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Resistance of K-RasB^{V12} proteins to farnesyltransferase inhibitors in Rat1 cells

(protein prenylation/geranylgeranyl transferase I/transformed cells/benzodiazepine-5B/L-739,749)

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ABSTRACT **Benzodiazepine (BZA)-5B, a CAAX farnesyltransferase inhibitor, was previously shown to block the farnesylation of H-Ras and to reverse the transformed morphology of Rat1 cells expressing oncogenic H-Ras^{V12}. Nontransformed Rat1 cells were not affected by BZA-5B, suggesting that they produce a form of Ras whose prenylation is not blocked by this compound. The likely candidate is K-RasB, which differs from H-Ras primarily in the terminal 24 amino acids. In the current study we examined the effect of BZA-5B on the prenylation of a chimeric oncogenic Ras protein designated H/K-RasB^{V12}, consisting of the first 164 amino acids of H-Ras^{V12} followed by the last 24 amino acids of K-RasB. BZA-5B failed to block the prenylation of this chimera and was thus unable to reverse the transformed morphology of Rat1 cells in which it was expressed. Another potent inhibitor of H-Ras farnesylation, L-739,749, also failed to block prenylation of H/K-RasB^{V12}. Similar results were obtained in transfected cells expressing a widely used version of K-RasB^{V12} containing a 10-amino acid extension at its NH$_2$ terminus. Neither BZA-5B nor L-739,749 reversed the transformed morphology of cells expressing H/K-RasB^{V12}. The resistance of K-RasB to farnesyltransferase inhibition provides a likely explanation for the resistance of nontransformed cells to the growth inhibitory effects of BZA-5B and L-739,749.**

Ras proteins have been the subject of intense research since 1982 when a mutant H-Ras allele was shown to transform cells to a malignant phenotype (1). Mutations have subsequently been identified in all three Ras genes (H-, K-, and N-Ras) in ≈30% of all human tumors, including 50% of colon cancers and >90% of pancreatic cancers (2). The vast majority of these mutations occur in the K-Ras gene.

The function of normal and oncogenic Ras proteins is dependent upon their localization to the inner face of the plasma membrane (3). This is accomplished by a series of posttranslational modifications, beginning with the farnesylation of an invariant cysteine residue located 4 amino acids from the COOH terminus. This cysteine lies within the consensus CAAX sequence, where C is the farnesylated cysteine, A is an aliphatic amino acid, and X is usually methionine or serine. After farnesylation, the three terminal amino acids are removed by a protease, and the newly exposed cysteine COOH group is methylated. These modifications render the COOH terminus hydrophobic, thus promoting the interaction of Ras proteins with the plasma membrane (3). Oncogenic Ras proteins with mutations in the CAAX consensus that prevent farnesylation fail to associate with the plasma membrane and lack transforming potential (4).

The three mammalian Ras genes produce four proteins of 188 (K-RasB) or 189 (H-Ras, K-RasA, and N-Ras) amino acids in length. The A and B forms of K-Ras arise from alternative splicing of the fourth exon, which encodes the CAAX box (1). All Ras proteins are closely related in structure, including 100% identity over the first 86 amino acids. The proteins diverge slightly between amino acids 87 and 164, with nine substitutions between H-Ras and K-RasB, six of which are conservative. The region between amino acid 165 and the COOH terminus, known as the variable domain, shows no sequence similarity among the Ras proteins other than a consensus CAAX sequence at the extreme COOH terminus (1).

A single farnesyltransferase (FTase) consisting of a 49-kDa α subunit and a 46-kDa β subunit attaches farnesyl groups to all known Ras proteins (5–8). A related enzyme, geranylgeranyl transferase I (GGTase-I), shares the α subunit of FTase but contains a distinct 43-kDa β subunit (8). GGTase-I transfers a 20-carbon geranylgeranyl group to proteins that contain a CAAX consensus in which X is usually leucine (8, 9).

FTase binds CAAX sequences with high affinity, even when they are presented to the enzyme as tetrapeptides (5, 10). The four Ras proteins terminate in the following CAAX consensus sequences: CVIM (K-RasB), CIIM (K-RasA), CVVM (N-Ras), and CVLS (H-Ras) (1). When these tetrapeptides were studied as competitive inhibitors of FTase, CVIM (K-RasB) was found to have a 20-fold higher affinity for the enzyme than CVLS (H-Ras) (11). Leucine-terminated tetrapeptides, such as those found in substrates for GGTase-I, were poorly recognized by FTase. Conversely, none of the Ras-derived CAAX tetrapeptides was efficiently recognized by GGTase-I (9, 12).

We and our collaborators described a peptidomimetic inhibitor of FTase, designated BZA-5B, in which the two central aliphatic amino acids of the CAAX sequence are replaced by a benzodiazepine (BZA) group (13). When added to intact cells, BZA-5B prevented the farnesylation of H-Ras and reversed the transformed morphology of Rat-1 cells that express oncogenic H-Ras (13). Surprisingly, BZA-5B had no effect on the growth or morphology of nontransformed Rat1 cells or on the activation of the mitogen-activated protein kinase pathway in response to epidermal growth factor (14). Rat1 cells transformed by the Src oncogene were also resistant to the growth inhibitory effects of BZA-5B (13). Inasmuch as these responses are generally thought to require Ras function, these results suggested that Rat1 cells may produce a form of Ras whose prenylation is not blocked by BZA-5B.

To address this possibility, we recently studied the prenylation of recombinant K-RasB *in vitro* (15). We chose K-RasB because most Ras mutations in human tumors occur in the K-*ras* gene and because the B isoform is the predominant version produced from this gene (1, 2). We found that FTase has a 50-fold higher affinity for K-RasB than for H-Ras (15). The BZA peptidomimetic inhibited the farnesylation of K-RasB *in vitro* but only at concentrations that were 8-fold higher than required to inhibit H-Ras farnesylation (15). Surprisingly, we also found that K-RasB, but not H-Ras, was an efficient substrate *in vitro* for GGTase-I (15). The high affinity of

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Abbreviations: BZA, benzodiazepine; FPP, farnesyl pyrophosphate; FTase, farnesyltransferase; GGPP, geranylgeranyl pyrophosphate; GGTase-I, geranylgeranyl transferase.

K-RasB for FTase and its ability to be a substrate for GGTase-I are due to the combination of a COOH-terminal methionine and a stretch of lysines that is located immediately upstream of the farnesylated cysteine. Oncogenic K-RasB appears to be farnesylated and not geranylgeranylated in transfected cells (16). Nevertheless, the *in vitro* observations suggested that cellular K-RasB might become geranylgeranylated when farnesylation is blocked by an FTase inhibitor. Geranylgeranylated K-RasB is known to have transforming potential similar to the farnesylated version (17).

A non-BZA peptidomimetic inhibitor of H-Ras farnesylation, L-739,749, was shown to inhibit the growth of K-RasB-transformed fibroblasts in soft agar and to inhibit the growth of tumors in nude mice derived from K-RasB transformed Rat1 cells (18). Such growth inhibition has not been shown directly to result from inhibition of farnesylation of K-RasB, and it might be secondary to some other effect of the inhibitor. The best way to resolve this problem would be to examine the effect of FTase inhibitors on the steady-state levels of prenylated K-RasB in cultured cells. Unfortunately, current antibodies are not able to distinguish K-RasB from other forms of Ras in cells that express normal amounts of these proteins.

In the current study, we prepared a chimeric Ras construct consisting of the first 164 amino acids of H-Ras^{V12} followed by the last 24 amino acids of K-RasB. We expressed this chimera, designated H/K-RasB^{V12}, in Rat1 cells and found that its prenylation was not inhibited by either BZA-5B or L-739,749, whereas both compounds efficiently blocked the prenylation of H-Ras. Similar results were obtained in transfected cells expressing authentic K-RasB^{V12} containing a 10-amino acid extension at its NH_2 terminus. These results provide a likely explanation for the resistance of nontransformed Rat1 cells to the growth inhibitory effects of BZA-5B.

METHODS

Materials. L-739,749 (18) was synthesized at Genentech and provided by Kenneth J. Weese and James C. Marsters, Jr. pZIP-K-RasB^{V12} (16) was provided by Channing Der (University of North Carolina at Chapel Hill). All other reagents were from previously described sources (14).

Plasmids. To construct pRcCMV-H/K-RasB^{V12}, a 315-bp PCR fragment was amplified from pRcCMV-H-Ras (19) with a 5′ primer (24-mer) beginning at codon 71 and a 3′ primer (58-mer) spanning codons 158–164 of H-Ras, followed by codons 165–176 of K-RasB. This fragment was used as a template in a second PCR reaction with the same 5′ primer and a 3′ primer (76-mer) encoding amino acids 170–188 of K-RasB, followed by a stop codon and a *Not* I restriction site. The ≈360-bp product of this reaction was digested with *Bst*XI and *Not* I, and the resulting 240-bp fragment was ligated into the corresponding region of pRcCMV-H-Ras^{V12} (14) to yield pRcCMV-H/K-RasB^{V12}. The COOH-terminal methionine of H/K-RasB^{V12} was changed to leucine by PCR mutagenesis (14) to yield pRcCMV-H/K-RasB^{V12,L188}. The regions amplified by PCR were sequenced on both strands.

Cell Culture. Transfection of Rat1 cells, addition of inhibitors, and analysis of morphology were done as described (14).

Subcellular Fractionation and Immunoblotting. Cells were seeded on day 0 at 5×10^4 cells per 100-mm dish in 10 ml of medium A [Dulbecco's modified Eagle's medium containing penicillin (100 units/ml), streptomycin (100 μg/ml), and 10% calf serum (Colorado Serum)]. Cells were refed daily on days 1–5 with 10 ml per dish of medium B [medium A/1% (vol/vol) dimethyl sulfoxide and 100 μM dithiothreitol] containing the indicated concentration of drug. On day 5 (20 hr before harvest), 100 μM compactin was added as indicated. The cells were harvested on day 6 by scraping in phosphate-buffered saline (PBS), pelleted by centrifugation (4°C), and resuspended in 0.15 ml of buffer A [10 mM Tris·HCl at pH 7.5, 10% (vol/vol) sucrose, 0.5% (vol/vol) aprotinin, 0.5 mM phenylmethylsulfonyl fluoride, and 5 μg/ml each of pepstatin and leupeptin]. After 15 min on ice, the cells were lysed by 20 passages through a 25-gauge needle, and NaCl was added to reach 0.1 M. The lysates were centrifuged (4°C) for 1 min at $10^3 \times g$, and the supernatants were centrifuged for 30 min at $10^5 \times g$. The supernatants (S-100) were transferred to a new tube, and the pellets ($10^5 \times g$ pellet) were resuspended in 0.15 ml of buffer A/0.1 M NaCl. Protein concentration was determined with the BCA protein assay reagent (Pierce), and 20 μg of protein from each fraction was resolved by SDS/PAGE (20) on 12.5% gels and transferred to nitrocellulose filters. The filters were analyzed by immunoblotting with pan Ras Ab-3 and visualized by enhanced chemiluminescence (14).

Metabolic Labeling and Immunoprecipitation. Cells were seeded in duplicate on day 0 at 5×10^4 cells per 60-mm dish in 3 ml of medium A. Cells were refed daily on days 1–4 with 3 ml of medium B containing no addition or 200 μM of inhibitor. On day 5, one set of dishes was refed as above, and the other set was refed with 1.5 ml per dish of medium B containing the same additions plus 100 μM compactin. After a 30-min incubation, 150 μCi of [^{3}H]mevalonolactone (60 Ci/mmol; 1 Ci = 37 GBq) were added to the dishes containing compactin, and the incubation continued for 4 hr. Cells were rinsed three times with phosphate-buffered saline (PBS) and disrupted with 0.3 ml of lysis buffer (14). The lysates were transferred to 1.5-ml Eppendorf tubes and centrifuged (4°C) for 1 min at 12,000 × *g*, and the protein content of each detergent-soluble fraction was measured as described above. Ras proteins were immunoprecipitated from an equivalent amount of each detergent-soluble fraction with 15 μg of v-H-Ras (Ab-1)-agarose (13), and the precipitated material was analyzed by SDS/PAGE on 12.5% gels. The gels were treated with ENTENSIFY, dried, and exposed to Kodak X-Omat AR film at −80°C.

RESULTS

Before initiating the current studies, we made a concerted effort to develop a Rat1 cell line that overexpresses authentic K-RasB^{V12}. All of these attempts failed, even though we routinely obtained cell lines overexpressing H-Ras^{V12} using the same vector. As an alternative, we prepared a plasmid encoding a chimeric H-Ras/K-Ras protein. This plasmid was derived from our previously described H-Ras^{V12} plasmid by replacing the COOH-terminal 25 amino acids of H-Ras^{V12} with the corresponding 24 amino acids from K-RasB. This chimeric protein, designated H/K-RasB^{V12}, differs from authentic K-RasB by only nine amino acids, six of which are conservative substitutions and none of which are near the COOH terminus. We previously showed that a chimeric H-Ras protein containing the last 14 amino acids of K-RasB is prenylated *in vitro* in a manner indistinguishable from authentic K-RasB (15). Therefore, we believe that the prenylation of H/K-RasB^{V12} *in vivo* faithfully mimics that of authentic K-RasB.

Fig. 1 shows immunoblots of cytosol (S-100) and membrane ($10^5 \times g$ pellet) fractions from Rat1 cells that stably overexpress H-Ras^{V12} or H/K-RasB^{V12}. When the cells were grown in the presence of solvent alone or BZA-7B, an inactive analogue of BZA-5B, both H-Ras^{V12} and H/K-RasB^{V12} were found entirely in the pellet fractions (Fig. 1 *A* and *B*). Growth in increasing concentrations of the FTase inhibitor BZA-5B led to a decrease in the amount of prenylated H-Ras^{V12} in the pellet and a corresponding accumulation of nonprenylated H-Ras^{V12} in the S-100 fraction (lanes 2–5). Compactin, which blocks the synthesis of prenyl groups, had a similar effect (lane 6). In contrast, BZA-5B at concentrations up to 200 μM did not produce any decrease in the amount of H/K-RasB^{V12} in the pellet or any accumulation of the nonprenylated version in

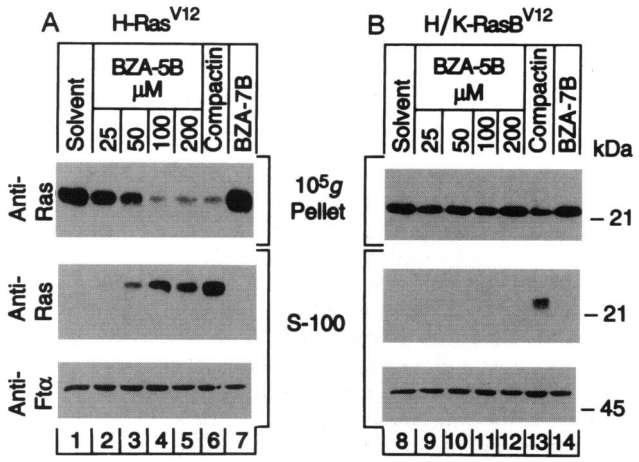

FIG. 1. BZA-5B inhibits prenylation of H-Ras^{V12}, but not H/K-RasB^{V12}. Rat1 cells transfected with H-Ras^{V12} (*A*) or H/K-RasB^{V12} (*B*) were grown for 5 days in medium B containing the indicated concentration of BZA-5B or 200 μM BZA-7B. Dishes in lanes 6 and 13 received 100 μM compactin 20 h before harvest. The $10^5 \times g$ supernatant (S-100) and pellet ($10^5 \times g$ pellet) fractions were prepared as described, and 20 μg of protein from each fraction was subjected to SDS/PAGE. Ras proteins were visualized by immunoblotting with pan Ras Ab-3 (Anti-Ras). S-100 fractions were also analyzed by immunoblotting with an antibody against the α subunit of FTase (anti-FTα) (21). Exposure time was 1.5 min.

the S-100 (lanes 9–12). Only compactin induced the appearance of nonprenylated H/K-RasB^{V12} in the S-100 (lane 13).

Fig. 2 shows the appearance of Rat1 cells transfected with expression vector alone or with vectors encoding H-Ras^{V12} or H/K-RasB^{V12}. The cells were grown with BZA-5B (*Right*) or its inactive analog, BZA-7B (*Left*). As shown previously (14), the appearance of untransformed Rat1 cells was not affected by growth for 4 days in the presence of 200 μM BZA-5B (*B*). The transformed morphology of Rat1 cells transfected with H-Ras^{V12} was almost completely reversed by BZA-5B (*C* and

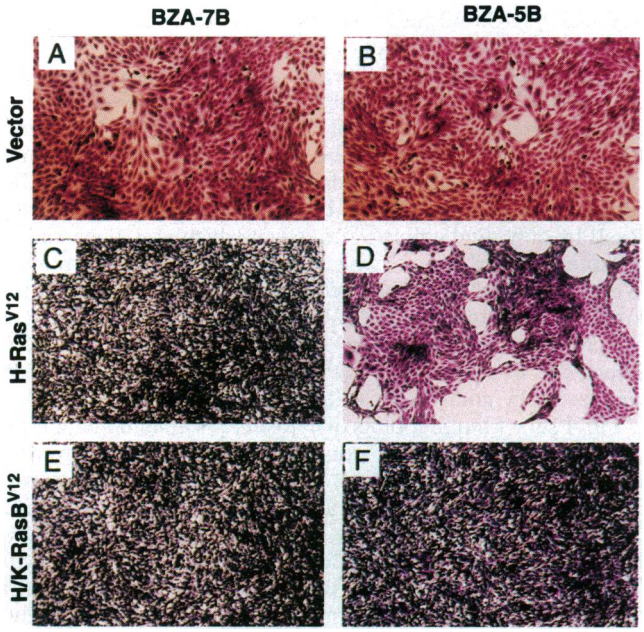

FIG. 2. BZA-5B reverses the transformed morphology of Rat-1 cells transfected with H-Ras^{V12}, but not H/K-RasB^{V12}. Rat1 cells transfected with the indicated construct were grown for 4 days in medium B containing 200 μM BZA-7B (*A*, *C*, and *E*) or BZA-5B (*B*, *D*, and *F*). Cells were fixed, stained with crystal violet, and photographed. (×20.)

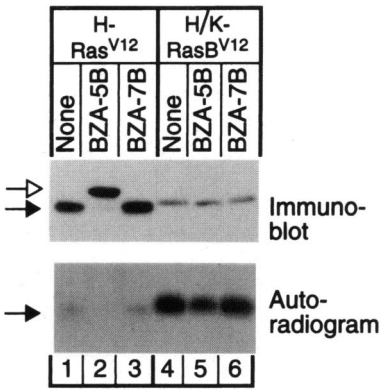

FIG. 3. Differential incorporation of [^{3}H]mevalonate into H-Ras^{V12} and H/K-RasB^{V12}. Rat1 cells transfected with the indicated construct were grown for 4 days in medium B containing 200 μM of BZA-5B or BZA-7B. (*Upper*) Detergent-soluble fractions were prepared as described, and 50 μg of protein from each dish was subjected to SDS/PAGE, after which Ras proteins were visualized by immunoblotting with pan Ras Ab-3. Exposure time was 30 sec. (*Lower*) Cells were labeled with [^{3}H]mevalonolactone, and Ras proteins were immunoprecipitated from 250 μg of each detergent-soluble fraction with v-H-Ras (Ab-1)-Agarose. Immunoprecipitated Ras proteins were subjected to SDS/PAGE, and the gel was treated with Entensify, dried, and exposed to film for 4 days.

D). In contrast, the appearance of Rat1 cells transfected with H/K-RasB^{V12} was not affected by BZA-5B (*E* and *F*).

We next compared the efficiency of incorporation of prenyl groups derived from [^{3}H]mevalonate into H-Ras^{V12} vs. H/K-RasB^{V12}. Cells were grown for 4 days in the presence of solvent alone, BZA-5B, or BZA-7B and then either labeled with [^{3}H]mevalonate for the final 4 hr or harvested without radiolabeling. As shown in Fig. 3 *Upper* (immunoblot), the steady-state level of H-Ras^{V12} was several-fold higher than that of H/K-RasB^{V12}. Consistent with the results in Fig. 1, BZA-5B decreased the amount of prenylated H-Ras^{V12} and increased the nonprenylated version, indicated by its slower mobility during SDS/PAGE (lane 2). Again, H/K-RasB^{V12} resisted inhibition by BZA-5B (lanes 4 and 5). In parallel lysates, ^{3}H-labeled Ras proteins were visualized by immunoprecipitation followed by autoradiography (Fig. 3 *Lower*). Much less ^{3}H was incorporated into H-Ras^{V12} as compared with H/K-RasB^{V12} (compare lanes 1 and 4, *Lower*), even though the total amount of H-Ras^{V12} was higher (*Upper*). As expected, BZA-5B inhibited the incorporation of ^{3}H into H-Ras^{V12} (*Lower*, lane 2). It also caused a slight but reproducible reduction in the amount of ^{3}H incorporated into H/K-RasB^{V12} (*Lower*, lane 5). However, this degree of inhibition was not sufficient to cause any change in the steady-state level of prenylated H/K-RasB^{V12} as measured by immunoblotting (*Upper*, lanes 4–6).

To confirm the selective prenylation of H/K-RasB^{V12} at low concentration of prenyl groups, we incubated the cells in the presence or absence of compactin plus various concentrations of unlabeled mevalonate for 18 h. We included a Rat1 cell line transfected with H/K-RasB^{V12,L188}, in which the COOH-terminal methionine was changed to leucine, making it a substrate for GGTase-I (14, 17). The amount of each Ras protein in the membrane ($10^5 \times g$ pellet) and cytosol (S-100) fractions was determined by immunoblotting. In the absence of compactin, all three proteins were found almost entirely in the membrane fraction (Fig. 4, compare lanes 1 and 8 with 9 and 16). Incubation with compactin in the absence of exogenous mevalonate caused nonprenylated versions of all three proteins to appear in the S-100 (Fig. 4 *D–F*, lane 10). Mevalonate at 100 μM was required to fully restore prenylation of H-Ras^{V12}, as measured by the disappearance of the nonprenylated version from the S-100 (Fig. 4*D*, lane 14). In contrast, the

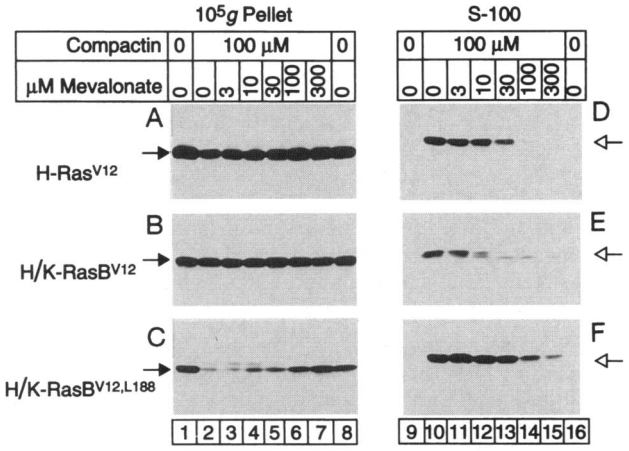

FIG. 4. Differential requirement for exogenous mevalonate to overcome a compactin-induced block in prenylation of H-Ras^{V12}, H/K-RasB^{V12}, and H/K-RasB^{V12,L188}. Rat1 cells transfected with the indicated construct were grown for 3 days in medium A and then refed with medium A containing the indicated concentrations of compactin and unlabeled mevalonate. After incubation for 18 h, the cells were harvested, and the pellet (*A–C*) and S-100 (*D–F*) fractions were prepared. Aliquots of protein from each fraction (20 μg) were analyzed by SDS/PAGE and immunoblotted as described in Fig. 1. Closed and open arrows denote prenylated and nonprenylated versions of each protein, respectively. Exposure time was 1 min.

prenylation of H/K-RasB^{V12} was fully restored at 30 μM mevalonate (Fig. 4*E*, lane 13). The small amount of H/K-RasB^{V12} in the S-100 that remained at higher mevalonate concentrations (lanes 13–15) was the faster migrating, prenylated version of the protein. Surprisingly, the prenylation of H/K-RasB^{V12,L188} was not fully rescued even at 300 μM mevalonate (Fig. 4*F*, lane 15).

We next examined the effects of L-739,749, a non-BZA peptidomimetic related to the tetrapeptide CIFM (18), on the prenylation of H/K-RasB^{V12}. This compound is a potent inhibitor of H-Ras prenylation, and it has also been shown to inhibit the growth of K-RasB-transformed Rat1 cells in soft agar (18). However, the ability of L-739,749 to directly inhibit K-RasB prenylation has not been reported. We included in this experiment a Rat1 cell line transfected with a widely used K-RasB plasmid that overexpresses a K-RasB^{V12} protein of ≈24 kDa, owing to 10 amino acids of vector-derived sequence at its NH_2 terminus (16). We have designated this chimeric protein *K-RasB^{V12}. Fig. 5 shows immunoblots of cytosol

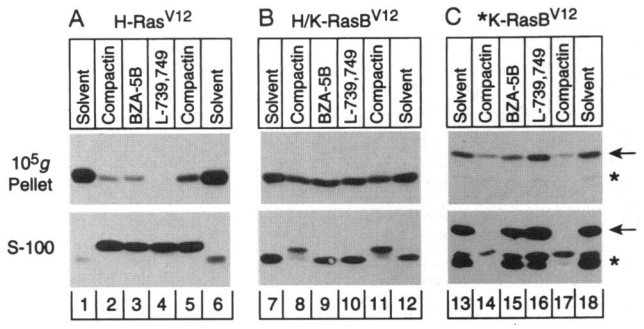

FIG. 5. Prenylation of H/K-RasB^{V12} and *K-Ras^{V12} is resistant to both BZA-5B and L-739,749. Rat1 cells transfected with the indicated construct were grown for 4 days in medium B containing 100 μM BZA-5B or L-739,749. Dishes in lanes 2, 5, 8, 11, 14, 17 received 100 μM compactin 20 h before harvest. The S-100 and pellet fractions were prepared as described, except that the immunoblots were developed with the SuperSignal CL-HRP substrate system (Pierce). Exposure times were 5 sec for the pellet in *A–C*, 3 sec for S-100 in *A*, 30 sec for S-100 in *B*, and 30 min for S-100 in *C*. In *C*, arrows denote transfected *K-Ras^{V12}; asterisks (*) denote endogenous Ras proteins.

(S-100) and membrane (10^5 g pellet) fractions from Rat1 cells that stably overexpress H-Ras^{V12}, H/K-RasB^{V12}, or *K-RasB^{V12}. When the cells were grown in the presence of solvent alone, all three proteins were found primarily in the pellet (Fig. 5 *A–C*, lanes 1, 6, 7, 12, 13, 18). The small amount of each protein in the S-100 migrated as the prenylated version. Incubation with compactin caused nonprenylated versions of both H-Ras^{V12} and H/K-RasB^{V12} to appear in the S-100 (lanes 2, 5, 8, 11). Compactin caused a slight decrease in the amount of *K-RasB^{V12} in the pellet and a disappearance of *K-RasB^{V12} from the S-100 (lanes 14, 17). This disappearance may indicate rapid degradation of unprenylated *K-RasB^{V12}. BZA-5B (100 μM) depleted prenylated H-Ras^{V12} from the pellet and caused accumulation of unprenylated H-Ras in the cytosol (lane 3). The same concentration of L-739,749 produced an even greater depletion of prenylated H-Ras^{V12} from the pellet (lane 4). In contrast, neither BZA-5B nor L-739,749 depleted prenylated H/K-RasB^{V12} or *K-RasB^{V12} from the pellet, nor did they cause an accumulation of the nonprenylated versions in the S-100 (lanes 9, 10, 15, 16).

Fig. 6 shows the appearance of Rat-1 cells transformed by H-Ras^{V12} or H/K-RasB^{V12} after treatment for 4 days with solvent, 100 μM BZA-5B, or 100 μM L-739,749. The transformed morphology of Rat1 cells expressing H-Ras^{V12} was almost completely reversed by incubation with either BZA-5B or L-739,749 (compare *A* with *B* and *C*). In contrast, neither compound reversed the morphology of Rat1 cells transfected with H/K-RasB^{V12} (compare *D* with *E* and *F*).

DISCUSSION

The current findings are consistent with the hypothesis that nontransformed cells resist the growth inhibitory effects of BZA-5B because they continue to prenylate their endogenous K-RasB. Both benzodiazepine (BZA-5B) and non-BZA (L-739,749) peptidomimetics failed to inhibit the prenylation of H/K-RasB^{V12}. They also failed to inhibit the prenylation of *K-RasB^{V12}, a version of K-RasB that contains 10 novel amino acids at the NH_2 terminus. Because we obtained similar findings with H/K-RasB^{V12} and with *K-RasB^{V12}, we believe that this behavior reflects the behavior of endogenous K-RasB in these cells.

It is possible that K-RasB is farnesylated *in vivo* by the classic FTase and that the two inhibitors are simply not sufficiently potent to prevent this prenylation. Alternatively, K-RasB may be prenylated *in vivo* by another prenyl transferase that cannot prenylate H-Ras and is resistant to the two inhibitors. We recently found that BZA-2B, the active form of BZA-5B, can inhibit the farnesylation of K-RasB *in vitro*, but an 8-fold higher

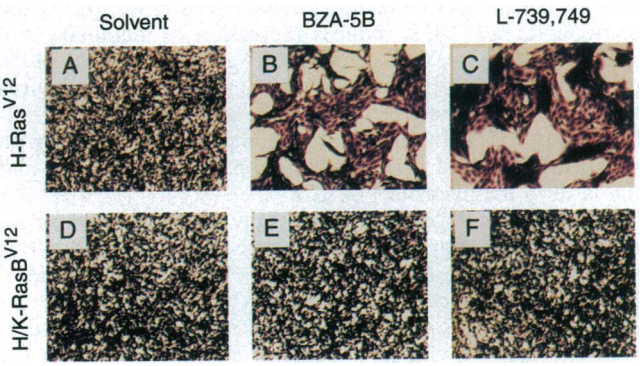

FIG. 6. Transformed morphology of Rat1 cells transfected with H/K-RasB^{V12} is resistant to L-739,749. Rat1 cells transfected with the indicated construct were grown for 4 days in medium B containing no addition (solvent, *A* and *D*) or 100 μM BZA-5B (*B* and *E*) or 100 μM L-739,749 (*C* and *F*). Cells were fixed, stained with crystal violet, and photographed. (×20.)

concentration was required to inhibit farnesylation of K-RasB as compared with H-Ras (15). It seems unlikely that the 8-fold difference observed *in vitro* would lead to complete resistance to BZA-5B in intact cells. These findings therefore suggest that K-RasB might be prenylated by at least one other enzyme in addition to the classic FTase.

One alternative enzyme that might prenylate K-RasB *in vivo* is GGTase-I. We recently reported that this enzyme can prenylate K-RasB, but not H-Ras, *in vitro* (15). Consistent with this finding, Lerner *et al.* (22) recently described an inhibitor of GGTase-I that blocks the prenylation of *K-RasB^{V12} in transfected NIH 3T3 cells. However, these observations are inconsistent with a previous study that identified the prenyl group attached to transfected *K-RasB in mammalian cells as farnesyl (16). This was done by labeling the cells with [^{3}H]mevalonate, after which K-RasB was isolated by immunoprecipitation, the prenyl group was cleaved with methyl iodide, and its identity was determined by C_{18} reversed phase chromatography. Using an identical procedure, we also identified the ^{3}H-prenyl group attached to H/K-RasB^{V12} as farnesyl, both in the absence and presence of BZA-5B (data not shown). However, the [^{3}H]mevalonate labeling experiments must be conducted in the presence of limiting mevalonate. Under these conditions, geranylgeranylation of proteins is selectively reduced compared to farnesylation (23). Our observation that the geranylgeranylation of H/K-RasB^{V12,L188} in the presence of compactin requires a much higher concentration of mevalonate than does prenylation of H-Ras^{V12} or H/K-RasB^{V12} (Fig. 4*F*) is consistent with this finding. This result suggests that even if H/K-RasB^{V12} is a substrate for GGTase-I, its detection by radiolabeling with mevalonate may not be possible.

Recent *in vitro* experiments demonstrated that GGTase-I transfers farnesyl as efficiently as geranylgeranyl to RhoB, one of its known substrates (24). Although GGTase-I does not transfer farnesyl efficiently to K-RasB *in vitro* (unpublished data), we cannot rule out the possibility that this might occur in intact cells in which the concentration of GGPP is limiting, a condition that exists during the mevalonate-labeling procedure. Alternatively, K-RasB might be geranylgeranylated by GGTase-I under *normal* conditions *in vivo* and only become farnesylated when mevalonate, and thus FPP and GGPP are limiting. This would explain the identification of the labeled prenyl group on K-RasB as farnesyl and might also explain the minimal inhibition of [^{3}H]mevalonate incorporation into K-RasB that we (Fig. 3) and others (19) have observed in the presence of FTase inhibitors.

L-739,749 inhibits the anchorage-independent growth of Rat1 cells transformed by oncogenic versions of H-Ras, N-Ras, and K-RasB (25). In contrast, it had no effect on the growth of Rat1 cells transformed by v-*raf* or v-*mos* oncogenes, both of which are believed to function independently of Ras. A related compound, L-777,832, induces regression of mammary and salivary tumors in transgenic mice harboring a viral H-*ras* oncogene under control of the mouse mammary tumor virus long terminal repeat (26). L-744,832 and L-739,749 are different prodrugs of the same parent compound, L-739,750, with the former having an isopropyl ester on the COOH group and the latter a methyl ester (18, 26).

The ability of L-739,749 to selectively inhibit the anchorage-independent growth of cells transformed by H-, K-, and N-Ras suggests that these growth-inhibitory effects are due to inhibition of Ras function and that this compound blocks the prenylation of all Ras proteins (25). However, direct measurements of the prenylation of K-RasB in cultured cells in the presence of L-739,749 or L-444,832 have not been published. In our experiments, L-739,749 did not prevent the prenylation of H/K-RasB^{V12} or *K-RasB^{V12}, suggesting that its ability to inhibit the anchorage-independent growth of K-RasB-transformed Rat1 cells might be due to a block in prenylation of one or more proteins other than K-RasB. This conclusion is consistent with recent results in which L-744,832 was reported to inhibit the anchorage-independent growth of ≈70% of all human cancer cell lines with no relation to the presence or absence of a mutated Ras (25).

The implications of the current findings for the utility of FTase inhibitors as anticancer drugs in humans is presently unclear. The *in vivo* animal studies conducted to date suggest that FTase inhibitors might be effective against some tumors even without inhibiting K-RasB prenylation. However, the precise mechanism by which this occurs is unknown, and it seems likely that a compound that efficiently blocked the prenylation of K-RasB would be much more effective.

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