Ras mutation status of cells does not correlate with FTI sensitivity and that other farnesylated, Ras-related proteins are necessary for Ras transformation (37) supports this hypothesis. Our data show that the signaling pathway(s) used by K-Ras4B to induce soft agar colony growth is sensitive to FTI although K-Ras4B itself is FTI-resistant. Further, we show that an FTI-resistant mutant of H-Ras (H-(K_6)-CVIM) can induce colony growth that is not inhibited by FTI, indicating that H-Ras and K-Ras4B may induce transformation by mechanisms that do not overlap completely.

The Polybasic Domain Increases Ras Affinity for FTase—The results of our prenylation assays, soft agar assays, and luciferase assays demonstrated the independent role of the polybasic domain in conferring on Ras proteins functional resistance to FTI. However, each of these assays was performed in transiently or stably transfected cells in which FTI resistance could result from increased Ras affinity for FTase and/or alternative prenylation. Therefore, it was not clear whether, in the absence of alternative prenylation, the M-C*AAX* motif would itself contribute to increased Ras affinity for FTase or whether this role was exclusive to the polybasic domain. Although James *et al.* (20) had shown that an M-C*AAX* (-CVIM) or a polybasic domain each increased affinity of H-Ras (H-CVIM) for FTase by 6-fold, these increases in affinity unexpectedly did not result in increased resistance to the benzodiazepine peptidomimetic FTI, BZA-2B.

To clarify this point, we assessed the affinity of several Ras mutants for FTase by *in vitro* prenylation assays. Bacterially expressed Ras mutants, which are unprenylated due to the absence of FTase in *E. coli*, were isolated and used as substrates in a reaction also containing [^{3}H]farnesyl pyrophosphate and purified FTase. The prenylation of each substrate protein was performed in the presence of a peptide-competitive FTI (L-744,832) at several concentrations in order to determine the FTI concentration at which 50% of the maximal prenylation reaction could be inhibited (IC_{50}). The results of these assays are summarized in Table I. Together, the polybasic domain (6K) and an M-C*AAX* (-CVIM) render either H-Ras (H-(K_6)-CVIM) or K-Ras resistant to FTI at all concentrations tested (IC_{50} > 625 nM), confirming that these motifs in combination contribute to Ras affinity for FTase and account for the FTI resistance of K-Ras4B. However, replacement of the polybasic domain with five consecutive glutamines (K-(Q_5); IC_{50} = 80 nM) while keeping the CVIM sequence intact reduces the Ras/FTase association to levels approximating that of H-Ras (IC_{50} = 10 nM). Based on the crystal structure of FTase complexed with a K-Ras4B C*AAX* peptide and a farnesyl diphosphate analogue (38), it seems likely that increased affinity between FTase and Ras results from the ionic interaction of the Ras polybasic domain and an acidic region on the surface of FTase near the active site. Residual physical association between K-(Q_5) and FTase probably results from hydrogen-bonding interactions between the polar glutamine residues and this negatively charged region of FTase. If the corresponding sequence of H-Ras is substituted for the glutamines, affinity is reduced further (K-(H); IC_{50} = 25 nM), showing that the polybasic domain alone accounts for most of the affinity of Ras for FTase. The subsequent removal of the CVIM from K-(H) to give K-(H)-CVLS (IC_{50} = 20 nM) has no additional effect on association with FTase, further confirming that the M-C*AAX* motif does not significantly contribute to the Ras/FTase interaction.

Table I

The polybasic domain increases Ras affinity for FTase

In vitro prenylation reactions containing bacterially expressed, unprenylated Ras proteins (1 μM), [^{3}H]farnesyl pyrophosphate (1 μM), and purified recombinant FTase (50ng) were performed in the presence of various FTI concentrations (5-625 μM) or vehicle (Me_2SO) for 10 min at 30 °C. Proteins were precipitated with trichloroacetic acid, immobilized on membranes, and washed free of unreacted radiolabel. Incorporation of [^{3}H]farnesyl into protein was determined by scintillation counting and normalized to maximal incorporation determined in the absence of FTI and allowing the reaction to go to completion. The concentration of substrate-competitive FTI necessary to block 50% of maximal farnesylation (IC_{50}) reflects Ras/FTase association.

Ras substrate	IC_{50}
	nM
H-Ras	10
H-(K_6)-CVIM	>625
K-Ras	>625
K-(Q_5)	80
K-(H)	25
K-(H)-CVLS	20

Alternative Prenylation of N- or K-Ras Is Functionally Tolerated—Finally, in light of evidence that the identity of the prenyl group may influence signaling and/or subcellular localization of Ras (29, 39, 40), we wanted to confirm the functional equivalence of farnesylated and geranylgeranylated Ras. If Ras-F and Ras-GG do not have identical functions in cellular signaling, then the well established effect of FTIs on K-Ras-transformed cells may result from this difference rather than (or in addition to) FTI inhibition of non-Ras farnesylated proteins. In order to rule out the former possibility, we first confirmed the ability of alternatively prenylated wild-type N- and K-Ras (N-Ras-GG and K-Ras-GG) to support the growth of NIH 3T3 cells (Fig. 5). Our observations that N- and K-Ras support cell growth when alternatively prenylated support the current belief that FTI-induced geranylgeranylation of N- and K-Ras has no discernible effect on their function, is tolerated by cells, and permits these Ras isoforms to protect normal cells from FTI toxicity. That wild-type H-Ras-GG fails to support cell growth contrasts with the ability of the oncogenic H-Ras-GG mutants that we tested to generate stable NIH 3T3 cell lines and induce anchorage-independent growth (Fig. 4). This sharp functional distinction in proliferation may reflect the observation that inactive and active H-Ras have been shown to partition into distinct plasma membrane microdomains with distinct functional consequences (39, 40).

To further confirm the functional equivalence of farnesylated and geranylgeranylated K-Ras, we compared the gene expression profiles of transformed human embryonic kidney cells (HEKs), stably expressing the oncogenic form of either K-Ras-F or K-Ras-GG, by microarray analysis on arrays containing 18,000 human genes and expressed sequence tags (University of North Carolina Lineberger Comprehensive Cancer Center,

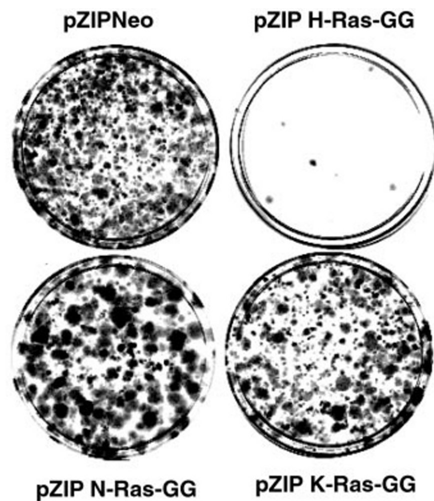

FIG. 5. **Alternative prenylation of N- and K-Ras is functionally tolerated.** NIH 3T3 cells were transfected with wild-type N-Ras, K-Ras, or H-Ras modified to be preferentially geranylgeranylated (188/189L) or with empty vector (pZIP). Transfected cells were selected in 400 μg/ml G418, and colonies were stained with crystal violet.

Genomics Core Facility). We observed no significant differences at the mRNA level between the set of genes expressed in either cell line (data not shown), suggesting that signaling by K-Ras is independent of the identity of the prenyl group. This is consistent with reports in which RhoA signaling was shown to be independent of the identity of the prenyl group (41, 42). We conclude that K-Ras-F and K-Ras-GG are functionally equivalent and that the effect of FTIs on K-Ras-transformed cells probably results from inhibition of other farnesylated proteins. Therefore, it is especially important to identify unambiguously, as we have helped to do here, the sequence elements in K-Ras4B that render it resistant to FTI, in order to define criteria by which other relevant FTIs targets may be identified.

DISCUSSION

Our studies demonstrate a previously unreported contribution of the polybasic domain characteristic of K-Ras4B to the functional resistance of Ras proteins to FTIs independent of M-C*AAX*-induced alternative prenylation. Either motif alone, when placed into H-Ras, prevents FTI-induced inhibition of prenylation, reduces the sensitivity of Ras-transformed NIH 3T3 fibroblasts to FTI-induced inhibition of anchorage-independent growth, and prevents FTI-induced block of Ras-dependent signaling through the transcription factor Elk-1. We have also shown that this effect is the result of enhanced physical association between FTase and Ras mediated by the polybasic domain. We propose a model in which a methionine-containing C*AAX* motif allows Ras to bypass FTI-induced functional inhibition by permitting alternative prenylation of Ras proteins by GGTase, and the polybasic domain renders FTIs less effective as competitive inhibitors by increasing Ras affinity for FTase.

These results may have important implications for the identification of relevant FTI targets that may include both Ras and non-Ras farnesylated proteins. There are ~250 human proteins with literal carboxyl-terminal C*AAX* motifs. However, most of these contain amino acids at the -AAX positions that make them poor substrates for FTase (43–45). Of those that are potential FTase substrates (Table II), many, including numerous members of the Ras superfamily, are known to be farnesylated, whereas others are presumed to be farnesylated but have not yet been specifically evaluated. At present, several C*AAX*-containing, farnesylated human proteins are currently under investigation as FTI targets (reviewed in Refs. 3, 5, 6, 13, and 46), including RhoB, the centromere-binding proteins CENP-E and CENP-F, the phosphatases PRL1, -2, and -3, the chaperone protein DnaJ, and Rheb. Our data demonstrating the importance of the polybasic domain in FTI resistance support the possibility that these proteins, which lack a polybasic domain, should be sensitive to FTIs and may account for the effects of FTIs on transformed cells *in vitro* and *in vivo*. Indeed, Rheb has been shown to be FTI-sensitive (47, 48) and appears not to be subject to alternative prenylation (48). PRL1 to -3 are possible exceptions, since each contains either two or three basic amino acids just upstream of the C*AAX* motif. However, it is not clear from our studies whether three basic amino acids can confer FTI resistance to the same degree as the polybasic domain of K-Ras4B that contains six consecutive basic amino acids.

Among those farnesylated proteins not yet under intensive study, we predict that the absence of a polybasic domain in RhoD, inositol-1,4,5-trisphosphate 5-phosphatase IV, and Rap2A would render these proteins FTI-sensitive. In contrast, the presence of basic amino acids in Rnd1, Rnd2, Rnd3/RhoE, TC10, and inositol-1,4,5-trisphosphate 5-phosphatase I might impart significant FTI resistance to these proteins.

Similar predictions can be made for C*AAX*-containing proteins whose prenylation status has not yet been explicitly determined but whose C*AAX* sequences and homology to other farnesylated proteins imply that they too will be farnesylated. For example, the putative tumor suppressor NOEY2/ARHI/RhoI, which lacks a polybasic motif, is more likely to be FTI-sensitive than AGS1, which contains three basic amino acids. In addition, a search of the SwissProt database shows that there are ~40 other human proteins unrelated to the Ras superfamily whose C*AAX* motifs could support farnesylation. Among these are cyclin G2 (CFPS), members of the transforming growth factor β (CKCS), inhibin (CGCS), and bone morphogenetic protein (CGCH) families, many of which lack a polybasic domain, making them potential FTI targets if they are farnesylated. Given the current confusion surrounding the identity of relevant FTI targets, it will be important to determine whether any of these proteins is farnesylated, affected by FTIs, and can account for the observed biological effects of FTIs. In any case, it is likely that FTIs will affect numerous farnesylated proteins simultaneously to produce complex, cell type-specific effects.

Complicating the identification of relevant FTI targets is the potential alternative geranylgeranylation of M-C*AAX* proteins in the presence of FTI. This phenomenon, well documented here and elsewhere for N-Ras (CVVM) and K-Ras (CVIM) (8, 9, 21), may also apply to other farnesylated proteins such as RhoE (CTVM) to render these proteins FTI-resistant regardless of the presence or absence of a polybasic motif. There is also evidence implicating the prenyl group in the association of Ras family proteins with both downstream effectors (29) and upstream guanine nucleotide exchange factors (49), suggesting that geranylgeranylated proteins may not always be functionally equivalent to their farnesylated counterparts and, therefore, that alternatively prenylated proteins including N-Ras and K-Ras may be relevant FTI targets. However, our observation that both farnesylated and geranylgeranylated K-Ras12V produce nearly identical gene expression profiles when stably expressed in human embryonic kidney cells supports the widely held belief in the functional equivalence of the farnesylated and geranylgeranylated forms of this protein and clearly implicates other farnesylated proteins in the inhibition of K-Ras-transformed cells by FTIs.

Many proteins that are normally geranylgeranylated, such as members of the Rho family, also contain polybasic domains.

TABLE II
Farnesylated proteins can be distinguished by the presence or absence of a polybasic domain

Human proteins containing a C*AAX* motif were compiled from the SwissProt database. Those with C*AAX* motifs containing amino acids that presumably preclude farnesylation and those in which *X* represents leucine (L, coding for geranylgeranylation) were eliminated. For each protein, the C*AAX* sequence and the sequence immediately upstream of the C*AAX* motif corresponding to the polybasic domain of K-Ras4B are shown. The accession numbers can be used to search the "protein" database at the Web site for the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). *, an accession number for the "nucleotide" database. ND, prenylation has not been explicitly reported. Proteins are grouped according to current knowledge concerning their potential for alternative prenylation and their relevancy as FTI targets and are listed alphabetically within each group.

Protein name	Accession no.	"Polybasic" region	C*AAX*	Alt Pren?
Farnesylated proteins under evaluation as potential FTI targets				
CENP-E	CENE_HUMAN	HASSGKDVPE	CKTQ	N
CENP-F	CENF_HUMAN	AGLESKGSEN	CKVQ	N
DnaJ	DJA1_HUMAN	EDDEHHPRGGVQ	CQTS	N
H-Ras	RASH_HUMAN	DESGPGCMSCK	CVLS	N
PRL-1 (PTPCAAX1)	NP_003454	KDSNGHRNN	CCIQ	N
PRL-2 (PTPCAAX2/OV-1)	NP_003470	RFRDTNGH	CCVQ	N
PRL-3	NP_116000	KDPHTHKTR	CCVM	?
Rheb	RHEB_HUMAN	DGAASQGKSS	CSVM	N
RhoB	RHOB_HUMAN	KRYGSQNGCINC	CKVL	Both
Confirmed or likely farnesylated proteins				
AGS1 (activator of G-protein signaling)	NP_057168	SQAKDKER	CVIS	?
G-protein, γ11 subunit	GBGB_HUMAN	DKNPFKEKGS	CVIS	N
G-protein, γ-T1 subunit (transducin)	GBG1_HUMAN	EDKNPFKELKGG	CVIS	N
G-protein, γ-T2 subunit	GBGU_HUMAN	DKNPFKEKGG	CLIS	N
G-protein-coupled receptor (putative)	GP41_HUMAN	QGCGTGGQVA	CAES	N
Inositol-1,4,5-trisphosphate 5-phosphatase I	I5P1_HUMAN	GKPHAHVHKC	CVVQ	N
Inositol-1,4,5-trisphosphate 5-phosphatase IV	*AF187891	QQALQSQNSSTI	CSVS	N
Lamin A/C	LAMA_HUMAN	SSPRTQSPQN	CSIM	?
Lamin B1	LAM1_HUMAN	QGTPRASNRS	CAIM	?
Lamin B2	LAM2_HUMAN	QGDPRTTSRG	CYVM	?
K-Ras4A	RASK_HUMAN	KTPCGVKIKK	CIIM	Y
K-Ras4B	RASL_HUMAN	KKKKKKSKTK	CVIM	Y
NOEY2/ARHI/RhoI	RHOI_HUMAN	MPNTTEKLLDK	CIIM	?
N-Ras	RASN_HUMAN	DGTQGCMGLP	CVVM	Y
Phosphorylase B kinase α subunit	KPB1_HUMAN	YVQEFLPHSI	CAMQ	?
Phosphorylase B kinase β subunit	KPBB_HUMAN	EGEVKPNNDDP	CLIS	?
Peroxisomal farnesylated protein (PXF/HK33)	PXF_HUMAN	SGPPGASGEQ	CLIM	?
Rap2A	RAP2_HUMAN	DKDDPCCCSA	CNIQ	N
RhoD	RHOD_HUMAN	WRRITQGF	CVVT	N
Rhodopsin kinase	RK_HUMAN	GSSSSSKSGM	CLVS	N
Rnd1 (Rho6)	RHO6_HUMAN	TFKKEKAKS	CSIM	?
Rnd2(RhoN/Rho7)	RHON_HUMAN	EIHKDRAKS	CNLM	?
Rnd3(RhoE/Rho8)	RHOE_HUMAN	TDLRKDKAKS	CTVM	?
TC10	NP_036381	KRIGSRCINC	CLIT	N
Unknown prenylation status				
Bone morphogenetic protein 5-8 precursors	BMP5(6,7,8)_HUMAN	K/RKY/HRNMVVRA	CGCH	ND
Bone morphogenetic protein 11 precursor	GDFB_HUMAN	YGKIPGMVVDR	CGCS	ND
Cyclin G2	CGG2_HUMAN	FFFNFKVAQTL	CFPS	ND
Inhibin β a-(c-, e-) chain precursor	IHBA(C,E)_HUMAN	K/TDI/VQ/PN/DMI/VVEE/A	CGCS	ND
MAPKAP kinase-2	AAD09136	NKRRKKQAQSSSASQG	CNNQ	ND
Neuropeptide Y receptor type 5	NY5R_HUMAN	IKADLVSLIH	CLHM	ND
Phosphorylase B kinase α-liver	KPB2_HUMAN	YLQELLPNSG	CQMQ	ND
Serine/threonine protein kinase 11	ST11_HUMAN	SSKIRRLSA	CKQQ	ND
TGFβ1 (β2, β3) precursor	TGF1(2, 3)_HUMAN	EQLSNMI/VVRS	CKCS	ND
WNT-3A	WN3A_HUMAN	NARAERREK	CRCV	ND

This observation is of particular interest in light of the fact that the negatively charged surface of FTase with which the polybasic domain interacts resides primarily within the α-subunit of the enzyme (38) that is common to both FTase and GGTase. Therefore, geranylgeranylated proteins that contain polybasic domains may be more resistant to Ras-competitive GGTIs than those that do not, suggesting that these proteins can also be usefully defined according to the presence or absence of a polybasic domain.

Previously, *in vitro* studies using purified FTase and Ras substrates showed that neither an M-C*AAX* nor a polybasic domain increase FTase resistance to the peptidomimetic FTI benzodiazepine (20). However, since FTIs such as L-744,832 compete with substrate for binding to enzyme, it seemed likely that if the polybasic domain increases Ras affinity for FTase, it alone should contribute to functional FTI resistance in Ras proteins. Our work confirms this possibility by showing that a polybasic domain alone is sufficient to render Ras partially functionally resistant to the FTI L-744,832, as demonstrated by the ability of polybasic domain-containing Ras mutants to produce colonies in soft agar and to signal through the transcription factor Elk-1 in the presence of FTI. Moreover, we have shown that this resistance is probably the result of increased affinity of Ras for FTase. This conclusion is consistent with previous reports (21, 50) that have shown that K-Ras4B has an affinity for FTase 10-fold greater than N-Ras and 20-fold greater than H-Ras, suggesting that the M-C*AAX* motif does not contribute greatly to Ras affinity for FTase, whereas the polybasic domain of K-Ras4B does.

Our observation that anchorage-independent growth of NIH 3T3 cells stably expressing K-Ras4B is partially sensitive to FTI although this Ras isoform contains both a methionine-containing C*AAX* motif and a polybasic domain, becomes alternatively prenylated, and signals efficiently through Elk-1 in the presence of FTI suggests that H-Ras and K-Ras4B are functionally distinct, a hypothesis for which considerable evi-

dence has accumulated (31–34, 51). In our studies, K-Ras and H-(K_6)-CVIM appear to induce anchorage-independent growth via incompletely overlapping mechanisms that are not equally affected by FTI. The fact that H-(K_6)-CVIM and K-Ras both activate Elk to the same degree in the presence of FTI while generating anchorage-independent colonies that are not equally sensitive to FTI further supports the notion that each Ras isoform utilizes additional, distinct signaling pathways in order to produce a transformed phenotype. These pathways may themselves differentially utilize other farnesylated proteins potentially adding still more complexity. However, since H- and K-Ras4B have identical effector binding domains but highly variant COOH termini, differences in preferred signaling pathways may also result from FTI-sensitive differences in the subcellular localization of each Ras isoform (32, 34, 40, 52). It is interesting to note that we have created an unnatural Ras protein (H-(K_6)-CVIM) that is more functionally resistant to FTI at inducing anchorage-independent growth than any naturally occurring Ras variant. It is increasingly likely that biological differences among the Ras isoforms reflect, in part, differences in subcellular localization and trafficking mediated by the divergent COOH termini of these proteins. If this is the case, studies comparing the signaling mechanisms of K-Ras4B and H-(K_6)-CVIM, which may be similarly modified and localized within the cell, will help distinguish inherent differences in utilization of signaling pathways from differences in subcellular localization.

Several physical and biological characteristics of K-Ras4B have been shown to be dependent on the polybasic domain. The polybasic domain has been shown to be required for 1) K-Ras4B to transform NIH 3T3 and Rat-1 cells (53), 2) K-Ras4B membrane localization (33), 3) formation of ionic interactions between the polybasic domain and membrane anionic lipids to stabilize membrane association (33, 54), and 4) K-Ras4B association with microtubules (55), an interaction that has implications for differential subcellular transport and localization of Ras isoforms. Our work adds a new function to this list by demonstrating the role of the polybasic domain in increasing Ras affinity for FTase, thereby resulting in enhanced functional Ras FTI resistance and suggesting that FTase may distinguish among Ras proteins in part through the polybasic domain.

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