A Mutant Form of Human Protein Farnesyltransferase Exhibits Increased Resistance to Farnesyltransferase Inhibitors*

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Protein farnesyltransferase (FTase) is a key enzyme responsible for the lipid modification of a large and important number of proteins including Ras. Recent demonstrations that inhibitors of this enzyme block the growth of a variety of human tumors point to the importance of this enzyme in human tumor formation. In this paper, we report that a mutant form of human FTase, Y361L, exhibits increased resistance to farnesyltransferase inhibitors, particularly a tricyclic compound, SCH55622, which is a competitive inhibitor of FTase with respect to the CAAX (where C is cysteine, A is an aliphatic amino acid, and X is the C-terminal residue that is preferentially serine, cysteine, methionine, glutamine or alanine) substrates. The Y361L mutant maintains FTase activity toward substrates ending with CIIIS. However, the mutant also exhibits an increased affinity for peptides terminating with CII, a motif that is recognized by geranylgeranyltransferase I (GGTase I). The Y361L mutant also demonstrates activity with Ha-Ras and Cdc42Hs proteins, substrates of FTase and GGTase I, respectively. In addition, the Y361L mutant shows a marked sensitivity to a zinc chelator HPH-5 suggesting that the mutant has altered zinc coordination. These results demonstrate that a single amino acid change at a residue at the active site can lead to the generation of a mutant resistant to FTase inhibitors. Such a mutant may be valuable for the study of the effects of FTase inhibitors on tumor cells.

Protein farnesyltransferase (FTase) catalyzes the transfer of a farnesyl group onto a conserved cysteine residue four amino acids from the C terminus of a number of proteins, such as Ras, that are involved in cell growth and morphogenesis (1–4). This modification is critical for membrane association and subsequent protein-protein interactions of these proteins. FTase is a heterodimeric enzyme consisting of α- and β-subunits. The α-subunit of FTase is shared with the related prenyltransferase, protein geranylgeranyltransferase type I (GGTase I), whereas the β-subunits of FTase and GGTase I are approximately 30% homologous. The FTase enzyme recognizes the CAAX motif (C is cysteine, A is an aliphatic amino acid, and X is the C-terminal residue that is preferentially serine, cysteine, methionine, glutamine or alanine) that is found at the C termini of the substrate proteins. GGTase I enzyme also recognizes a CAAX motif; however, the terminal X amino acid is predominantly leucine or phenylalanine. This motif is referred to as the CAAL motif.

The three-dimensional structures of the rat FTase enzyme have recently been resolved without substrate bound (5, 6), with bound substrate farnesyl pyrophosphate (FPP) (7), and with bound FPP and CAAX peptide analogs (8). In the structure without bound substrate, a non-cognate peptide provided by an adjacent β-subunit was modeled into the active site of the enzyme. The structure showed that the α- and β-subunits are largely composed of α-helices. The β-subunit forms a barrel-like structure, and one side of this barrel is wrapped by the α-subunit in a crescent shape. One molecule of zinc ion is bound to the heterodimer, and the binding of zinc is coordinated by β-subunit residues Asp-297, Cys-299, His-362, and a water molecule (5–7). The resolution of the rat enzyme has enabled the evaluation of the mutagenic analysis of several amino acid residues of the yeast and human FTase enzyme that are conserved among prenyltransferase β-subunits (10–13).

In previous studies from our laboratory, we identified residues of yeast FTase involved in substrate recognition (14, 15). The residues identified were at two regions of the β-subunit of the FTase enzyme. Amino acid changes at positions 159, 362, and 366 of the yeast enzyme exhibited altered substrate recognition. The crystal structures suggest that the corresponding residues of rat FTase are located in α-helices that surround the central cavity formed by the β-barrel structure (5, 6). All three amino acid residues are located along one side of a hydrophobic pocket formed by the α-subunit (6). In particular, position 362 (position 361 in the rat enzyme) is believed to stabilize the peptide substrate binding (5, 8).

Inhibitors of farnesyltransferase (FTIs) have recently emerged as promising anti-cancer drugs (1, 2). FTIs block anchorage-independent growth of transformed cells and induce their morphological reversion (16–20). In addition, they are capable of inducing apoptosis of transformed cells (21–23). A survey of a variety of human cancer cell lines has shown that 70% of cancer cells are sensitive to the FTIs (24). Studies utilizing a number of animal model systems showed that FTIs block tumor growth and potentiate regression of tumors (22, 25–27). Remarkably, these inhibitors have little effect on untransformed cells. Moreover, FTIs do not exhibit significant toxicity in animal studies (2). These inhibitors are currently being assessed in clinical trials (28).

In this study, we sought to identify a novel type of FTase
FTI-resistant Farnesyltransferase inhibitors. These mutants should provide valuable tools to probe important elements of the active site structure required for the recognition of the inhibitors and substrate proteins. In addition, such mutants should be invaluable for assessing whether in vivo effects of the inhibitors, such as morphological changes (19, 20, 29) and altered cell cycle progression (22, 30), are in fact due to the inhibition of FTase. A hint for identifying mutant FTase resistant to FTIs came from our previous work on yeast FTase. As described above, mutants having a single amino acid change at either residue 159, 362, or 366 exhibited altered substrate recognition. They acquire the ability to recognize the CAAL motif, which is normally recognized by GGTagase I (14, 15). Since many of the inhibitors act to compete with FTase substrates, we speculated that mutants resistant to FTIs could be found among such mutants that exhibited altered substrate recognition. To test this idea, we introduced mutations into human FTase that corresponded to the yeast mutations and characterized these mutant enzymes.

We show in this paper that an amino acid change at residue 361 has a significant effect on the sensitivity of FTase to FTIs. In particular, the mutant shows more than several thousand-fold increase in the resistance to a tricyclic FTI, SCH56582 (19). The mutant also exhibits altered sensitivity to a zinc chelator, suggesting that conformational changes have occurred at the active site of the enzyme.

**EXPERIMENTAL PROCEDURES**

Materials—Farnesyltransferase inhibitors SCH44342 and SCH56582 (19, 31) were provided by Dr. W. R. Bishop (Scherer-Plough). B108 (32) was provided by Dr. A. Garcia (Eisai Institute). BMS193269 (33) was provided by Dr. V. Manne (Bristol-Myers Squibb Co.). a-Hydroxy-farnesyl-phosphonic acid (34) was purchased from Sigma. Manumycin (35) was provided by Dr. M. Hara (Kyowa Hakko Kogyo). Zinc chelators, HPH-5 and HPH-6 (36), were provided by Dr. M. Otsuka (Kumamoto University). Monoclonal antibodies to human a- and b-subunits were obtained from Signal Transduction Laboratories (Lexington, KY).

**Purification of FTases—**Protein farnesyltransferases were purified by expressing both subunits as fusion proteins in E. coli (7). The plasmid pWHA was provided by Dr. K. Miyazono, Cancer Institute, Tokyo) was inserted as an EcoRI to XhoI fragment into a modified form of pGEX-5X-3 (Amersham Pharmacia Biotech) which has a deleted BamHI site and an HA epitope in frame with glutathione S-transferase (GST). The a-subunit (provided by Dr. K. Miyazono) was inserted as an EcoRI to XhoI fragment into pMAL-c2, a maltose binding vector (New England Biolabs). The constructs were co-transformed into DH5a, and FTase was purified using glutathione-agarose as described previously (15). The Y361L mutation was introduced into the b-subunit gene by PCR. The primers for mutagenesis were 5'-GGCAC TGGCGGTAGATTC ATCAACACCGTCTACGATCCGCACT-3' and 5'-AGGCAGTACGGTTCGTAGGA ATCCACGGGAGCTGCGC-3'. The amplified PCR product encoding the mutation at position 361 was sequenced and inserted into the wild type gene by restriction digestion. Additional mutations at positions 361 and 362 were introduced by site-directed mutagenesis with overlap expansion using PCR (37) with the following forward and reverse primers for Met-361, Ile-362, and Ala-362: 5'-GGCAAGTCTCGCTGATTC ATCAACACCGGACCTAGA CACCTGACTGCTC-3' and 5'-AGGCAGTACGGTTCGTAGGA ATCCACGGGAGCTGCGC-3'. All constructs were sequenced to verify the presence of the appropriate mutation. The wild type and the mutant enzymes used in this study were greater than 90% homogeneous as judged by SDS-polyacrylamide gel electrophoresis. The Bradford dye assay (Bio-Rad) was used to determine protein concentration.

**Prenyltransferase Assays—**Protein prenyltransferase assays were carried out essentially as described (38). The reaction mixture contained 50 mM Tris-HCl (pH 7.4), 10 mM MgCl2, 5 mM ZnCl2, 5 mM dithiothreitol. The substrates used were either GST (glutathione S-transferase) fused with short peptides containing C-terminal CAAX motifs (e.g. CIIS, CIIL, CIIM, CIIC, and CIHA for short peptides encoding cysteine, isoleucine, isoleucine, and either serine, leucine, methionine, cysteine, or alanine, respectively) or alternatively full-length substrates of GST-Ha-Ras or GST-Cdc42Hs (38). The prenyl substrates used were [3H]FPP (22.5 Ci/mmol; 1 Ci = 37 GBq) or [3H]Farnesylpyrophosphate (19.3 Ci/mmol). The amount of the wild type and mutant enzymes was optimized and was between 50 and 100 ng per assay, resulting in an overall enzyme concentration of approximately 100 nm. Time course assays for the wild type and mutant FTases were performed with various substrate combinations to determine the appropriate incubation times required for the acquisition of initial rate values. Determination of IC50 values for the inhibitors was performed under the above reaction conditions for 10 min at 37 °C in the presence of the indicated concentrations of FTase inhibitors. For experiments using zinc chelators, ZnCl2 was not included in the reaction mixture.

**Expression of Human FTase Genes in Yeast—**Saccharomyces cerevisiae haploid strains used in this study were the following: YOT559-3C (MATa cal1-1 leu2 trp1 ura3 ade2), YPH250Du (MATa dpr1:URA3 lys2 leu2 trp1 ura3 ade2 his3), RS51-3A (MATa ram2 his3 ura3 ade8 trp1 can1), and SP1 (MATa his3 leu2 ura3 trp1 ade8 can1). Yeast media used were YPD medium and SC-trp medium, synthetic minimal media supplemented with adenine, uracil, and 0.5% casamino acids (39). Yeast trypsin inhibitor was carried out by the lithium (19.4). Yeast media was provided by ligating a 1.8-kilobase blunt-ended fragment containing the human FTase a gene into a PvuII site of pWHB downstream of the yeast FTase b gene. The plasmid pWHB was constructed by insertion of the human FTase a gene into a blunt-ended Nde1 site in frame with the HA epitope of the yeast expression vector pWHA, which is a modified version of pAS1 with a TRP-selectable marker. Expression of the human genes in yeast is constitutive under the regulation of the strong promoter for the yeast gene glyceraldehyde-3-phosphate dehydrogenase. Mutant b genes were produced by replacement of the wild type sequence with the appropriate mutant form into unique Nde1/AflII sites in the b gene. Yeast cells expressing human FTase WT or Y361L were grown in SC-Trp media to an absorb of 1. The cells were harvested and broken with glass beads containing 50 mM Tris (pH 7.4) and 1% Triton X-100. The supernatants were collected, and protein concentration was determined by Bradford assay, and equal amounts were subjected to 10% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with either a monoclonal antibody specific for the human FTase a-subunit or an anti-HA antibody for detection of the human b-subunit.

**RESULTS**

**Human FTase Mutant Y361L Exhibits Increased Affinity for CIIL Substrate—**Our strategy to identify FTase mutants resistant to FTIs is to search for FTase mutants that exhibit altered recognition of the CAAX motif. We have previously identified such mutants of yeast FTase (14, 15). In that study, we showed that residues Ser-159, Tyr-362, and Tyr-366 play critical roles in the recognition of the CAAX motif (15). A single amino acid change at any of these residues led to the increased recognition of the CAAL motif normally recognized by GTGase I. We first examined whether analogous mutations in human FTase resulted in similar effects on its substrate affinity by introducing analogous amino acid changes into the human FTase. Residues Tyr-362 and Tyr-366 of yeast FTase correspond to residues Tyr-361 and Tyr-365 of the human enzyme. These residues are conserved in FTases, whereas leucine or phenylalanine is found at the corresponding residues of GGTase I. Mutagenesis of yeast FTase indicated that an alteration of Tyr-362 to leucine resulted in increased CAAL recognition (15). Based on these observations, we introduced a tyrosine to leucine change at residue 362 and 365. Ser-159 of yeast FTase corresponds to Pro-152 of the human enzyme. Position 152 was mutated to leucine, since methionine is found at the corresponding residue of the b-subunit of human GTGase I. The mutant and the wild type enzymes were prepared by co-expressing the a- and b-subunits in E. coli. For some experiments, we also used enzymes purified from baculovirus-infected S9 cells. The results were essentially the same using either preparation.
Fig. 1. Utilization of GST-CIIS and GST-CIIL by the wild type and the mutant FTases. Varying concentrations of GST-CIIS or GST-CIIL peptides were incubated with 100 nm WT or mutant P152M, Y361L, or Y365L enzyme FTases for 10 min at 37 °C. 1 μl [3H]FPP was used, and radioactivity incorporated was counted as described (38).

A

B

Fig. 1A shows the ability of the mutant and the wild type enzymes to utilize GST-CIIS, a prototypical CAAX motif peptide, as a substrate. As can be seen, P152M, Y361L, and Y365L mutants retained approximately 50% of FTase activity compared with that of the wild type enzyme. When GST-CIIL, a CAAL motif peptide, was used as a substrate, we observed a significant difference between the mutants and the wild type. Y361L incorporated [3H]farnesyl into the GST-CIIL substrate efficiently. In contrast, much less radioactivity was incorporated into the GST-CIIL substrate with the wild type enzyme. P152M and Y365L mutants also showed low activity with the GST-CIIL substrate. Kinetic analyses were performed to assess the affinity of the enzyme and the catalytic activity for peptide substrates. Table I summarizes the kinetic parameters of the mutant and the wild type enzymes for CIIS and CIIL. The mutant at 36 °C at a level comparable to that seen with previously characterized mutants of yeast FTase (data not shown).

The relationship between the affinity of the Y361L mutant for the CIIL substrate and its catalytic activity was determined. As shown in Fig. 2A, incorporation of [3H]farnesyl to Ha-Ras protein by the mutants Y361L, Y365L, and P152M was approximately 50% that of the wild type enzyme. In contrast, significant incorporation of [3H]farnesyl to Cdc42Hs protein was detected with the Y361L mutant. On the other hand, the P152M and Y365L mutants as well as the wild type enzyme exhibited a low level of activity toward Cdc42Hs protein (Fig. 2B). Therefore, of the three human FTase mutants, Y361L is remarkable for its ability to recognize the CAAL motif, while maintaining recognition of the CAAX motif as well.

Protein Substrate Affinity of Human FTase Mutants—Since the alteration of a single amino acid residue potentiated discrete recognition, we examined the potential for differences in binding and catalytic efficiency with related CAAX tetrapeptide motif proteins. In addition to the above CIIS and CIIL peptides, substrate fusion proteins ending with CIIM, CIIC, and CIIA were prepared. In addition, two other mutants of Tyr-361 were prepared by in vitro mutagenesis; tyrosine 361 was changed to isoleucine and methionine. Previous observations with yeast FTase indicated that these mutations resulted in increased CAAL recognition (15). As a negative control, we also prepared a mutant with histidine 362 changed to alanine, which is adjacent to residue 361 and coordinates the zinc ion (5). This alteration reduces FTase catalytic activity (10). Results of these analyses are presented as changes in substrate utilization of the mutants relative to the wild type enzyme (Fig. 3). The Y361L and Y363M mutants exhibited marked increase in their efficiency to utilize the CIIL substrate. The ability to utilize other substrates, CIIS, CIIM, CIIC or CIIA, remains comparable to that of the wild type enzyme. The Y361I mutant also shows a slight increase in its ability to utilize the CIIL substrate. On the other hand, the H362A mutant shows a drastically reduced ability to utilize these substrates, reflecting decreased FTase activity. This histidine 362 functions as one of the ligands for zinc coordination (5), and changing this to alanine is expected to reduce its activity. It is interesting to note that the affinity for substrates terminating with the CIIM, CIIC, and CIIA motifs as measured by $K_m$ values did not differ appreciably between the wild type and Tyr-361 mutant enzyme FTases (data not shown).

Increased affinity of the Y361L mutant for the CIIL substrate, a normal substrate for GGTase I, suggests that the mutant can function as GGTase I. This was demonstrated to be the case using temperature-sensitive yeast cells that require introduction of functional GGTase I to grow at restrictive temperatures. As shown in Fig. 4A, expression of the Y361L mutant suppresses the temperature-sensitive growth of the yeast cal1 mutant (38) which is defective in GGTase I due to a mutation in GGTase I β-subunit. For this experiment, we overexpressed both α- and β-subunits of human FTase in the cal1 strain. Expression of either subunit alone did not support growth at restrictive temperatures (data not shown). Western analysis confirmed the production of α- and β-subunits in the cal1 strain (data not shown). Yeast FTase mutants of the β-subunit (Dpr1), S159N and Y362L, known to suppress cal1 phenotypes (15), were used as positive controls. Human FTase Y361L was capable of suppressing the growth of the cal1 mutant at 36 °C at a level comparable to that seen with previously characterized mutants of yeast FTase β-subunit, S159N and Y362L (15). In contrast, the wild type human FTase, as well as the wild type yeast FTase, did not suppress the growth defect at 36 °C. Fig. 4B shows that the suppression was observed with the Y361M mutant also but not with the Y361I mutant nor with the H362A mutant. The human FTase β-subunit Y361L, Y361M, Y361I, or H362A was co-expressed with the Dpr1, S159N and Y362L (15). In the case using temperature-sensitive yeast cells that require introduction of functional GGTase I to grow at restrictive temperatures, as shown in Fig. 4A, expression of the Y361L mutant suppresses the temperature-sensitive growth of the yeast cal1 mutant (38) which is defective in GGTase I due to a mutation in GGTase I β-subunit. For this experiment, we overexpressed both α- and β-subunits of human FTase in the cal1 strain. Expression of either subunit alone did not support growth at restrictive temperatures (data not shown). Western analysis confirmed the production of α- and β-subunits in the cal1 strain (data not shown). Yeast FTase mutants of the β-subunit (Dpr1), S159N and Y362L, known to suppress cal1 phenotypes (15), were used as positive controls. Human FTase Y361L was capable of suppressing the growth of the cal1 mutant at 36 °C at a level comparable to that seen with previously characterized mutants of yeast FTase β-subunit, S159N and Y362L (15). In contrast, the wild type human FTase, as well as the wild type yeast FTase, did not suppress the growth defect at 36 °C. Fig. 4B shows that the suppression was observed with the Y361M mutant also but not with the Y361I mutant nor with the H362A mutant. The human FTase β-subunit Y361L, Y361M, Y361I, or H362A was co-expressed with the FTase α-subunit. The limitation of growth by the Y361I mutant may be a threshold effect, since this mutant displayed a weak affinity for CIIL substrate peptides (see Fig. 3). All the human FTase β mutants except H362A were capable of complementing temperature-sensitive growth of yeast dpr1 (FTase β-subunit) and ram2 (FTase α-subunit) mutants defective in FTase when co-expressed with the human α-subunit (Fig. 4, C and D), confirming that they retain wild type FTase activity in vivo.

Of the other human FTase mutants, the P152M mutant was capable of complementing dpr1 mutant but was incapable of
FTI-resistant Farnesyltransferase

TABLE I
Kinetic parameters of human WT and mutant FTases

|       | Kₘ (µM) | kₘ (s⁻¹) | kₘ/Kₘ (µmol⁻¹’s⁻¹) |
|-------|---------|----------|--------------------|
| WT    | 0.38 ± 0.02 | 0.064 ± 0.004 | 1.6 × 10⁴ |
| P152M | 0.41 ± 0.07 | 0.059 ± 0.010 | 1.4 × 10⁵ |
| Y361L | 0.52 ± 0.03 | 0.035 ± 0.007 | 6.7 × 10⁴ |
| Y365L | 0.50 ± 0.06 | 0.046 ± 0.011 | 9.2 × 10⁴ |

FIG. 2. Utilization of GST-Ha-Ras and GST-Cdc42hs by the wild type and the mutant FTases. Varying concentrations of GST-CIIS or GST-CIIL substrate protein, an enzyme concentration of 100 nM and 1 µM [³H]FPP for 10 min at 37 °C, Kₘ and kₘ values were obtained from Lineweaver-Burk plots of the wild type and mutant enzymes. The values reported are the average including standard deviation of three independent experiments.

Y361L Mutant Exhibits Increased Resistance to FTase Inhibitors, in Particular to Tricyclic Inhibitors—Because the Y361L mutant shows altered CAA recognition, were not suppressing cal1 /^α/ phenotype (data not shown).

With another tricyclic FTI, SCH44342, IC₅₀ values for the wild type enzyme were approximately 2 µM, whereas the IC₅₀ value for the Y361L mutant was 400 µM (Fig. 5B). Again, the P152M and Y365L mutants remained sensitive to SCH44342 similar to the wild type enzyme. The increased resistance of the Y361L mutant to SCH56582 over SCH44342 appears to be due to the presence of a bromine at position 3 of the tricyclic ring, since this is the only difference between the two tricyclic compounds. The incorporation of a bromine atom at this position may either result in a more structurally compromised inhibitor molecule which is unable to enter the active site of the Y361L mutant or may interfere with the electronegative character of the molecule. This suggests that even discrete differences in inhibitor compounds are detectable by the mutant FTase.

In addition to tricyclic inhibitors, the mutant exhibits significant resistance to peptidomimetic inhibitors, B1088 and
FTI-resistant Farnesyltransferase

BMS193269 (Table II). B1088 is a peptidomimetic derived from a tetrapeptide CVFM, and it acts as a competitor of the CAAX substrate (32). B1088 inhibited the wild type FTase with an IC50 of 0.002 μM, whereas the Y361L mutant was inhibited with an IC50 of 0.03 μM, a 15-fold increase. With BMS193269, we observed that the mutant is 28-fold more resistant compared with the wild type enzyme. We also tested two other compounds, α-hydroxyfarnesyl phosphonic acid and manumycin, both of which act as competitive inhibitors of FTase with respect to the prenyl substrate, FPP. The mutant exhibits a modest increase in the resistance to α-hydroxyfarnesyl-phosphonic acid. However, the mutant showed almost similar levels of sensitivity to manumycin compared with the wild type enzyme. Taken together, these results suggest that the Y361L mutant shows varying degrees of resistance to different FTase inhibitors and that the mutant exhibits the most dramatic resistance to tricyclic inhibitors. In addition, the mutant appears to respond to slight structural differences among the FTase inhibitors.

Y361L Mutant Exhibits Increased Sensitivity to a Zinc Chelator—Whereas the mutant Y361L enzyme exhibits resistance to some FTase inhibitors, it shows markedly increased sensitivity to zinc chelators, HPH-5 and HPH-6. These compounds are efficient zinc chelators that were designed based on the metal chelating domain of bleomycin (41). HPH-5 is a tetradeionate ligand that strongly chelates divalent zinc ion through its two nitrogens and two sulfurs (36). HPH-5 is a tetradenine ligand that strongly chelates divalent zinc ion through its two nitrogens and two sulfurs (36). HPH-5 is a tetradenine ligand that strongly chelates divalent zinc ion through its two nitrogens and two sulfurs (36). HPH-5 is a tetradenine ligand that strongly chelates divalent zinc ion through its two nitrogens and two sulfurs (36). HPH-5 is a tetradenine ligand that strongly chelates divalent zinc ion through its two nitrogens and two sulfurs (36). HPH-5 is a tetradenine ligand that strongly chelates divalent zinc ion through its two nitrogens and two sulfurs (36). HPH-5 is a tetradenine ligand that strongly chelates divalent zinc ion through its two nitrogens and two sulfurs (36). HPH-5 is a tetradenine ligand that strongly chelates divalent zinc ion through its two nitrogens and two sulfurs (36). HPH-5 is a tetradenine ligand that strongly chelates divalent zinc ion through its two nitrogens and two sulfurs (36). HPH-5 is a tetradenine ligand that strongly chelates divalent zinc ion through its two nitrogens and two sulfurs (36). HPH-5 is a tetradenine ligand that strongly chelates divalent zinc ion through its two nitrogens and two sulfurs (36). HPH-5 is a tetradenine ligand that strongly chelates divalent zinc ion through its two nitrogens and two sulfurs (36). HPH-5 is a tetradenine ligand that strongly chelates divalent zinc ion through its two nitrogens and two sulfurs (36). HPH-5 is a tetradenine ligand that strongly chelates divalent zinc ion through its two nitrogens and two sulfurs (36). HPH-5 is a tetradenine ligand that strongly chelates divalent zinc ion through its two nitrogens and two sulfurs (36). HPH-5 is a tetradenine ligand that strongly chelates divalent zinc ion through its two nitrogens and two sulfurs (36). HPH-5 is a tetradenine ligand that strongly chelates divalent zinc ion through its two nitrogens and two sulfurs (36). HPH-5 is a tetradenine ligand that strongly chelates divalent zinc ion through its two nitrogens and two sulfurs (36). HPH-5 is a tetradenine ligand that strongly chelates divalent zinc ion through its two nitrogens and two sulfurs (36). HPH-5 is a tetradenine ligand that strongly chelates divalent zinc ion through its two nitrogens and two sulfurs (36). HPH-5 is a tetradenine ligand that strongly chelates divalent zinc ion through its two nitrogens and two sulfurs (36). HPH-5 is a tetradenine ligand that strongly chelates divalent zinc ion through its two nitrogens and two sulfurs (36). HPH-5 is a tetradenine ligand that strongly chelates divalent zinc ion through its two nitrogens and two sulfurs (36). HPH-5 is a tetradenine ligand that strongly chelates divalent zinc ion through its two nitrogens and two sulfurs (36). HPH-5 is a tetradenine ligand that strongly chelates divalent zinc ion through its two nitrogens and two sulfurs (36). HPH-5 is a tetradenine ligand that strongly chelates divalent zinc ion through its two nitrogens and two sulfurs (36).
enzymes were used instead. FTase assays were carried out without ment. Essentially similar results were obtained when E. coli from baculovirus-infected Sf9 HPH-5. The wild type and the Y361L mutant FTases were purified from Procedures" in the presence of increasing concentrations of HPH-5. were carried out for 10 min at 37 °C as described under "Experimental rescue of HPH-5 inhibition by the addition of ZnCl2. FTases purified also preincubated with HPH-5 for 5 min at 37 °C prior to the addition to His-362 is likely to have potentiated the increased sensitiv-

diamine showed no inhibition, whereas 1,10-phenanthroline values relative to HPH-5 and HPH-6. Dipyridyl and ethylenediamine (Table III). These metal chelators exhibited drastically reduced efficacy as inhibitors of WT or mutant FTase as seen by the 800–13,000-fold increase in IC50 values relative to HPH-5 and HPH-6. Dipyridyl and ethylenediamine showed no inhibition, whereas 1,10-phenanthroline showed some inhibition at 4 mM. However, there was no difference in sensitivity between the wild type and the mutant enzymes. The tyrosine 361 residue mutated in the FTI-resistant mutant is located adjacent to histidine 362 which is one of three residues of the β-subunit involved in chelating a zinc ion in the enzyme complex (5). The introduction of this mutation adjacent to His-362 is likely to have potentiated the increased sensitiv-

![Graph](image)

**TABLE III**

| Zn chelators       | IC50 WT | IC50 Y361L |
|---------------------|---------|------------|
|                     | μM      | μM         |
| HPH-5               | 620     | 1.9        |
| HPH-6               | 700     | 5.1        |
| 1,10-Phenanthroline | 4,000   | 4,300      |
| Dipyridyl           | 100,000 | 65,000     |
| Ethylenediamine     | 15,500  | 12,000     |

In this paper, we report, for the first time, identification of a mutant form of FTase resistant to FTase inhibitors. The mutant has increased resistance to FTIs, particularly to tricyclic inhibitors. Since the tricyclic inhibitors act as competitors of the CAAX substrate, these results suggest that the mutant is capable of recognizing subtle differences between the inhibitors and the substrate protein. This idea is further supported by the observation that the mutant is capable of recognizing differences between two tricyclic compounds, SCH56582 and SCH44342. These two compounds differ only in the presence of a bromine on the tricyclic ring of SCH56582 (31). Resistance to other classes of FTIs, specifically CAAX peptidomimetics, was also observed to a lesser extent, indicating that inhibitors generally bind differently to the active site of FTase. An important feature of our mutant is that it exhibits FTase activity toward normal substrates. The k_cat/K_m values of the Y361L mutant FTase for CIIS substrate are comparable to those of the wild type enzyme, whereas the k_cat/K_m for CIIL substrate is 10-fold greater for the mutant. In addition, the Y361L mutant enzyme can functionally replace yeast FTase in vivo as seen with the complementation of yeast FTase-defective mutants, dpr1 and ram2. A mutation of Arg-202 to alanine was previously identified that suggested discrete recognition differences among classes of peptidomimetic FTIs (10). In this mutant, however, the affinity for substrate peptides was completely abrogated as indicated by a greater than 400-fold increase in the K_m value for CAAX substrate.

Mutagenesis of yeast FTase has provided several clues regarding substrate recognition (10–15). Extensive mutagenesis of regions conserved among prenyltransferases has suggested the importance of residues that are in close proximity to the catalytic zinc ion in the three-dimensional crystal structure. However, previous studies with yeast FTase suggested Tyr-362 greatly impacted the recognition of peptide substrates (15). As shown in this paper, the corresponding residue Tyr-361 of human FTase also plays a critical role in the recognition of the CAAX substrate. Alteration of this residue to a hydrophobic residue such as leucine or methionine results in increased affinity for the CAAL motif proteins. This suggests that the residue Tyr-361 is located in or close to the CAAX peptide-binding site in the ternary substrate-bound enzymatic complex.

Recent structure determinations support the above idea (5–8). The structure published by Park et al. (5) shows that this residue is located very close to the presumed CAAX peptide-binding site in a hydrophobic pocket lined with aromatic residues. Moreover, Dunten et al. (6) have published an FTase structure that differs from the previous structure determination in the placement of the peptide and prenyl substrates in the active site cavity; however, the residues Pro-152, Tyr-361, and Tyr-365 are located along one side of the hydrophobic pocket in the center of the β-subunit barrel wherein the substrates presumably bind. Most recently, Strickland et al. (8) have shown that the ternary structure of rat FTase complexed with acetyl-Cys-Val-Ile-seleno-Met-COOH and a-hydroxyfarnesylphosphonic acid results in major rearrangements of active site side chains upon substrate binding. Tyr-361 and Pro-152 lie in narrow pockets in which the Ile and Met side chains are sequestered, respectively. This again suggests that these residues effect substrate recognition, while having little effect on catalysis. The conservation of position 361 among all FTases, but its lack of conservation among prenyltransferases including GGTTase I, also suggests a significant recognition role rather than catalytic role.
According to various three-dimensional structures, Tyr-361 is located very near the FPP substrate molecule and the peptide substrate in the presumed active site (Fig. 7A). When an energy minimized substitution of residue 361 with leucine was performed, the orientation of Leu-361 was pointed away from the FPP molecule, suggesting a relaxed active site in which a tetrapeptide containing $X = $ leucine could be accommodated (Fig. 7B). In contrast to the result with Y361L, alteration of residues Pro-152 or Tyr-365 resulted in only a minor increase in the CAAL affinity. Mutations at these residues in the yeast FTase display an increased affinity for the CAAL motif. Along with Tyr-361, residues Pro-152 and Tyr-365 reside in an interior hydrophobic pocket formed by the $b$-subunit, wherein the peptide and prenyl substrates bind (6). Therefore, not all mutations identified in yeast FTase exert similar effects in human FTase. Further mutagenic analysis of positions 152 and 365 are required to determine the importance of these sites in the human FTase enzyme.

Another interesting characteristic of the Y361L mutant is that it exhibits increased sensitivity to zinc chelators HPH-5 and HPH-6. The restoration of FTase enzymatic activity upon addition of excess $\text{ZnCl}_2$ at the start of the enzymatic reaction, rather than after the zinc chelator was preincubated with the FTase enzyme, suggests a complex is formed by HPH compounds bound to zinc at the active site. We believe that zinc affinity is not significantly altered in the mutant, since the mutant and the wild type enzymes were inhibited by similar concentrations of general metal chelators such as 1,10-phenanthroline, dipyridyl, and ethylenediamine. Taken together with the FTI results, it appears that the active site of the enzyme is altered in the mutant in a way that makes the mutant enzyme inaccessible to tricyclic FTIs but more accessible to HPH-5 and HPH-6. Structural analysis of the active site after an energy minimized mutation is imposed at position 361 (Fig. 7) reveals a definite realignment of the leucine at 361 away from the zinc ion, resulting in a relaxed configuration that may make the mutant enzyme more susceptible for inhibitor chelation and the observed sensitivity toward the HPH compounds. Alternatively, introduction of this mutation may shift the orientation of adjacent zinc ligand side chains such as His-362. However, further structural and mutational analysis of Tyr-361 and adjacent residues may be necessary to evaluate this idea.

Because the Y361L mutant exhibits FTI-resistant FTase activity, we speculated that introduction of the mutant gene into transformed cells may render cells resistant to FTI. To test this idea, we transiently expressed the Y361L mutant FTase $\beta$-subunit together with the $\alpha$-subunit in v-K-ras transformed NRK (KNRK) cells. The $\beta$-subunit was fused with the green fluorescent protein to observe selectively the transfected cells. Previously, we have shown that KNRK cells undergo dramatic morphological changes by SCH56582 (20). However, these morphological changes were not observed when KNRK cells expressed the mutant FTase. In contrast, KNRK cells expressing the wild type FTase at a similar level to that of the Y361L mutant still underwent FTI-induced morphological changes. These results support the possibility that the FTI-induced morphological changes may be potentiated by the inhibition of FTase in KNRK cells. There are other effects of FTI on KNRK cells which include inhibition of anchorage-independent growth, decrease of S-phase cells, accumulation of G1-phase cells, and induction of apoptosis (20, 23). It will be interesting to examine whether these FTI effects are altered by the expression of the Y361L mutant FTase. In addition, the Y361M and Y361I mutants, which demonstrated varying degrees of CAAL recognition, were resistant to SCH56582. These may be utilized to disseminate further the phenotypic characteristics resulting from FTI treatments. This line of investigation may provide critical results to pinpoint biologically significant effects induced by FTIs.

Our finding that a single amino acid change causes FTase to become completely resistant to FTase inhibitors raises general issues about the use of FTase inhibitors for cancer treatments. FTIs need to be administered continuously, since the termination of treatment with the inhibitors in a mouse mammary tumor model system led to recurrence of tumors (26). We are currently trying to obtain other FTase mutants resistant to FTIs. Collecting a variety of mutants resistant to the FTIs may provide information on how FTI-resistant mutants could be generated, and this knowledge could be important in predicting how readily such mutants arise.

It has not escaped our attention that our finding of the inhibition of FTase activity by a zinc chelator HPH-5 raises a possibility that a novel type of FTase inhibitor based on zinc chelation could be developed. Because the zinc ion participates in FTase catalysis (5–9), such an approach may be effective. In this regard, it is intriguing that HPH-5 may form a complex with the enzyme rather than simply chelating a zinc ion away from the enzyme. This suggests that a compound with specificity toward FTase might be obtained by the use of derivatives of HPH-5. Further work is needed to develop derivatives of HPH-5 that demonstrate suitable potency against the wild type human FTase.

$^2$ K. Del Villar and F. Tamanoi, unpublished results.
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Note Added in Proof—Recently, Strickland et al. (Strickland, C. L., Weber, P. C., Windsor, W. T., Wu, Z., Le, H. V., Albanese, M. M., Alvarez, C. S., Cesarz, d. del Rosario, J., Deskus, J., Mallams, A. K., Njorgo, F. P., Piwinski, J. J., Remiszewski, S., Rossman, R. R., Taveras, A. G., Vihubhlan, B., Doll, R. J., Girjavallabhan, V. M., and Ganguly, A. K. (1999) J. Med. Chem. 42, 2125–2135) reported crystal structures of FTase complexed with SCH compounds. The results show the close proximity of residue 361 to SCH44342.

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FTI-resistant Farnesyltransferase

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