Negative Growth Selection against Rodent Fibroblasts
Targeted for Genetic Inhibition of Farnesyl Transferase

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Abstract
The Ras oncoprotein must be modified by farnesyl transferase (FTase) for biological activity. Therefore, inhibition of FTase may offer a means to block ras induced cell transformation. To address this hypothesis, we have introduced antisense and dominant inhibitory FTase expression plasmids into a panel of normal, mutant ras-, and mos-transformed rodent fibroblasts in an effort to genetically suppress FTase activity. Antisense FTase constructs reduced colony formation efficiency ~29% in normal and ~41% in ras-transformed cells relative to control plasmids. In contrast, antisense FTase plasmids did not exhibit a statistically significant effect on colony formation efficiency in mos-transformed transfectants. FTase aN199K is a mutant form of the a subunit of FTase that exhibits dominant inhibitory activity versus native FTase. Only mos-transformed transfectants exhibited expression of aN199K RNA in 15 of 16 fibroblast lines that were randomly selected and characterized. Our data suggest that genetic inhibition of FTase may result in a selection against animal cell growth.

Introduction
Cellular transformation by Ras is dependent upon the integrity of its COOH terminus, which is a target for modification by FTase.2 FTase is a heterodimeric enzyme which adds a C15 isoprenyl moiety to the COOH termini of a set of cellular proteins, including Ras. Oncogenic Ras proteins which lack farnesyl groups transform poorly because they are not efficiently associated with cell membranes, where they must be localized in order to function properly (reviewed in Refs. 1–4).

On this basis, it has been proposed that FTase represents a novel therapeutic target in the many types of human tumors where Ras may play an etiological or contributing role. However, the biological foundation for this proposal is uncertain. First, although experiments performed in yeast and animal cells indicate that farnesylation of Ras is necessary for its biological activity, it has not yet been demonstrated that inhibition of FTase will prove sufficient to block Ras action. This is an important issue since the recognition specificity of another prenyl transferase, GGTase I, overlaps to some degree with FTase (5-7). Second, the consequences for normal and transformed cell physiology in animal cells where FTase activity is inhibited have not been examined. Metabolic labeling experiments with tritiated isoprenoid precursors have indicated that the number of farnesylated cellular proteins is relatively small (reviewed in Ref. 8), but some of these may be essential to cell growth [e.g., nuclear lamins (9)] or necessary for important cell functions [e.g., forms of prenylated, a G-protein that transmits the visual signal in the retina (10, 11)]. Finally, if inhibition of FTase can be shown to suppress Ras function, it will be important for therapeutic reasons to determine whether the inhibition is cytotoxic or cytostatic for transformed cell growth. The issues outlined above are central to determining the utility of FTase as a target for developing anticancer agents.

To begin to address these issues, we sought to genetically inhibit FTase activity in normal and transformed rodent fibroblasts. Both antisense and dominant negative approaches have been explored. We report here (a) the identification of a mutant form of the FTase a subunit that exhibits the in vitro characteristics predicted for a dominant negative action in vivo, and (b) that there appears to be a growth selection against cells targeted for suppression of FTase following transfection of antisense and dominant negative expression vectors.

Results
Inhibition of Cell Colony Formation following Transfection of Antisense FTase β Subunit Expression Plasmids. FTase activity requires both the a and β subunits of the enzyme. We chose to target the β subunit of FTase for suppression by an antisense RNA approach because this subunit is unique to the enzyme, unlike the a subunit, which is shared by FTase and GGTase I (12–14). An antisense human FTase β subunit cDNA was introduced into the mammalian expression vector hygCMV, which contains a drug-selectable marker for hygromycin resistance (hygro) and the strong CMV early promoter for expression of antisense RNA. The human FTase β cDNA in hygCMV (antis.), which is described elsewhere (15), encompassed the complete β coding region and did not include 3’ noncoding sequences.

We wished to examine the effects of antisense FTase β expression on the phenotype of Ras-transformed cells. For transfection, three types of Rat6 fibroblast lines were used, referred to as K-Ras, Neo, and Mos cells. K-Ras cells were generated by transforming Rat6 cells with the mutant K-ras12 gene, and Mos cells were generated by transforming Rat6 cells with the v-mos oncogene. Neo cells were derived by transfection of Rat6 cells with an expression plasmid containing only the neo gene. The Neo and Mos lines
were used to control for antisense effects on normal established fibroblasts (Neo) or on cells transformed by another oncogene (Mos). Mos is proposed to act independently of Ras in signal transduction pathways leading to cell growth because Mos activity is not inhibited by microinjection of anti-Ras antibodies in vivo (16). Construction and characterization of the Rat6 lines is described in "Materials and Methods."

Following selection for hygromycin resistance, we observed an \( \sim 29\% \) reduction in the efficiency of colony formation in Neo and \( \sim 41\% \) reduction in K-Ras cell lines relative to the control hygCMV vector (see Table 1). Within a 95% confidence interval \( (P < 0.05, \text{one-sided}) \), these data were statistically significant as analyzed by Student's t test. In contrast, an \( \sim 20\% \) reduction in Mos cell colony number was not found to be statistically significant (see "Materials and Methods" for a description of the statistical analysis). The results represent at least three independent experiments, each performed in triplicate, with at least two independent clonal lines of each cell type. We attempted to rescue colony formation efficiency in K-Ras cells by cotransfection of hygCMV-\( \beta \)antis.) with oncogenic K-ras, v-raf, or v-mos expression plasmids. The biological activity of the v-Raf and v-Mos proteins encoded on the latter two plasmids are independent of Ras function (16). These plasmids had no effect on the suppressive phenomenon (data not shown). This result suggested that activation of a Ras pathway may sensitize cells to anti-FTase targeting in a manner that is dominant to the potential transforming signals transduced by v-Raf and v-Mos. Taken together, the data indicated that there was a negative growth selection against Neo and K-Ras cells following transfection of hygCMV-\( \beta \)antis.), but that Mos cells were less sensitive to this protocol.

To verify expression of the transfected antisense \( \beta \) RNA, colonies were pooled and grown into mass culture for Northern analysis (see Fig. 1). The morphology of the relatively lower number of colonies transfected with the antisense \( \beta \) plasmids was noted to be similar to those transfected with control plasmids. Control pools generated by transfection of hygCMV exhibited an endogenous \( \beta \) mRNA of \( \sim 3 \) kb, similar in size to that previously reported in rat cells (17). Antisense pools exhibited an additional RNA of \( \sim 1.7 \) kb that was consistent with the size of the predicted antisense RNA

![Fig. 1. Expression of antisense FTase \( \beta \) RNA in transfected cells. Northern analysis was performed with \( \sim 2 \mu g \) of poly(A) \( ^{+} \) RNA from pooled colonies of each cell transfected, using the human FTase \( \beta \) and L32 cDNAs as hybridization probes. L32 cDNA encodes a ubiquitously expressed large ribosomal subunit protein (18) that controls for RNA loading.](image)

expressed from hygCMV-\( \beta \)antis.). To control for RNA loading, blots were also hybridized with a probe for L32, which encodes a large ribosomal subunit protein (18). A prominent feature of the Northern data was the low level of steady-state antisense RNA, comparable to that of the relatively rare endogenous FTase \( \beta \) subunit message (17), despite the use of the strong CMV promoter to drive expression from hygCMV-\( \beta \)antis.). There was no suppression of either FTase activity or Ras prenylation (as assayed by mobility shifts in immunoprecipitation gels) observed in extracts of antisense pooled colonies relative to control pooled colonies; similar results were obtained from four cell lines generated by clonal expansion of mutant Ras cell colonies.

We reasoned that since hygCMV-\( \beta \)antis.) suppressed relative colony formation and since pooled colonies transfected with this plasmid poorly expressed the antisense RNA, there might be a negative growth selection against cells expressing high levels of antisense RNA. Therefore, in an effort to obtain inducible, high-level expression of antisense \( \beta \) RNA, we generated a panel of Rat6 cell lines with antisense \( \beta \) genes

Table 1. Efficiency of colony formation following transfection of Neo, Mos, and K-Ras cells with FTase \( \beta \) antisense plasmids

<table>
<thead>
<tr>
<th>Cell type</th>
<th>No. of trials</th>
<th>( n )</th>
<th>Mean colony formation efficiency, hygCMV-( \beta )antis.)( ^{+} ) (Mean colony formation efficiency, hygCMV-( \beta )antis.)( ^{+} ) (90% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neo</td>
<td>5</td>
<td>3</td>
<td>71 (55-87)( ^{a} )</td>
</tr>
<tr>
<td>Mos</td>
<td>4</td>
<td>3</td>
<td>80 (58-102)( ^{b} )</td>
</tr>
<tr>
<td>K-Ras</td>
<td>4</td>
<td>3</td>
<td>59 (31-86)( ^{c} )</td>
</tr>
</tbody>
</table>

\( ^{a} \) Expressed as a percentage of the mean number of colonies observed following transfection with the control plasmid hygCMV.\( ^{+} \) expressed as a percentage of the mean number of colonies observed following transfection with the control plasmid hygCMV.

\( ^{b} \) Statistically significant decrease in colony formation efficiency by hygCMV-\( \beta \)antis.) with 90\% (\( P < 0.1 \), two-sided) or 95\% (\( P < 0.05 \), one-sided) confidence.

\( ^{c} \) G. C. Prendergast, unpublished data.
expressed from the steroid-inducible mouse mammary tumor virus promoter. Several lines induced steady-state antisense RNA to ~10 times the level of endogenous β within 6 h of the addition of dexamethasone to culture media. However, we observed that the antisense RNA level decreased within 2–5 days of steroid treatment to approximately that of the sense message, without any change in FTase activity during this period. The rapidity of the phenomenon could not be accounted for by desensitization of the mouse mammary tumor virus promoter. This suggested that the decrease in antisense RNA was due to an epigenetic effect in the cell population. In K-Ras cells containing the inducible antisense gene, there was no change in morphology following steroid induction. However, a reduction in the size of colonies grown in soft agar in the presence of steroid relative to uninduced controls was observed. 3 This observation and the epigenetic suppression of antisense β RNA that occurred following steroid induction argued that the antisense FTase β gene was functioning in a manner consistent with the colony formation experiments. Taken together with those analyses, the data argued that high-level expression of antisense FTase β RNA is incompatible with cell growth.

Characterization of a Yeast Mutant FTase α Subunit That Supports GGTase I but not FTase Activity. To corroborate the antisense effort, we developed a second genetic approach using a mutant FTase subunit that would produce a dominant negative effect in vivo. Previous work in the yeast Saccharomyces cerevisiae identified RAM2 as encoding a mammalian FTase α subunit homologue (12, 13). A second-site suppressor mutation of yeast Ras function in RAM2 (ram2.1) yielded a strain which lacked FTase activity but retained ~34% of its GGTase I activity relative to wild-type (5). Since the FTase α subunit is shared by both FTase and GGTase I in S. cerevisiae and mammalian cells (12–14, 19), we hypothesized that the ram2.1 mutation might result in specific loss of FTase catalytic function but not dimerization capability, properties that could confer a dominant negative activity.

The alteration in the product of the ram2.1 gene, Ram2.1, was identified as a N143K mutation (15). Wild-type Ram2 or mutant Ram2.1 was expressed in Escherichia coli together with either Ram1 [yeast FTase β subunit (12)] or Cdc43 [yeast GGTase I β subunit (19)] for sources of the prenylprotein transferases. The enzymes were enriched from E. coli cell lysates by immunoaffinity chromatography (see “Materials and Methods”). The presence of the Ram1 or Cdc43 protein in these enzyme preparations was confirmed by Western blot analysis (data not shown) and used to normalize the amount of enzyme used in the assays containing Ram2 or Ram2.1 proteins. As expected, coexpression of recombinant Ram1 with Ram2 gave FTase activity, and coexpression of Cdc43 with Ram2 gave GGTase I activity. In contrast, the recombinant Ram2.1 protein was unable to generate FTase activity when coexpressed with Ram1. Coexpression of Ram2.1 with Cdc43 protein generated GGTase I activity (see Table 2), arguing for a specific loss of FTase catalytic function in the Ram2.1 subunit. We concluded that the Ram2.1 protein exhibits differential effects on FTase and GGTase I activity when coexpressed with the different prenyltransferase β subunits.

Human FTase αN199K Exhibits in Vitro Characteristics That Predict a Dominant Negative Activity when Overexpressed in Vitro. Since there is significant homology between yeast and mammalian FTase subunits (13), a similar mutation at the homologous position in the human FTase α subunit would be expected to behave like the mutant yeast Ram2.1 protein. This mutant, αN199K (15), would be predicted to possess a dominant negative activity against FTase in vivo if, in vitro, it (a) gave rise to defective FTase activity, like the yeast mutant, and (b) retained the ability to dimerize with the wild-type human FTase β subunit.

We have previously demonstrated that recombinant human αN199K is defective for FTase activity when coexpressed with wild-type FTase β subunit (15). Consistent with the possibility that this reflected a loss of catalytic rather than dimerization function of αN199K, we observed that titration of the mutant αN199K protein with constant amounts of wild-type human α and β subunits resulted in the suppression of FTase activity.4 However, to directly examine the dimerization capability of this mutant, wild-type human FTase β subunit was cotranslated in vitro with αN199K or wild-type α subunit and subjected to coimmunoprecipitation with an antipeptide FTase α antibody (see Fig. 2). We observed that the wild-type FTase β subunit was coimmunoprecipitated with both the α and αN199K subunits. This indicated that the mutant αN199K subunit retained its ability to dimerize with the wild-type FTase β subunit in vitro. It was not possible to examine the ability of the αN199K subunit to generate GGTase I activity because the human GGTase I β subunit has not yet been cloned. However, based on the

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activity of the analogous yeast mutant, one would predict that αN199K would retain the ability to generate GTase I activity with the GTase I β subunit. Taken together with the results of the experiments with the yeast subunits, these data suggested that αN199K would exert a dominant negative activity against F1ase but not GTase I following overexpression in vivo.

Selection against High-Level Expression of F1ase αN199K mRNA in Rodent Fibroblasts. For expression in animal cells, αN199K was inserted downstream of the CMV promoter in the plasmid DHFRhyg-CMV to produce DHFRhyg-αN199K (see Fig. 3A). This vector contains a hygromycin gene for drug selection and a DHFR gene for amplification of the plasmid in vivo by mttx treatment. As a control vector, a frameshift mutation was engineered into this plasmid to produce DHFRhyg-αN199KΔ. The frameshift was generated by deleting a region corresponding to the NH2 terminus of a codon within the αN199K gene, resulting in truncation of the polypeptide at amino acid residue 16.

A panel of Neo, Mos, K-Ras, and H-Ras Rat6 lines was developed using the normal and frameshifted αN199K ex- pression vectors and was analyzed by Northern blotting. We observed that, with the exception of one K-Ras line, only the Mos lines expressed detectable levels of αN199K RNA (see Table 3). The one positive K-Ras line expressed αN199K RNA at a level similar to the endogenous α message and to the Mos lines. RNA from the negative control frameshifted αN199K genes did not accumulate to detectable levels but could be observed in mttx-resistant (see below). These data suggested that Mos cells might be more tolerant to expression of αN199K.

In an effort to amplify αN199K RNA levels in the expressing Mos lines, cells were exposed to 10 μM mttx, which in principle causes amplification of the DHFR gene and nearby linked DNA. Cell populations which grew out after ~4 weeks were analyzed by Northern blotting, with probes for F1ase and L32 (to control for differences in RNA loading). The data from two αN199K Mos lines (αN1 and αN3) and a control αN199KΔ line before and after mttx treatment are shown in Fig. 3B. RNA from untransfected Rat6 cells was also analyzed to identify the endogenous rat α message. As expected, amplification of an exogenous DHFR RNA of the predicted size was observed in all mttx lines. Expression of the frameshift mutant αN199KΔ RNA was not detected before mttx treatment, but after selection, a message of the predicted size was observed. The reason that the αN199KΔ RNA is undetectable before mttx selection is unclear.

In contrast to the observed amplification of the mutant αN199KΔ RNA, expression of the αN199K RNA in the Mos-αN1 and Mos-αN3 lines was suppressed following mttx selection. Similar results were seen in the one K-Ras line expressing αN199K RNA (data not shown). Taken together, these data suggested that there was a selection against high-level expression of αN199K RNA in rodent fibroblasts.

Discussion

F1ase is one enzyme in a related family of prenyl-protein transferases that add C95 (farnesyl) or C20 (geranylgeranyl) moieties to protein COOH termini (reviewed in Refs. 2–4). The importance of these enzymes in cell physiology is underscored by the growing number of cellular proteins whose proper localization and function has been found to depend on isoprenylation. Following farnesylation of Ras and other proteins, there occur in vivo at least two further modifications, proteolysis of the three COOH-terminal amino acids and carboxymethylation of the COOH-terminal modified cysteine. However, unlike prenylation, these modifications have been shown to be dispensable for Ras transformation.

Table 3: Expression of F1ase αR2.1 in Rat6 fibroblasts

<table>
<thead>
<tr>
<th>Parental line for transfection</th>
<th>Lines expressing αN199K RNA</th>
<th>Change in RNA level in mttx cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>DHFR αN199K</td>
</tr>
<tr>
<td>H-Rasα/R2.1</td>
<td>0/4</td>
<td>NA</td>
</tr>
<tr>
<td>K-Ras</td>
<td>1/5</td>
<td>NA</td>
</tr>
<tr>
<td>Neo</td>
<td>0/4</td>
<td>NA</td>
</tr>
<tr>
<td>α-Mos</td>
<td>3/3</td>
<td>+</td>
</tr>
</tbody>
</table>

Fig. 3. Expression of αN199K in untreated and methotrexate-selected Mos cell lines. A, expression construct. The human F1ase dominant negative subunit αN199K was placed under the control of the cytomegalovirus early region promoter in DHFRhyg-CMV, which also contains the hph (hygromycin) gene for selection in hygromycin, and the DHFR gene for amplification of the plasmid by methotrexate. AMpret, ampicillin resistant; K, thymidine kinase. B, Northern analysis was performed with ~2 μg of poly(A) RNA from selected Mos cell lines before and after selection with 10 μM methotrexate (mttx), Mos-αN1 and Mos-αN3 were generated by transfection of DHFRhyg-αN199K, whereas Mos-α was constructed by transfection of the frameshift mutant construct DHFRhyg-αN199KΔ (see text). The blot was hybridized with human F1ase α, L32, and DHFR cDNAs.
Genetic Approaches to Inhibit FTase in Animal Cells.

Taken together, the results from both antisense and dominant negative approaches using αN199K suggest that there may be a negative growth selection against rodent fibroblasts targeted for FTase suppression. Relative colony formation was inhibited ~29% in normal cells and ~41% in K-Ras-transformed cells by antisense FTase β expression plasmids. Pooled transfectants that grew out exhibited low levels of antisense β RNA similar in abundance to the rare endogenous β mRNA (17), despite the use of a strong promoter to express the antisense RNA. We have shown that a human FTase α subunit mutant, αN199K, has in vitro characteristics that predict a dominant negative activity against FTase following overexpression in vivo. Results consistent with the antisense work were found subsequent to transfection of FTase αN199K dominant negative vectors. αN199K expression was consistently observed in Mos cells (the same cell lines that were refractory to significant inhibition of colony formation by antisense β plasmids), but not normal cells or those transformed by mutant Ras. Use of a dominant negative-based strategy to coamplify the dominant negative gene with DHFR in αN199K-expressing cells resulted in suppression rather than augmentation of its mRNA levels. This suggested a general selection against cells with high-level expression of αN199K.

αN199K as a Dominant Negative Mutant. We have demonstrated that the human αN199K subunit has in vitro properties that predict a dominant negative effect against cellular FTase activity in vivo. An especially attractive feature of this mutant is that in its yeast form it retains the ability to complex with the GGTase I β subunit and produce GGTase I activity. A conclusive demonstration that αN199K will act in this manner awaits the cloning of the human GGTase I β subunit, which has not been isolated. However, given our data, we have argued that αN199K would act in a dominant negative manner against FTase but not GGTase I activity in animal cells. The inability to obtain Neo and Ras cell lines which efficiently expressed αN199K suggested that there was a negative growth selection against cells that accumulated αN199K mRNA. This result was consistent with the possibility that αN199K was acting in a dominant negative manner. The αN199K mutant might be useful to assess the biological requirements and function for farnesylation in other systems, where its dominant negative activity could be verified directly.

Negative Growth Selection against Fibroblasts Targeted for Suppression of FTase. It is difficult to provide a molecular interpretation of our data because of (a) the apparent negative growth selection against cells targeted for FTase suppression by both antisense and dominant negative protocols, and (b) the lack of understanding of the regulation of cellular prenyl transferase activity. The relative resistance of Mos cells to the inhibitory effects of the protocols that we have used could be explained at least two ways. First, the Mos oncoprotein may directly or indirectly control prenyl transferase activity, perhaps allowing Mos cells to react epigenetically and escape any effects of the antisense and dominant negative-acting elements. Second, Mos may act downstream of any requirements for prenylated proteins in the mitogenic signal transduction pathways. If so, the differential effect in Mos cells may indicate that cell viability per se is not targeted with the same sensitivity as processes upstream of Mos that are required for growth in Neo and Ras cells. This interpretation would be encouraging because it would tend to favor the possibility that important farnesylated proteins, such as lamins, may be less sensitive to loss of FTase function than signal transduction proteins, such as Ras, that would be targeted by anti-FTase cancer therapies.

The possibility that there is a negative growth selection against cells targeted for suppression of FTase would be consistent with observations in the yeast S. cerevisiae. Deletion of RAM1 results in loss of FTase activity but does not affect other prenyl-protein transferases since they do not utilize the Ram1 β subunit. ram1 cells are still viable and grow at reduced temperatures, although they do not proliferate at normal temperatures (12). In contrast to RAM1, deletion of RAM2 is lethal (12). Since FTase and GGTase I share the Ram2 α subunit, the lethality presumably reflects the elimination of both enzyme activities. Thus, yeast cells can tolerate loss of FTase (albeit at reduced growth temperatures) but not loss of additional prenyl-protein transferase activities. From these data, one would predict that loss of prenyl transferase activity in animal cells would inhibit growth or viability. In the experiments reported here, these phenotypes would be indistinguishable.

Our results suggest a selection against monolayer growth of both normal and ras-transformed rodent fibroblasts targeted for genetic inhibition of FTase. The relative resistance of Mos cells to the same protocols suggests that there may be a differential response to loss of FTase which is dependent upon the physiology of the cell. We did not observe a significant differential response between normal and ras-transformed cells. However, one cannot rule out the possibility that there is a subtle difference which is not amenable to identification by genetic approaches. A pharmacological approach to address this issue would be useful since dosage levels could be controlled more effectively. Given our findings, one would anticipate some general toxicity of pharmacological agents directed against FTase if the enzyme was constitutively inhibited. Further work in this area is required to assess the therapeutic index of potential FTase inhibitors, relative to other antitumor drugs.

Materials and Methods

Cell Biology. Rat6 and H-RasVal12-transformed fibroblasts were a gift from K-M. Wong and I. B. Weinstein (Columbia University, New York, NY). Neo cells were generated by transfection of Rat6 with RSVneo (encoding the neomycin resistance gene, neo’) and selection with the aminoglycoside G418. K-Ras cells were constructed by transfection of Rat6 fibroblasts with pZIPneoKras4B(V12) (21), which encodes neo’ and human mutant K-Ras4BVal12. Mos cells were generated by cotransfection of RSVneo and LTR-v-mos (courtesy of D. Blair). The colony formation assay was performed with at least two clones of each cell line. The Neo lines used were morphologically indistinguishable from the Rat6 parent, whereas the K-Ras and Mos lines had transformed phenotypes. All lines were verified for expression of neo and the K-Ras transgene by Northern analysis and/or immunoprecipitation with the anti-Ras antibody Y13-259.

Transfections were carried out as described (22) using 20 µg of plasmid DNA. Following selection for 12 days in 200 µg/ml hygromycin B (Calbiochem), cells were either scored by fixation in 0.02% Coomassie Blue R250-50% methanol or ring cloned and expanded into mass culture to generate cell lines. Methotrexate-resistant cell lines were produced by seeding ~5 x 10⁵ cells into ribonucleotide- and deoxy-
ribonucleotide-free minimum essential medium α plus 10% dialyzed fetal bovine serum (GIBCO) and 10 μM methotrexate (Sigma).

Plasmid Constructions. Full-length human FTase α, αN199K, and β CDNA that were used for cloning have been described elsewhere (15). hygCMV-β(antis.) was generated by inserting the antisense orientation at a unique BglII site downstream of the CMV promoter in hygCMV α 1.3 kb human FTase β cDNA. This cDNA was generated by polymerase chain reaction and contains only a protein coding sequence without noncoding regions. For in vitro expression, human FTase α, αN199K, and β CDNA were inserted into pBS+. For expression in mammalian cells, DHFRhyg-αN199K was generated by inserting the BamHI-linkered cDNA from pBSαN199K at a unique BglII site in DHFRhygCMV. A 0.3 kb-base pair Smal fragment in the NH2-terminal αN199K coding region was deleted to create a frameshift mutant, DHFRhyg-αN199K, which truncates the encoded polypeptide at amino acid 16.

Statistical Analysis of the Antisense Colony Formation Data. Cell colony formation efficiency following transcription with hygCMV-β(antis.) was expressed as the mean percentage of the cell colony number scored following transcription with the control vector hygCMV. Student’s t test was used to assign a confidence value to the data and normalize for the different number of trials performed with each cell type. The range of values possible within a 90% (P < 0.1, two-sided) or 95% (P < 0.05, one-sided) confidence interval was computed (shown in Table 1) by multiplying the standard error in the mean by a multiple obtained from a standard table of percentiles of the Student’s t distribution (the value of the multiple decreases as the number of trials performed increases). If the possible range of values encompasses 100% of the control, then the data are statistically significant within the selected confidence interval, with 90% (P < 0.1, two-sided) or 95% (P < 0.05, one-sided).

Northern Analysis. Poly(A)+ RNA was selected from total cytoplasmic cell RNA using a commercial preparation kit (Pharmacia). Approximately 2 μg/lane were analyzed on Northern gels essentially as described (23), using FTase β or α cDNAs, L32 cDNA encoding a ubiquitously expressed large ribosomal subunit protein (18), or DHFR cDNA as a hybridization probes.

Immunoprecipitation. A polyclonal rabbit anti-peptide antiseraum, anti-901, was raised to the bovine FTase α peptide KHSRESDPPTNVQ coupled to thyroglobulin (Sigma). The antisera cross-reacts with human FTase α despite the amino acid differences at the underlined residues. Crude anti-901 serum was purified by binding to Protein A-Sepharose for use in immunoprecipitation. α, αN199K, and β CDNAS subcloned in pBS+ were transcribed in vitro using T7 polymerase. RNAs were translated in 50-μl reactions containing wheat germ extract and 50 μCi [35S]methionine (NEN), as recommended by the vendor (Promega). Immunoprecipitation was performed by mixing 30 μl programmed extract, 30 μl Protein A-Sepharose (Pharmacia), 300 μl IP buffer (10 mM Tris-Cl, pH 7.4–250 mM NaCl-5 mM EDTA-0.2% Nonidet P-40), and 2.5 μg anti-901. Where indicated, anti-901 was blocked by incubation for 30 min at 4°C with peptide (2:1 w/w). Following a 90-min incubation at 4°C with gentle shaking, immunoprecipitates were washed four times with IP buffer, boiled for 3 min in SDS gel loading buffer, analyzed by SDS-polyacrylamide gel electrophoresis on an 8% gel, and treated for fluorography (ENLIGHTENING, Dupont) as directed by the vendor.

In Vitro Assay of S. cerevisiae FTase and GGTase I. Expression of Ram2 was from the plasmid pBH57-EF, which is pBH57 (12), which had been modified such that the codons for Glu–Glu–Phe were added to the COOH-terminal end of the RAM2 coding sequence. Ram2.1 was expressed from pBH57-2.1-EF, which is pBH57-EF into which the D143N mutation from the ram2.1 gene was placed (15). Ram1 was expressed from pBH56, which is similar to pBH63 (12). Cdc43 was expressed from pUC9-CDC43, which is pUC9 into which a Pst-I/EcoRI polymerase chain reaction fragment containing the CDC43 coding sequence (24) had been cloned. The BH57 derivatives Ram2 and Ram2.1 confer chloramphenicol resistance, whereas pBH56 and pUC9-CDC43 encode resistance to ampicillin. E. coli strains expressing Ram2 with Ram1, Ram2 with Cdc43, and Ram2.1 with Cdc43 were made and used as sources of the corresponding prenyl-protein transferases. The enzymes were enriched from E. coli cell lysates by immunoaffinity chromatography using a column coupled to the YL1/2 antibody (15), which recognizes the Glu–Glu–Phe epitope engineered on Ram2 and Ram2.1.

FTase activity was assayed in 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid pH 7.5–5 mM MgCl2-5 mM dithiothreitol-10 mM ZnCl2-0.1% (w/v) polyethylene glycol (average molecular weight, 20,000), with 1 μM [3H]farnesyl diphosphate (26.9 Ci/mmol) and 3 μM Ras-CLV5 protein as substrates, as described (25). GGTase I activity was assayed as for FTase using 2 μM [3H]geranylgeranyl diphosphate (15 Ci/mmol) and 3 μM Ras-CAL in substrates. The reaction velocities were determined and calculated as pmol incorporated (FPN or GPP) per pmol enzyme per s.

Acknowledgments

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References