Polyisoprenylation of Ras in Vitro by a Farnesyl-Protein Transferase*

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Farnesylation of Ras occurs in vitro on a Cys residue in the C-terminal sequence -Cys-Val-Leu-Ser (termed a CAAX box). This modification is required for Ras membrane localization and cell transforming activity. Using [3H]farnesyl-PP, as precursor and Escherichia coli-expressed Ras, forms of Ras having the CAAX sequence were radiolabeled upon incubation with the cytosolic fraction of bovine brain. Forms of Ras having a deletion of the CAAX sequence or a Cys to Ser substitution in this sequence were not substrates. Radioactivity incorporated into Ras by bovine brain cytosol was released by treatment with iodomethane but not with methanolic KOH indicating a thioether linkage. High pressure liquid chromatography analysis of the cleavage products on a C-18 column showed a major peak of radioactivity that co-eluted with a farnesol -Ser-Val-Leu-Ser were not inhibitory. These results required for membrane localization of Ras and is referred to as a CAAX box1 (9).

All Ras proteins are modified by the isoprenoid farnesyl at the C-terminal Cys via a thioether linkage (3–5). Mutational studies have shown that the Cys of the CAAX box is required for all of the C-terminal processing steps suggesting that polyisoprenylation is the first step (3, 9). Other proteins have been identified that are modified by farnesyl or geranylgeranyl moieties (10–13).

It has been speculated that a putative farnesyl-protein transferase using farnesyl-PP, (FPP) as precursor is responsible for Ras farnesylation (14). Recently, Vorburger et al. (15) demonstrated that mevalonate derivatives could be incorporated into lamin B2, which has a CAAX sequence, using a rabbit reticulocyte lysate. In the present study, we show that Ras can be farnesylated in broken cell preparations by a farnesyl-protein transferase activity.

MATERIALS AND METHODS

Polyisoprenylation of Ras—[3H]Mevalonic acid (35 Ci/mmol) and [3H]FPP (20 Ci/mmol) were purchased from Du Pont-New England Nuclear. [3C]FPP (49.7 mCi/mmol) was prepared enzymatically from partially purified prenyl transferase, [4-14C]isopentyl-PP, (47.5 mCi/mm, Amersham Corp.), and geranyl-PP: the methodology for this procedure will be described elsewhere. Reactions contained 280 pmol (10 μCi) of [3H]mevalonic acid, 500 pmol (0.025 μCi) of [3C]PP, or 25 pmol (0.5 μCi) of [3H]FPP. In a standard 50 μl reaction, 250 pmol of Ras purified from Escherichia coli (17) was added to 35 μl of rabbit reticulocyte lysate (Promega, L4210) or a tissue extract (100,000 x g supernatant, 10 mg/ml). Tissue extracts were prepared as described previously (18) in buffer containing 50 mM Tris-Cl, pH 8.0, 1 mM EGTA, 1 mM MgCl₂, 1 mM dithiothreitol, 10 μg/ml aprotinin, 0.5 μM phenylmethylsulfonyl fluoride. Buffer conditions were adjusted by the addition of 0.1 volume of 500 mM Tris-Cl, pH 8.0, 50 mM MgCl₂ or 0.1 volume of 500 mM NaHepes, pH 7.5, 50 mM MgCl₂, 50 mM dithiothreitol. In the Tris-buffered assays, the reactions were incubated for 1 h at 24 °C. Reaction velocities in the Hepes-buffered assays were at least 4 times greater, and incubations were for 10 min at 24 °C.

Ras proteins used as substrates were expressed in E. coli and purified (17, 18). Ras proteins having different CAAX hxx sequences were constructed with oligonucleotide linkers as described (17). E. coli-expressed Rho was a gift of J. C. Licali (Instituto de Investigaciones Biomedicas, Madrid). Geranyl and trans,trans-farnesol were purchased from Aldrich. FPP, geranyl-PP, and dimethylallyl-PP, were synthesized as described (16). Geranylgeraniol and geranylgeranyl-PP, were generous gifts of Dr. Tetsuo Takigawa (Kuraray Co., Ltd., Tokyo) and Prof. Kyozo Ogura (Tohoku University, Sendai), respectively.

Peptides were prepared with an Applied Biosystems model 430A peptide synthesizer, purified by reverse-phase HPLC, and characterized by amino acid analysis and fast atom bombardment mass spectrometry. Chemical farnesylation was achieved by reacting trans,trans-farnesyl bromide (Aldrich) with unprotected peptide. Peptide solutions were prepared with 10 mM dithiothreitol. Dimethyl sulfoxide, which is compatible with the enzyme assay, was required to solubilize the farnesylated peptide.

Enzyme reactions were analyzed by SDS-PAGE on 13.7% polyacrylamide gels and visualized by autoradiography after fluorographic

1 The abbreviations used are: CAAX box, a sequence motif in the farnesilation acceptor site; EGTA, [ethylenebis(oxyethylenenitrilo)]-tetractetraic acid; FPP, farnesyl-PP; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC high pressure liquid chromatography.

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enhancement with Enlightning (Du Pont-New England Nuclear). For immunoprecipitation of radiolabeled Ras, reactions were first extracted with Triton X-114 using the procedure of Bordier (19). After phase separation at 37 °C, the upper aqueous phase was removed, and the detergent phase was diluted by the addition of 160 μl of cold phosphate-buffered saline plus 150 μl of Ras monoclonal antibody Y13-259 coupled to Sepharose-protein A beads (2, 3).

Chemical Cleavage and Identification of the Isoprenoid Moiety on Ras—Following Triton X-114 extraction and immunoprecipitation of radiolabeled Ras, the protein was treated using procedures described previously (5, 20). Chemical cleavage was initiated with 100 μl of CHL (Aldrich) for 16 h at 37 °C in the dark. An aliquot (30 μl) of 35% NaCO₃ was added, followed by an overnight room temperature incubation. Reactions were extracted 3 times with 400 μl each of CHCl₃/methanol (9:1). The lower organic phases were pooled, evaporated under nitrogen, resuspended in 200 μl of solvent A (50% CH₃CN, 25 mM phosphate-buffered saline plus 150 μl of Ras monoclonal antibody Y13-259), and an aliquot was applied to a 0.5 x 15-cm HPLC column (Vydac). Elution was achieved at 1 ml/min using a 22.5-ml linear gradient of 0–15% solvent B (100% CH₃CN, 25 mM H₂PO₄), followed by a steeper gradient (56.5 ml at 1 ml/min) of 15–100% solvent B. The elution profile was monitored by measuring absorbance at 210 nm. Fractions (250 μl) were collected and measured for radioactivity in 2.5 ml of Redi-Safe scintillation fluid (Beckman).

RESULTS AND DISCUSSION

Farnesylation of Ras in Vitro—To evaluate in vitro polyisoprenylation of Ras, we have used two engineered Saccharomyces cerevisiae RAS gene products, [Leu⁺]RAS1(term.) and [Leu⁺]RAS1(term.)*CVLS (Ref. 17). Both proteins contain the first 184 amino acids of yeast RAS1 which does not have any Cys residues. [LeuGR]RAS1(term.) ends with a Pro ring at the unique C-terminal region of [Leu⁺]RAS1(term.)CVLS suggesting that modification was occurring at 5-MeVal. Treatment of both proteins with farnesol as described under "Materials and Methods" using [¹⁴C]FPP incorporated 40-200-fold more radiolabel incorporation was observed (not shown).

![Fig. 1. SDS-PAGE of Ras labeled with either [³H]mevalonate or [¹⁴C]FPP.](image)

A. Ras labeled with [³H]mevalonate or [¹⁴C]FPP. B. Ras labeled with [³H]mevalonate or [¹⁴C]FPP. [Leu⁺]RAS1(term.)*CVLS and [Leu⁺]RAS1(term.) were labeled in a reticulocyte lysate with [³H]mevalonate (lanes 1–3) or [¹⁴C]FPP (lanes 4–6) as described under "Materials and Methods." Aliquots of the total reaction (A) or Y13-259 immunoprecipitates of the total reaction (B) were analyzed on 13.7% SDS-polyacrylamide gels, and proteins were visualized by fluorography. Lanes: 1 and 4, minus Ras; 2 and 5, [Leu⁺]RAS1(term.)*CVLS; 3 and 6, [Leu⁺]RAS1(term.). Mᵦ, molecular size in kilodaltons.

Farnesylation of Ras in Vitro-To evaluate in vitro polyisoprenylation of Ras, we have used two engineered Saccharomyces cerevisiae RAS gene products, [Leu⁺]RAS1(term.) and [Leu⁺]RAS1(term.)*CVLS (Ref. 17). Both proteins contain the first 184 amino acids of yeast RAS1 which does not have any Cys residues. [LeuGR]RAS1(term.) ends with a Pro ring at the unique C-terminal region of [Leu⁺]RAS1(term.)CVLS suggesting that modification was occurring at 5-MeVal. Treatment of both proteins with farnesol as described under "Materials and Methods" using [¹⁴C]FPP incorporated 40-200-fold more radiolabel incorporation was observed (not shown).

Activity was not detected in the membrane fraction. Incorporation of [¹⁴C]FPP into Ras was also detected using a bovine brain cytosolic fraction (Fig. 2). No incorporation was observed with forms of Ras having a deletion of the CAAX box (lane 2) or a Cys to Ser substitution ([Leu⁺]RAS1(term.)*CVLS, lane 3). Two ras-related 20-kDa G-proteins were also tested. YPT1 (C-terminal sequence Gly-Gly-Cys-Cys) was not radiolabeled (lane 4) whereas Rho (-Cys-Val-Val-Leu) was an effective substrate (lane 3). Ras proteins having an extended CAAX box (-Cys-Val-Leu-Ser-Ala) or a shortened sequence (-Cys-Val-Leu) were poor substrates (not shown); at 5 μM, no radiolabel incorporation was detected. At 100 μM, 40- and 200-fold less radiolabel incorporation was observed for [Leu⁺]RAS1(term.)*CVLSA and [Leu⁺]RAS1-(term.)*CVLS respectively, compared with the control [Leu⁺]RAS1(term.)*CVLS.

A product identification was performed as described under "Materials and Methods" using Ras protein radiolabeled by [¹⁴C]FPP and bovine brain cytosol. Recovery of radioisotope following treatment with iodomethane was 65%. Recovery of [¹⁴C]FPP standard in a parallel reaction lacking Ras was 61% indicating that the 35–40% loss occurred during the extraction procedures. The reaction products were analyzed by HPLC using a C-18 column as shown in Fig. 3. A major peak representing 86% of the recovered radioactivity co-eluted with the farnesol standard. A similar profile was observed for Ras labeled with [³H]mevalonate in rabbit reticulocyte lysates (not shown). No peaks were detectable for assays having [Leu⁺]RAS1(term.) suggesting that modification was occurring at the unique C-terminal region of [Leu⁺]RAS1(term.)*CVLS. Ras was positively identified as the acceptor protein by immunoprecipitation with the Ras-specific monoclonal antibody Y13-259 (Fig. 1B).

Ras labeled with [³H]mevalonate or [¹⁴C]FPP was quantitatively extracted into a Triton X-114 detergent phase as has been described previously for Ras modified in vivo (2, 3); Ras lacking incorporated radiolabel remained in the aqueous phase (data not shown). This result indicated that the in vitro modification conferred hydrophobic properties to Ras.

The activity or activities responsible for radiolabel incorporation into Ras remained in the reticulocyte cytosol fraction upon centrifugation at 100,000 x g. Polyisoprenylation of Ras using [¹⁴C]FPP could also be detected in 100,000 x g supernatant fractions prepared from rat brain, heart, kidney, liver, lung, skeletal muscle, spleen, and testis; the highest activity was observed using the supernatant prepared from brain. Activity was not detected in the membrane fraction. Incorporation of [¹⁴C]FPP into Ras was also detected using a bovine brain cytosolic fraction (Fig. 2). No incorporation was observed with forms of Ras having a deletion of the CAAX box (lane 2) or a Cys to Ser substitution ([Leu⁺]RAS1(term.)*CVLS, lane 3). Two ras-related 20-kDa G-proteins were also tested. YPT1 (C-terminal sequence Gly-Gly-Cys-Cys) was not radiolabeled (lane 4) whereas Rho (-Cys-Val-Val-Leu) was an effective substrate (lane 3). Ras proteins having an extended CAAX box (-Cys-Val-Leu-Ser-Ala) or a shortened sequence (-Cys-Val-Leu) were poor substrates (not shown); at 5 μM, no radiolabel incorporation was detected. At 100 μM, 40- and 200-fold less radiolabel incorporation was observed for [Leu⁺]RAS1(term.)*CVLSA and [Leu⁺]RAS1-(term.)*CVLS respectively, compared with the control [Leu⁺]RAS1(term.)*CVLS.

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Activity (cpm x 10^-12)

Fraction

\[ \text{IC}_{50} \]  

\begin{tabular}{|c|c|}
\hline
Compound & \text{IC}_{50} \ (\mu M) \\
\hline
Dimethylallyl-PP & 325 \\
Geranyl-PP & 67 \\
Farnesyl-PP & 0.7 \\
Geranylerganyl-PP & 1.4 \\
Farnesol & >100 \\
Geranylerganiol & >100 \\
\hline
\end{tabular}

\[ \text{S-(trans,trans)-Farnesyl- CVLS} \]

potently inhibited the assay. We do not know whether Ras is polyisoprenylated when geranylerganyl-PP is the precursor; however, Ras is not significantly modified by geranylerganylnl \textit{in vivo} (5) or \textit{in vitro} (this work) when \( [\text{H}] \) mevalonate is used as a precursor of polyisoprene biosynthesis. Geranylerganyl-PP, and dimethylallyl-PP, were less potent than competitors. Farnesol and geranylerganiol were inactive up to 100 \( \mu M \) indicating that the pyrophosphate moiety is required for competition.

Several peptides having the Harvey Ras CAAX sequence were also tested (Table I). The smallest peptide, CVLS, was nearly as potent as the larger peptides indicating that the critical interactions between Ras and the farnesyl-protein transferase are confined to the 4-residue CAAX box region. The sulphydryl moiety is apparently essential for competition because peptides having a Cys to Ser substitution were inactive. Peptides lacking the CAAX sequence, free Cys, \( \text{S)-(trans,trans)-farnesyI-CVLS} \), and unrelated peptides having a single Cys residue were not competitors when tested up to 100 \( \mu M \) (not shown). Initial experiments with peptide SSGCVLS indicate that it is a substrate for farnesylation. The predicted product of the smallest active peptide, \( \text{S-(trans,trans)-farnesyI-CVLS} \), was approximately 13-fold less potent than peptide alone (Table I).

**CONCLUSIONS**

The results in this study have identified an activity in the cytosolic fraction of mammalian cells which utilizes FPP to attach a farnesyl moiety onto Ras. The substrate specificity and peptide competition experiments indicate that the 4-residue CAAX box is the minimal sequence efficiently recognized by the enzyme. Although FPP was 2-fold more potent than geranylerganyl-PP, in competition experiments, it is possible that the farnesyl-protein transferase activity may also be involved with the modification of proteins with geranylerganyl. If this were true, we speculate that different sequences within the CAAX box (13) influence whether a protein is a substrate for farnesylation or geranylerganylation. Future studies with pure enzyme will help to resolve the specificity of the activity. We presume that the farnesyl-protein transferase detected in cell extracts is responsible for

**Properties of the Bovine Brain Farnesyl-Protein Transferase Activity—**Farnesylation of Ras was inhibited by EDTA; 5 mM MgCl\(_2\) served as an effective source of divalent cation. By varying the \([\text{H}]\)FPP concentrations from 0.03 to 30 \( \mu M \) and the [Leu\(^{64}\)]H Ras concentration from 0.03 to 250 \( \mu M \), we estimate apparent \( K_a \) values of 0.3 and 1–2 \( \mu M \) for FPP and Ras, respectively. Equivalent farnesylation was observed using Ras complexed with either guanosine-5'\'-O-(3-thiotriphosphate) or guanosine-5'\'-O-(2-thiodiphosphate). As shown in Fig. 4, the farnesyl-protein transferase activity eluted from a Superose-12 gel filtration column as a single peak having an apparent size of 190 kDa. A smaller activity peak having a size of 75 kDa was observed when the protease inhibitors leupeptin and antipain were not included in the chromatography buffer.

To determine specificity of the farnesyl transferase activity, several isoprenoids were tested as competitors of the farnesylation reaction (Table I). In assays having 0.5 \( \mu M \) \([\text{H}]\)FPP and 1 \( \mu M \) Ras, nonradioactive FPP and geranylerganyl-PP,
the physiological processing of Ras. Preliminary experiments\(^3\) with S. cerevisiae show that farnesyl-protein transferase activity is present in extracts prepared from wild-type cells, but activity is not detected in extracts from cells having the ram1 mutation that impairs Ras processing \(\text{in vivo}\) \(^{21,22}\). Clear assignment of the physiological importance of this activity will have to await rigorous biochemical analyses of yeast strains and mammalian cells having genetic defects in Ras processing.

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