Characterization of Ha-Ras, N-Ras, Ki-Ras4A, and Ki-Ras4B as in Vitro Substrates for Farnesyl Protein Transferase and Geranylgeranyl Protein Transferase Type I*

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Fang L. Zhang, Paul Kirschmeier, Donna Carr, Linda James, Richard W. Bond, Lynn Wang, Robert Patton, William T. Windsor, Rosalinda Syto, Rumin Zhang, and W. Robert Bishop‡

From the Departments of Tumor Biology and Structural Chemistry, Schering-Plough Research Institute, Kenilworth, New Jersey 07033

Ras proteins are small GTP-binding proteins which are critical for cell signaling and proliferation. Four Ras isoforms exist: Ha-Ras, N-Ras, Ki-Ras4A, and Ki-Ras4B. The carboxyl termini of all four isoforms are post-translationally modified by farnesyl protein transferase (FPT). Preylation is required for oncogenic Ras to transform cells. Recently, it was reported that Ki-Ras4B is also an in vitro substrate for the related enzyme geranylgeranyl protein transferase-1 (GGPT-1) (James, G. L., Goldstein, J. L., and Brown, M. S. (1995) J. Biol. Chem. 270, 6221–6226). In the current studies, we compared the four isoforms of Ras as substrates for FPT and GGPT-1. The affinity of FPT for Ki-Ras4B (Kₐ = 30 nM) is 10–20-fold higher than that for the other Ras isoforms. Consistent with this, when the different Ras isoforms are tested at equimolar concentrations, it requires 10–20-fold higher levels of CAAX-competitive compounds to inhibit Ki-Ras4B farnesylation. Additionally, we found that, as reported for Ki-Ras4B, N-Ras and Ki-Ras4A are also in vitro substrates for GGPT-1. Of the Ras isoforms, N-Ras is the highest affinity substrate for GGPT-1 and is similar in affinity to a standard GGPT-1 substrate terminating in leucine. However, the catalytic efficiencies of these geranylgeranylation reactions are between 15- and 140-fold lower than the corresponding farnesylation reactions, largely reflecting differences in affinity. Carboxyl-terminal peptides account for many of the properties of the Ras proteins. One interesting exception is that, unlike the full-length N-Ras protein, a carboxyl-terminal N-Ras peptide is not a GGPT-1 substrate, raising the possibility that upstream sequences in this protein may play a role in its recognition by GGPT-1. Studies with various carboxyl-terminal peptides from Ki-Ras4B suggest that both the carboxyl-terminal methionine and the upstream polyleucine region are important determinants for geranylgeranylation. Furthermore, it was found that full-length Ki-Ras4B, but not other Ras isoforms, can be geranylgeranylated in vitro by FPT. These findings suggest that the different distribution of Ras isoforms and the ability of cells to alternatively process these proteins may explain in part the resistance of some cell lines to FPT inhibitors.

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‡ To whom correspondence should be addressed: Dept. of Tumor Biology (K-15-4700), Schering-Plough Research Institute, 2015 Galloping Hill Rd., Kenilworth, NJ 07033. Tel.: 908-298-3050; Fax: 908-298-7115; E-mail: bob.bishop@spcorp.com.

1 The abbreviations used are: FPT, farnesyl protein transferase; FPP, farnesyl diphosphate; GGPT-1, geranylgeranyl protein transferase-1; GGPP, geranylgeranyl diphosphate; PAGE, polyacrylamide gel electrophoresis; SPA, scintillation proximity assay.
transfers a geranylated geranyl group from geranylgeranyl diphosphate (GGPP) to the cysteine residue of substrates containing a CAAX motif in which X is leucine. The substrates of GGPT-1 include RhoA, Rac-1, and the γ subunit of several heterotrimeric G proteins (13, 14). Although FPT and GGPT-1 display substrate selectivity, there are a few exceptions to these general rules. The Ki-Ras4B protein can be a substrate for both FPT and GGPT-1 in vitro (15). In addition, the small GTP-binding protein, RhO, can be either farnesylated or geranylgeranylated in vitro by GGPT-1 (16).

One role of farnesylation is to anchor Ras proteins to the membrane where they can participate in signal transduction. Abolishing isoprenylation disrupts Ras membrane association, thereby disrupting its function (7). It has been shown that prenylation is required for oncogenic Ras to transform cells (17, 18). Thus, inhibiting farnesylation may be a route for controlling the growth of Ras-transformed tumor cells. This has made FPT a very attractive target for anti-tumor drug discovery. Numerous inhibitors have been developed (11, 19), including FPP analogs (20), CAAX peptide analogs such as BZA-5B, L-731,734, B581, and Cys-AMBA-Met (21–24), and bisubstrate analogs (25). SCH44342 belongs to a novel class of tricyclic inhibitors. It is entirely nonpeptidic and has no sulfhydryl function, but it is a competitive inhibitor versus the CAAX protein substrate (26).

Inhibitors of FPT have been demonstrated to inhibit Ras farnesylation in cell culture and reverse cellular transformation induced by oncogenic Ras (21, 22, 26). Furthermore, such inhibitors can block tumor formation by Ha-, Ki-, and N-Ras transformed cells in nude mice (27, 28). Recently it was reported that several classes of FPT inhibitors block the anchorage-independent growth of human tumor cell lines; however, the sensitivity of a particular cell line to these inhibitors does not correlate to their Ras mutational status or their tissue of origin (29, 30). Similar observations have been made with the tricyclic FPT inhibitors. These observations raise questions as to the mechanism of this growth inhibition.

In the studies reported here, we examined for the first time all four full-length Ras isoforms as substrates for the prenyltransferases, FPT and GGPT-1. We found that the affinity of FPT for Ki-Ras4B is more than 20-fold higher than for the other forms of Ras. As a result, it requires 10–20-fold higher concentrations of SCH44342 or other CAAX-competitive inhibitors to block farnesylation of Ki-Ras4B than Ha-Ras. In addition, we found that not only Ki-Ras4B but also N-Ras and Ki-Ras4A are substrates for GGPT-1. The different distribution of Ras isoforms and the ability of cells to alternatively process these proteins may explain in part the resistance of some tumor cell lines to FPT inhibitors and may also contribute to the lack of cytotoxicity associated with these compounds.

EXPERIMENTAL PROCEDURES

Materials—Recombinant human FPT was expressed and purified (>95% pure) from the baculovirus/Si9 cell expression system according to procedures described previously (31). L-[3,4,5-3H]Farnesyl diphosphate (22.5 Ci/mmol) and L-[3,4,5-3H]geranylgeranyl diphosphate (19.5 Ci/mmol) were obtained from DuPont NEN. Zwittergent-3-14 was obtained from Fluka. FastMocTM chemistry. The side chain protecting groups were tert-butyloxycarbonyl for Ser, Thr, Asp, and Glu, tert-butyloxycarbonyl for Lys, and trityl for Cys and Gln. Solid-phase amino-terminal biotinylation after amino-terminal Fmoc (N-9-fluorenylmethoxycarbonyl) deprotection was performed manually, using n-biotin preactivated by O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate-O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate and 1-hydroxybenzotriazole (PerSeptive Biosystems). The peptides were cleaved and deprotected by trifluoroacetic acid with scavengers (80% trifluoracetic acid, 4% H2O, 4% phenol, 4% thioanisole, 4% ethanedithiol, 4% triisopropylsilane). The cleaved and deprotected peptides were separated from the resin by filtration and precipitated by anhydrous ethyl ether. The precipitated peptides were dissolved in H2O, rotary evaporated to remove the ether, and lyophilized. The crude peptides were finally purified by reversed phase high performance liquid chromatography, and the molecular weights were confirmed by mass spectroscopy.

Prenylation Chain Reaction Cloning and Expression of Ras Proteins—All Ras proteins were expressed as fusion proteins containing six histidine residues at the amino terminus. The coding region of human Ha-, N-, Ki-Ras4A, and Ki-Ras4B were amplified from appropriate cell lines by polymerase chain reaction and subcloned into QE vector to yield QE-Ras. The identity of all plasmids was confirmed by restriction mapping and DNA sequencing of the polymerase chain reaction-amplified fragments. Ha-Ras, Ha-Ras-CVLL, and N-Ras plasmids were transformed into SURE bacteria, while Ki-Ras4A and Ki-Ras4B were transformed into BL21 bacteria.

Purification of Ha-Ras, Ras-CVLL, and N-Ras Proteins—Six-histidine-tagged Ha-Ras, Ha-Ras-CVLL, and N-Ras proteins were purified from bacterial cells by nickel-affinity chromatography (Qiagen). All protein purification steps were performed at 4 °C. Cells were lysed by a microfluidizer in 64 mM Tris, pH 7.5, 5 mM MgCl2, 1 mM β-mercaptoethanol (buffer A) with 4 mM benzamidine, 2 µg/ml aprotinin, 2 µg/ml soybean trypsin inhibitor, 0.7 µg/ml pepstatin, 0.2 mM Pefabloc, and 0.5 µg/ml leupeptin. Insoluble material was removed by centrifugation at 15,000 × g for 20 min, and soluble protein was filtered (0.8 µm) and loaded onto an nickel-affinity column. The protein was eluted from the column with buffer A plus 100 mM imidazole. Imidazole was removed by a desalting column equilibrated with buffer A. Dithiothreitol was added to 1 mM, and the purified protein was stored in small aliquots at −80 °C. Ha-Ras, Ha-Ras-CVLL, and N-Ras obtained from this procedure are about 90% pure.

Purification of Ki-Ras4A and Ki-Ras4B Proteins—Six-histidine-tagged Ki-Ras4A and Ki-Ras4B proteins were purified by denaturing nickel-affinity chromatography followed by renaturation while bound to the column (32). Bacterial pellets were resuspended in denaturation buffer (6 M urea, 20 mM Tris, pH 8.0, 5 mM MgCl2, 30 mM GTP, 1 mM β-mercaptoethanol, 5% (v/v) glycerol, 50 mM NaCl with a protease inhibitor mixture) and lysed by using a microfluidizer. Insoluble material was removed by centrifugation as above, and soluble protein was filtered (0.8 µm) and loaded onto an nickel-affinity column. The column was washed overnight with renaturation buffer (20 mM Tris, pH 8.0, 5 mM MgCl2, 30 mM NaCl, 5% (v/v) glycerol, 50 µM β-mercaptoethanol). The column was then washed with renaturation buffer plus 100 mM NaCl, and bound proteins were eluted with renaturation buffer plus 100 mM imidazole. Imidazole was removed by a desalting column equilibrated with buffer A. Dithiothreitol was added to 1 mM, and the purified proteins were stored in small aliquots at −80 °C. Ki-Ras4A and Ki-Ras4B obtained from this procedure are about 90% pure.

Expression and Prenylation of Human GPT-1—GPT-1 and FPT share a common α subunit, but have distinct β subunits (33). To produce recombinant GPT-1, a cDNA clone for the human FPT α subunit was obtained from ATCC (ATCC63225). This cDNA was excised with BamHI and PvuII, and the resulting fragment was ligated into the vector p2-BAC (Invitrogen) to generate the construct p2B2N. A cDNA clone for the β subunit of rat GPT-1 in a pGem vector was obtained from Dr. Patrick Casey (Duke University Medical Center). This clone was humanized by mutating 10 rat residues to their human counterparts using the transformer site-directed mutagenesis kit (Clontech). The residues changed were: A2V, D7E, D91N, N110A, I133V, D143N, and the resulting fragment was ligated into the pGem vector to generate plasmid pGPT-1. The cDNA for GPT-1 β subunit was excised from pGem by digesting first with NotI, filling in with Klenow fragment, and digesting with HindIII. The resulting fragment was ligated into pBlueScript K+ to generate plasmid pBS-G. The cDNA for GPT-1 β subunit was excised from pBS-G by digesting with NotI and ApaI, and the resulting fragment was ligated into pA2BN to generate construct pG2-2B. This construct was used to produce recombinant baculovirus according to Summers and Smith (34). For GPT-1 production, log phase Sf9 cells (2 × 106/ml) were infected at a multiplicity of 2.
infection of 1.5 in a 10-liter Biologte tank and cultured for 3 days at 28 °C and 140 rpm in SP900-2 medium (Life Technologies, Inc.). Cells were harvested by centrifugation at 12,000 × g for 10 min at 4 °C. The purification of recombinant GGPT-1 was essentially the same as described previously (35). The final enzyme was about 50% pure.

**Determination of FPT and GGPT-1 Activity by Product Precipitation—** Activity of FPT and GGPT-1 with protein substrates was determined as described previously using acid precipitation of the prenylated product (9, 36). FPT reaction mixtures contained (in 200 μl): 50 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 20 mM KCl, 5 mM MgCl₂, 0.05–0.10, 0.2, 0.5, 1.0, 5.0, and 10.0 μM Ras substrates, and 0.5 μM [3H]FPP. GGPT-1 reaction mixtures were the same except [3H]GGPP was substituted for [3H]FPP and the reaction volume was changed to 50 μl. After pre-equilibration at 37 °C, reactions were initiated by addition of 1 nM FPT or 5 nM GGPT-1. For kinetic experiments, reactions were never allowed to proceed to more than 10% completion based on the limiting substrate.

**Determination of FPT and GGPT-1 Activity by Scintillation Proximity Assay (SPA)—** FPT and GGPT-1 activity with peptide substrates was determined by measuring the transfer of [3H]farnesyl or [3H]geranylgeranyl to biotinylated peptides as described previously (26, 37). The standard assay mixture contained 60 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 20 mM KCl, 50–2000 nM biotinylated peptide, 0.5 mM Zwittergent 3-14, 200 nM [3H]FPP (22.5 Ci/mmol) or [3H]GGPP (19.5 Ci/mmol), and 25 ng of purified recombinant FPT or 40 ng of GGPT-1 in a final volume of 50 μl. Assays were conducted at 25 °C for 20 min.

**RESULTS**

**Expression and Purification of Ras Proteins—** All four Ras isoforms (Ha-Ras, N-Ras, Ki-Ras4A, and Ki-Ras4B) were expressed in E. coli as amino-terminal His-tagged proteins and purified. The carboxyl-terminal sequences of each isoform are shown in Table I. The purified fusion proteins are about 90–95% pure and migrate at 28 kDa in SDS-PAGE (Fig. 1, top panel). The recombinant Ras proteins migrate slower than native Ras proteins due to the His-tag. To check the integrity of the CAAX box of these proteins, they were reacted with FPT in the presence of excess [3H]FPP under conditions where the reaction could proceed to completion. Following reaction with FPT, Ha-Ras and N-Ras proteins shift to a higher electrophoretic mobility form upon SDS-PAGE, characteristic of the prenylated proteins. Essentially all of the Ha-Ras and N-Ras undergo this gel shift, indicating that they possess intact CAAX boxes and can be farnesylated. Under the SDS-PAGE conditions employed here, Ki-Ras4A and Ki-Ras4B do not undergo a gel mobility shift following their farnesylation. To examine the extent of their farnesylation, gels were exposed to x-ray film. The autoradiographic intensity of Ki-Ras4A and Ki-Ras4B are comparable to those of Ha-Ras and N-Ras (Fig. 1, lower panel), indicating that these proteins are also intact and can serve as substrates for FPT.

**Prenylation and Geranylgeranylation of Ras Proteins—** All four Ras proteins serve as substrates for FPT (Fig. 2, A and B). The kinetic parameters Kₘ and kₐₐₜ for these proteins were measured at saturating FPP concentrations. The Kₘ values for Ha-Ras, N-Ras, and Ki-Ras4A are 0.6 μM, 0.4 μM, and 0.4 μM, respectively. The Kₘ for Ki-Ras4B is 0.03 μM. Thus, the affinity of FPT for Ki-Ras4B is about 20-fold higher than that for the other Ras isoforms. The kₐₐₜ values for FPT for Ha-, N-, and Ki-Ras4B are comparable (1.5–2.5 min⁻¹) while that for Ki-Ras4A is about 3-fold higher. In terms of catalytic efficiency, Ki-Ras4B is the best substrate for FPT among the Ras isoforms (Table II).

It was previously shown that Ki-Ras4B is an in vitro substrate for geranylgeranyl protein transferase-1 (GGPT-1) (15). We performed GGPT-1 assays to determine whether other Ras isoforms are also substrates for this enzyme. In agreement with previous reports, Ha-Ras is not a substrate for GGPT-1. However, like Ki-Ras4B, N-Ras and Ki-Ras4A are GGPT-1 substrates (Fig. 2C). Kₘ and kₐₜ values for these proteins were determined at saturating GGPP concentration. Among N-Ras and the two Ki-Ras isoforms, all of which terminate in methionine, N-Ras is the highest affinity GGPT-1 substrate (Kₘ = 2.1 μM). This is close to the Kₘ value for Ha-Ras-CVLL, a protein with the GGPT-1 consensus Leu in the X position of the CAAX box. The Kₘ values for Ki-Ras4A and Ki-Ras4B are about 10-fold higher than that for Ha-Ras-CVLL. The kₐₜ values for Ki-Ras4A and Ki-Ras4B are about 5-fold higher than those for N-Ras and Ha-Ras-CVLL. Overall, the catalytic efficiencies for the GGPT-1 reactions with N-Ras or either Ki-Ras isoform are similar and about 2-fold lower than that for Ha-Ras-CVLL (Table II). It is clear from these data that farnesylation of these substrates is the preferred reaction. In the case of Ki-Ras4B, the catalytic efficiency of its farnesylation reaction is about 140-fold higher than that of its reaction with GGPT-1, largely due to the affinity difference.

**Carboxyl-terminal Peptides Can Account for Most, but Not All, of the Properties of Ras Proteins—** The four Ras isoforms are highly homologous to each other, with most of the differences residing in the last 24 residues (2). To examine whether the carboxyl-terminal sequence differences account for the differences in substrate properties between these proteins, we prepared four biotinylated peptides comprising the 15 carboxyl-terminal residues of the various Ras proteins (Table I). These peptides were evaluated as substrates for FPT and GGPT-1 using an SPA assay. All four peptides are substrates for FPT (Fig. 3A). The apparent Vₘₐₓ for the Ki-Ras4A peptide is 3- to 4-fold higher than those for the other Ras peptides, consistent with assays utilizing whole protein substrates. The highest affinity peptide substrate was that derived from the Ki-Ras4B
These peptides were also tested as substrates for GGPT-1 (Fig. 3B). The Ki-Ras4A and Ki-Ras4B peptides are substrates for this enzyme. The GGPT-1 reaction with the Ki-Ras4B peptide proceeded with a higher affinity (about 3-fold) and a higher Vmax than the reaction with the Ki-Ras4A peptide. Surprisingly, the N-Ras peptide was not geranylgeranylated by GGPT-1, suggesting that upstream sequences present in the N-Ras protein may play a critical role in its recognition by GGPT-1.

Both the Polylysine Region and the Carboxyl-terminal Methionine Are Important for Geranylgeranylation of the Ki-Ras4B Peptide—To understand the critical features enabling the Ki-Ras4B peptide to serve as a substrate for GGPT-1, we prepared carboxyl-terminal peptides of different lengths (Table I). Biotin-GKKKKKKSKTKCVIM is the longest peptide tested, biotin-KKSKTKCVIM contains four less amino-terminal lysine residues, and biotin-TKCVIM is further truncated. In biotin-TKCVIS the last residue is changed from methionine to serine. GKKKKKKSKTKCVIM is a very good substrate for GGPT-1 (Fig. 4). Removing four of the six contiguous lysine residues greatly decreases the affinity of GGPT-1 for this peptide. Further removal of the KKSK sequence had little effect on its utilization; however, changing methionine to serine completely abolished its ability to serve as a GGPT-1 substrate (Fig. 4). Therefore, both the lysine residues and the carboxyl-terminal methionine contribute to the utilization of Ki-Ras4B by GGPT-1. This conclusion is consistent with the results of James et al. (15) who addressed this question using chimeric Ha/Ki-Ras proteins.

Geranylgeranylation of Ki-Ras4B by FPT—We also tested the ability of the four full-length Ras proteins to be used as substrates for FPT and GGPT-1 with different isoprene donors. GGPT-1 did not transfer farnesyl from FPP to any of the Ras proteins. Similarly, FPT did not transfer geranylgeranyl from GGPP to Ha-Ras, N-Ras, or Ki-Ras4A (data not shown). However, FPT can catalyze the transfer of geranylgeranyl from GGPP to Ki-Ras4B (Fig. 5A). The Km and kcat values for this reaction were 1.4 μM and 0.6 min⁻¹, respectively (Table II). This is similar to kinetic values reported by Pompliano et al. (38) for transfer of geranylgeranyl to yeast Ras-1 containing a started by adding 20 ng of FPT and proceeded for 4 min at 37 °C. Reactions were then stopped and processed as described under “Experimental Procedures.”

### Table II

| Protein Substrate | FPT activity | GGPT activity |
|-------------------|-------------|---------------|
|                   | Km (μM)    | kcat (μM·min⁻¹) | Km (μM)    | kcat (μM·min⁻¹) |
| Ras-CVLL          | NA         | NA            | NA         | NA              |
| Ha-Ras            | 0.6        | 1.2           | 2.0        | NA              |
| N-Ras             | 0.4        | 1.9           | 4.7        | 2.1             |
| Ki-Ras4A          | 0.4        | 5.0           | 12.4       | 8.8             |
| Ki-Ras4B          | 0.03       | 1.6           | 53.3       | 12.0            |
| Ki-Ras4B + GGPP   | 1.4        | 0.6           | 0.43       | NA              |
|                   | 1.4        | 0.6           | 0.43       | NA              |

FIG. 2. Prenylation of full-length Ras proteins. A, farnesylation of Ha-Ras, N-Ras, and Ki-Ras4A. The standard FPT reaction mixture was used. The concentration of FPP was 0.5 μM, while the protein substrate concentrations were varied as indicated. Reactions were started by adding 20 ng of FPT and proceeded for 4 min at 37 °C. Reactions were then stopped and processed as described under “Experimental Procedures.” B, farnesylation of Ki-Ras4A, N-Ras, and Ki-Ras4B. Assay conditions are the same as in A except that the protein substrate was Ki-Ras4B. C, geranylgeranylation of Ras proteins. The standard GGPT-1 reaction mixture was used. The concentration of GGPP was 0.5 μM, while the protein substrate concentrations were varied as indicated. The reactions were started by adding 20 ng of GGPT-1 and proceeded for 4 min at 37 °C. ○, Ras-CVLL; ●, N-Ras; □, Ki-Ras4A; ■, Ki-Ras4B; ▲, Ha-Ras.
CVIM terminus. This $K_m$ is about 47-fold larger than that of Ki-Ras4B in the normal FPT reaction, while its $k_{cat}$ is about one-third. This activity was confirmed using different Ras peptides (Fig. 5B). FPT can transfer geranylgeranyl from GGPP to the Ki-Ras4B peptide, but not to the Ha-Ras or N-Ras peptides. Weak activity was observed with the Ki-Ras4A peptide at the highest concentration tested. The $V_{max}$ for the Ki-Ras4B peptide is about one-half of that for the normal FPT reaction.

Inhibition of Ras Farnesylation—SCH44342 is a tricyclic inhibitor of FPT, which competes with various farnesyl acceptors with a $K_i$ of 0.24 $\mu$M. To evaluate its potency against the different isoforms of Ras, we measured its IC$_{50}$ using a constant substrate concentration for all four proteins. When Ras proteins were present at 1.2 $\mu$M, the IC$_{50}$ values of SCH44342 for Ha-Ras, N-Ras, Ki-Ras4A, and Ki-Ras4B were 0.6 $\mu$M, 0.9 $\mu$M, and 10 $\mu$M, respectively (Fig. 6A). The IC$_{50}$ for Ki-Ras4B is 10–20-fold higher than for the other Ras proteins, indicating that, as predicted for a competitive inhibitor, Ki-Ras4B farnesylation is more difficult to inhibit than the other Ras isoforms when present at equimolar concentration. These results are consistent with the affinity of the Ras isoforms for FPT.

SCH44342 did not inhibit any of the GGPT-1-catalyzed reactions except at very high concentrations (IC$_{50}$ $\geq$ 200 $\mu$M). This is in contrast to the results of James et al. (15) who reported that BZA-5B inhibited the reaction of GGPT-1 with Ki-Ras4B but not with Ha-Ras-CVLL. Interestingly, the geranylgeranylation of Ki-Ras4B catalyzed by FPT is inhibited by SCH44342 more potently than farnesylation of Ki-Ras4B by FPT (data not shown). This most likely reflects the reduced affinity of FPT for Ki-Ras4B when geranylgeranyl diphosphate is the isoprene donor.

DISCUSSION

In this work we directly compared all four full-length Ras proteins as substrates for the two isoprenyl protein transferases, FPT and GGPT-1. As FPT substrates, the affinity for Ki-Ras4B is 20-fold greater than that for the other Ras proteins (Table II; Fig. 2, A and B). This affinity difference between Ki-Ras4B and the other isoforms is also evident in the experiments using FPT inhibitors. The IC$_{50}$ values of SCH44342 for
Ha-Ras, N-Ras, and Ki-Ras4A are very similar, while the IC₅₀ value for Ki-Ras4B is 10–20-fold higher (Fig. 6A). Under the assay conditions employed, a greater difference was observed for the various carboxyl-terminal peptide substrates derived from these proteins (Fig. 6B). This observation may be significant in the clinical development of FPT inhibitors. To completely inhibit the farnesylation of Ki-Ras4B, significantly higher FPT inhibitor concentrations will be required than those needed for inhibiting Ha-Ras processing.

Early studies of FPT specificity employing tetrapeptides indicated that the Ki-Ras CAAX peptide (CVIM) was a more potent inhibitor of FPT activity than the Ha-Ras CAAX peptide (CVLS) (12). Studies in which various CAAX boxes were introduced into the yeast Ras-1 protein also indicated that the Ki-Ras CAAX sequence supported a higher affinity reaction (38). In these experiments the chimeric protein terminating in CVIM had a $K_m$ value of approximately 0.14 μM (39, 40). The authentic full-length Ki-Ras4B which we used has an apparent $K_m$ of 30 nM (Table II). Our data indicate that Ki-Ras4A and N-Ras behave more like Ha-Ras with respect to their affinity for FPT and sensitivity to competitive inhibitors.

The high affinity of FPT for Ki-Ras4B is not simply due to the presence of a carboxyl-terminal methionine, since N-Ras and Ki-Ras4A have 10-fold lower affinity despite having carboxyl-terminal methionines. It is likely that the stretch of lysine residues upstream of the Ki-Ras4B CAAX box, and not found in the other Ras isoforms, contributes to this high affinity. This is supported by the observation that Ki-Ras4B peptides lacking these lysines are inhibited by SCH44342 with IC₅₀ values.

**FIG. 5.** Geranylgeranylation of full-length Ras proteins and carboxyl-terminal peptides by FPT. A, prenylation of Ki-Ras4B by FPT. The standard FPT assay conditions were used except either [3H]FPP or [3H]GGPP was the isoprene donor. The assays were conducted at 37 °C for 4 min. B, geranylgeranylation of Ras peptides by FPT. The standard SPA assay conditions for FPT were used except that GGPP was substituted for FPP. The peptide concentrations were varied as indicated, and the assays were conducted at 25 °C for 20 min. ○, Ha-Ras peptide; ●, N-Ras peptide; □, Ki-Ras4A peptide; ■, Ki-Ras4B peptide.

**FIG. 6.** Inhibition of Ras farnesylation by SCH44342. A, inhibition of farnesylation of Ras proteins. The standard FPT assay conditions were employed except that various concentrations of SCH44342 (or Me₂SO vehicle control) was present. Ras proteins were used at 1.2 μM, and the assays were conducted at 37 °C for 4 min. FPT activity in the vehicle controls was designated as 100%. ○, Ha-Ras; ●, N-Ras; □, Ki-Ras4A; ■, Ki-Ras4B. B, inhibition of farnesylation of Ras peptides. The standard FPT SPA assay conditions were used except that various concentrations of SCH44342 or vehicle control were included. The concentration of all Ras peptides was 0.3 μM, and the assays were conducted at 25 °C for 20 min. The FPT activity in vehicle controls was designated as 100%. Symbols are the same as in A.
similar to that of the Ha-Ras peptide. The higher affinity that we observe with the authentic, full-length Ki-Ras4B (versus the yeast Ras-CVIM construct) probably reflects the presence of this region. Similar observations were made by James et al. (15) who found that Ki-Ras4B is about a 50-fold higher affinity substrate for FPT than is Ha-Ras. However, in those studies the apparent \( K_m \) values reported (10 \( \mu \)M for Ha-Ras and 0.2 \( \mu \)M for Ki-Ras4B) are unusually high compared with those found in the current studies and those reported by Pompliano et al. (39). Through construction of chimeric proteins James et al. (15) observed that addition of either the Ki-Ras4B CAAX box or the polybasic domain onto Ha-Ras increased its affinity for FPT.

The other significant finding in this work is that in addition to being FPT substrates, N-Ras and Ki-Ras4A, like Ki-Ras4B, serve as in vitro GGPT-1 substrates (Fig. 2C), while Ha-Ras is only a FPT substrate. This is the first demonstration that N-Ras and Ki-Ras4A are GGPT-1 substrates despite the fact that these proteins terminate in methionine rather than the leucine present in most substrates for this enzyme. However, it is now clear that the substrate specificity of GGPT-1 is not restricted to proteins terminating in Leu or Phe. Surprisingly, these proteins terminate in methionine rather than the

Recently, however, we observed that when COS monkey kidney proteins, Ki-Ras4A and Ki-Ras4B peptides were geranylgeranylated by FPT, that these upstream sequences in N-Ras and Ki-Ras4A have not served as FPT substrates, N-Ras and Ki-Ras4A, like Ki-Ras4B, are GGPT-1 substrates (Fig. 2). The other significant finding in this work is that in addition to being FPT substrates, N-Ras and Ki-Ras4A, like Ki-Ras4B, serve as in vitro GGPT-1 substrates (Fig. 2C), while Ha-Ras is only a FPT substrate. This is the first demonstration that N-Ras and Ki-Ras4A are GGPT-1 substrates despite the fact that these proteins terminate in methionine rather than the


This is the first demonstration that for its farnesylation, indicating that the farnesylation reaction is greatly favored. The Ki-Ras carboxyl-terminal peptide containing a CVIM terminus (38). FPT can bind both isoprenoid diphosphates, but it has much higher affinity for FPP. The finding that the \( K_m \) for Ki-Ras4B farnesylation is much smaller than that for its geranylgeranylation by FPT provides further evidence that isoprenoid diphosphate binding to FPT affects its binding affinity for protein acceptors.

FPT inhibitors, including the tricyclic compounds related to SCH44342, block anchorage-independent growth of a number of human tumor cell lines with a wide range of potency. The data reported here suggest that the type and level of Ras isoforms expressed in various cell types and the ability of cells to alternatively prenylate these proteins may contribute to such differences in sensitivity. We are currently examining the capacity of a variety of tumor cell lines which differ in sensitivity to FPT inhibitors to carry out the alternative prenylation reactions. Finally, as suggested by James et al. (15) the lack of cytotoxicity exhibited by FPT inhibitors may in part be explained by alternative prenylation of Ras isoforms and, perhaps, of other FPT substrates such as the nuclear lamins.

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Characterization of Ha-Ras, N-Ras, Ki-Ras4A, and Ki-Ras4B as in Vitro Substrates for Farnesyl Protein Transferase and Geranylgeranyl Protein Transferase Type I

Fang L. Zhang, Paul Kirschmeier, Donna Carr, Linda James, Richard W. Bond, Lynn Wang, Robert Patton, William T. Windsor, Rosalinda Syto, Rumin Zhang and W. Robert Bishop

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