Selective Inhibition of Farnesyl-Protein Transferase Blocks Ras Processing in Vivo*

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The ras oncogene product, Ras, is synthesized in vivo as a precursor protein that requires post-translational processing to become biologically active and to be capable of transforming mammalian cells. Farnesyltransferase appears to be a critical modification of Ras, and thus inhibitors of the farnesyl-protein transferase (FPTase) that catalyzes this reaction may block ras-dependent tumorigenesis. Three structural classes of FPTase inhibitors were identified: (a-hydroxyfarnesyl)phosphonic acid, chaetomellic acids, and zaragozic acids. By comparison, these compounds were weaker inhibitors of geranylgeranyl-protein transferases. Each of these inhibitors was competitive with respect to farnesyl diphosphate in the FPTase reaction. All compounds were assayed for inhibition of Ras processing in Ha-ras-transformed NIH3T3 fibroblasts. Ras processing was inhibited by 1 \( \mu \text{m} \) (a-hydroxyfarnesyl)phosphonic acid. Neither chaetomellic acid nor zaragozic acid were active in this assay. These results are the first demonstration that a small organic chemical selected for inhibition of FPTase can inhibit Ras processing in vivo.

The ras oncogene is found mutated in approximately 25% of human cancers (1, 2). Many laboratories have focused on the biochemistry and biology of ras-induced cellular transformation in anticipation that this information might prove useful in the identification of novel anticancer therapeutics (1, 2). One area of research that has recently attracted attention in this regard is the post-translational modifications of the ras gene product, Ras. These modifications are required for appropriate subcellular localization of Ras in the plasma membrane and for Ras to exhibit cell-transforming activity (3-5). The first and obligatory step in post-translational modification of the Ras precursor is farnesylation on the thiol group of the Cys residue located at the Ras COOH terminus. This Cys residue forms part of the prenylation recognition sequence, CAAX,\textsuperscript{1} found in many mammalian proteins. Transfer of a farnesyl moiety from farnesyl diphosphate to the Ras CAAX peptide is catalyzed by farnesyl-protein transferase (FPTase, Refs. 6-8). Several genetic studies have shown that inhibition of Ras farnesylation prevents Ras membrane localization and blocks Ras-induced cell transformation. For example, mutation of the Cys of CAAX to Ser renders Ras transformation-defective (9). Similarly, in the yeast Saccharomyces cerevisiae, mutation or disruption of the ram1 locus (which encodes the \( \beta \) subunit of FPTase) blocks the phenotypes normally observed in yeast with an activated [Val-19]RAS2 allele (10-14). These observations suggest that pharmacological inhibition of FPTase may also block Ras-dependent biological properties in mammalian cells (3-5). By contrast, subsequent modifications of farnesylated Ras including removal of AAX residues by proteolytic cleavage, methyl esterification of the new carboxyl terminus, and palmitoylation apparently are not obligatory for cell-transforming activity (15-17).

Following the identification of FPTase, it was quickly recognized that the substrates utilized by this enzyme, farnesyl diphosphate and the CAAX peptides, could serve as models for designing selective FPTase inhibitors (6, 7). Farnesyl diphosphate preferentially serves as substrate for FPTase versus other mammalian prenyl-protein transferases including geranylgeranyl-protein transferase type I (GGPTase-I) and GGPTase-II or Rab-GGPTase (19-25). Similarly, some CAAX sequences preferentially serve as substrates for FPTase while others function best as substrates of GGPTase-I or GGPTase-II (19-25). Although the Cys residue appears to be essential for binding of CAAX tetrapeptides to FPTase (6, 7), various amino acid changes can be introduced into the AAX positions which improve potency and/or confer nonsubstrate properties to the peptides (19, 26-29). Most importantly, the COOH-terminal amino acid of CAAX tetrapeptides determines which protein-prenyl transferase will bind the peptide. FPTase prefers CAAX sequences ending in Ser or Met, whereas GGPTase-I prefers CAAX sequences ending in Leu and GGPTase-II or Rab-GGPTase modifies proteins containing XXCC or XCXC motifs. Since most mammalian proteins are geranylgeranylated rather than farnesylated (30, 31), selective inhibitors of FPTase should exhibit fewer toxic side effects than nonselective inhibitors of both FPTase and GGPTase. Here, we report the identification of potent and selective inhibitors of FPTase in vitro and show that one FPTase inhibitor is capable of blocking Ras processing in vivo.

MATERIALS AND METHODS

Inhibitors—Chaetomellic acids A and B were isolated from a fermentation extract of the Coelomycete Chaetomella acutissima. The inactive inhibition was extracted with methyl ethyl ketone and subjected to chromatographic separation. Details of the fermentation, isolation, and structural elucidation of chaetomellic acids A and B will be described elsewhere. Zaragozic acid A (32, 33) was provided by K. E. Wilson (Merck Research Laboratories). Zaragozic acid A analog was a gift of R. W. Marquis and G. D. Berger (Merck Research Laboratories); the preparation of zaragozic acid A analog will be described elsewhere.\textsuperscript{2} (a-Hydroxyfarnesyl)phosphonic acid (28) was provided by N. J. Anthony and R. P. Gomez (Merck Research Laboratories). Lovastatin was supplied by A. Alberts (Merck Research Laboratories).

Prenyl-Protein Transferase Assays—Partially purified prepara-

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\textsuperscript{1} The abbreviation used are: CAAX, where C is Cys, A any aliphatic amino acid, and X, any other amino acid; FPTase, farnesyl-protein transferase; GGPTase, geranylgeranyl-protein transferase; PAGE, polyacrylamide gel electrophoresis; FPP, farnesyl diphosphate.

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Pharmacological inhibition of prenyl-protein transfers.

The indicated compounds were incubated at various concentrations in reactions having prenyl-protein transferses partially purified from bovine brain with substrates at near-Km concentrations under conditions prescribed under "Materials and Methods." Data are the average of 2-5 determinations. ND, not determined. GGPP, geranylgeranyl diphosphate. The peptide sequence is indicated using one letter amino acid abbreviations.

Table I. 

| Compound | IC50 (nM) |
|----------|----------|
|          | FPTase   | GGPTase-I | GGPTase-II |
| Chaetomellic acid A | 55      | 92,000    | 34,000     |
| Chaetomellic acid B | 185     | 54,000    | ND         |
| Zaragozic acid A | 216      | 620       | 66,000     |
| Zaragozic acid A analog | 12   | 1,710     | 16,800     |
| (o-Hydroxyfarnesyl) phosphonic acid | 30     | 35,800    | 87,000     |
| GGPP* | 280     | 800       | 660        |
| FPP* | 550     | 16,000    | 36,000     |
| CVIM* | 90      | 35,000    | >500,000   |

* Previous results are shown for comparison (19).
FIG. 2. Inhibition of FPTase by chaetomellic acid A and zaragozic acid A analog. Double-reciprocal plots of initial velocity versus the indicated substrate concentrations in the presence of different fixed concentrations of inhibitor. A, varying farnesyl diphosphate (FPP) concentration in the presence of 0 (□), 1.3 (●), 4 (○), and 12 (▲) nM chaetomellic acid A. Ras-CVIM concentration was held constant at 100 nM. B, varying Ras-CVLS concentration in the presence of 0 (▲), 2.33 (○), and 21 (□) nM chaetomellic acid A. FPP concentration was held constant at 20 nM. C, varying FPP concentration in the presence of 0 (□), 7 (●), and 35 (○) nM zaragozic acid A analog. Ras-CVIM concentration was held constant at 100 nM. D, varying Ras-CVLS concentration in the presence of 0 (□), 7 (●), and 21 (○) nM zaragozic acid A analog. FPP concentration was held constant at 20 nM. Assays were performed as described previously (28). In A and B, assays were done with recombinant human FPTase purified to homogeneity from E. coli (see footnote 4). Similar results were obtained with bovine FPTase (not shown). Assays in C and D were done with homogeneous FPTase purified from bovine brain (28).

at residue 59 (see Ref. 36). On SDS-PAGE, viral Ha-Ras appears as a doublet with the phosphorylated form migrating more slowly than the nonphosphorylated form (see Fig. 3, lanes 1 and 8). Approximately 25% of viral Ha-Ras is phosphorylated in these cells (36). This banding pattern is useful because it generates an internal standard for assessing relative band mobilities and intensities. In the processing assay, the mobility of Ras on SDS-PAGE is a function of farnesylation because it generates an internal standard for assessing relative band mobilities and intensities. In the processing assay, the mobility of Ras on SDS-PAGE is a function of farnesylation with processed Ras migrating more rapidly than unprocessed Ras. Lovastatin, an inhibitor of the rate-limiting step in the isoprenoid biosynthetic pathway, has previously been shown to block Ras processing in vivo and was used here as a positive control (Fig. 3, lanes 2 and 9) (17, 37, 38). Chaetomellic acid A and the zaragozic acids did not inhibit Ras processing when tested up to 100 μM for 24 h (Fig. 3, lanes 3–5). Shorter incubations of 4 h with these compounds in serum-free medium also did not reveal any inhibitory activity (not shown). In contrast, Ras processing was inhibited by (α-hydroxyfarnesyl)phosphonic acid at concentrations ≥1 μM (Fig. 3, lanes 6, 7, and 10). The observed inhibition of Ras processing with 1 μM (α-hydroxyfarnesyl)phosphonic acid processing (Fig. 3, lane 7) was only slightly less than that seen with 100 μM compound (Fig. 3, lane 6). Ras processing was not inhibited when (α-hydroxyfarnesyl)phosphonic acid was tested at 0.1 μM or below (not shown).

To confirm that the Ras mobility shifts observed in the compound-treated cells were due to inhibition of Ras processing, we demonstrated that the bands assigned as unprocessed Ras were soluble upon cell fractionation (not shown) and were not associated with the cell membrane fractions as is normally the case with farnesylated Ras (17, 38). We also attempted but were unable to radiolabel the unprocessed Ras in the compound-treated cells with [3H]mevalonic acid. [3H]Mevalonic acid is converted to [3H]farnesyl diphosphate in vivo and is then utilized by FPTase to farnesylate Ras under normal conditions (17, 38). As an additional control, we evaluated (α-hydroxyfarnesyl)phosphonic acid in RAT1 cells expressing a form of viral Ha-ras that has a mutation in the CAAX sequence (Ras-CVLS mutated to Ras-CVLL). This substitution changes Ras from a substrate for FPTase to a substrate for GGPTase-I. (α-Hydroxyfarnesyl)phosphonic acid at 100 μM did not inhibit the processing of Ras-CVLL, whereas 15 μM lovastatin effectively inhibited the processing of this geranylgeranylated protein (not shown). These results are consistent with our interpretation that the inhibition of Ras-CVLS processing by (α-hydroxyfarnesyl)phosphonic acid

| Inhibitor | Type of inhibition | K<sub>i</sub> (μM) |
|-----------|--------------------|-----------------|
| Chaetomellic acid A | Competitive | 3.5 ± 0.2 |
| Zaragozic acid A analog | Competitive | 0.5 ± 0.3 |
| (α-Hydroxyfarnesyl)phosphonic acid | Competitive | 5.2 ± 0.7 |

TABLE II

Inhibition patterns and constants for inhibitors of FPTase

The K<sub>i</sub> value is derived from a nonlinear least squares fit of the initial velocity data to either a purely competitive or purely noncompetitive inhibition model. Data for chaetomellic acid A and zaragozic acid A analog are from Fig. 2; data for (α-hydroxyfarnesyl)phosphonic acid are from Ref. 28.

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