Newly Synthesized Rho A, Not Ras, Is Isoprenylated and Translocated to Membranes Coincident with Progression of the G1 to S Phase of Growth-stimulated Rat FRTL-5 Cells

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Ras and Rho are involved in the regulation of signal transduction events governing cell growth and cell proliferation. Protein prenylation is essential for the activation and/or the translocation of these small GTPases; however, protein geranylgeranylation rather than farnesylation is required for G1/S transition. We studied prenylation and translocation of Ras and Rho A during G1/S progression in growth-stimulated rat thyroid FRTL-5 cells. Immunoblot analysis revealed that both Ras and Rho A were detected in membrane fractions at G0. Rho A was eliminated from the membrane fraction during G1 and was not detected on the membrane at mid-G1. Translocation of Rho A from the cytoplasm back to the membranes was observed during late G1 phase. In contrast, Ras remains in the membrane fraction through the cell cycle progression from G1 to S phase. The immunoprecipitation of Rho A from the membrane fraction demonstrated that newly synthesized Rho A, labeled by pulsing cells with [35S]methionine and [3H]coenzyme A, was geranylgeranylated and associated with the membrane in late G1. These results indicate that Rho A, not Ras, was eliminated from membrane fraction during G1 progression and that newly synthesized Rho A is geranylgeranylated and translocated to membranes during G1/S progression in growth-stimulated FRTL-5 cells.

Over the past five years, a number of small GTPases have been implicated in the signal transduction events governing cell growth and proliferation. Among the most thoroughly characterized is a small GTPase, Ras; Ras proteins function as relay switches transducing biological information from extracellular signals to the nucleus (1–3). Recently, a number of other small G-proteins, in particular members of the Rho family of GTPases, were also shown to be essential for cell cycle progression (4, 5).

The activity of Ras and Rho proteins requires their attachment to the inner leaflet of the plasma membrane (1, 6, 7). This process is initiated by the covalent attachment of a hydrophobic farnesyl or geranylgeranyl group to a cysteine at the fourth position from the COOH terminus of the proteins (8). Farnesyl or geranylgeranyl groups are derived from two major intermediate metabolites of the mevalonate, farnesylpyrophosphate, or geranylgeranylpyrophosphate, respectively. The enzymes that catalyze the attachment of farnesyl or geranylgeranyl moieties to proteins are now well characterized (9, 10). Farnesyltransferase transfers farnesyl from farnesylpyrophosphate to Ras (11), whereas geranylgeranyltransferase I attaches the lipid geranylgeranyl from geranylgeranylpyrophosphate to Rho proteins (12).

It is well known that activation of HMG-CoA1 reductase is essential for G1/S progression in various growth-stimulated cells (13, 14); thus, the contributions of farnesylated and/or geranylgeranylated proteins to cell cycle control have been intensively investigated. Recent experiments using specific inhibitors of geranylgeranyltransferase I and farnesyltransferase indicate that protein geranylgeranylation, not farnesylation, is required for the G1 to S phase transition of the cell cycle in mouse fibroblasts (15). Cyclin-dependent kinase (Cdk) enzymes are activated for entry into the S phase of the cell cycle (16). Elimination of Cdk inhibitor protein p27Kip1 during the G1 to S phase is required for the activation process (17). We demonstrated that geranylgeranylation of Rho proteins is essential for the degradation of p27Kip1 and facilitates the progression from G1 to S phase in growth-stimulated rat FRTL-5 cells (18). These data indicate a pivotal role of protein geranylgeranylation, not farnesylation, in G1/S transition, although the detailed mechanism is still unclear.

This investigation was performed to gain further insights into the requirement of geranylgeranylphosphate for G1/S transition in growth-stimulated FRTL-5 cells. We studied the following questions: When does the isoprenylation and membrane association of Ras and Rho small GTPases occur during G1/S phase progression in FRTL-5 cells? What correlation exists between growth-stimulated activation of HMG-CoA reductase and membrane association of Ras and Rho small GTPases?

EXPERIMENTAL PROCEDURES

Preparation of Liposomes of Isoprenoids—[3H]Geranylgeranylpyrophosphate ([3H]GGPP) was purchased from NEN Life Science Products. GGPP and farnesylpyrophosphate (FPP) were purchased from Sigma. Liposomes containing GGPP or FPP (200 µg) were prepared as described previously (18).

Cell Culture, Fractionation, and Assays—FRTL-5 cells (ATCC CRL-2095; E-mail: aizan@med.m.chiba-u.ac.jp).
8305), a strain of rat thyroid cells in continuous culture, were grown in Coon’s modified Ham’s F-12 medium supplemented with 5% calf serum and a six-hormone mixture consisting of thyrotropin (1 × 10⁻¹⁰ m), insulin (10 μg/ml), hydrocortisone (0.4 ng/ml), human transferrin (5 μg/ml), glycyl-L-histidyl-L-lysine acetate (10 ng/ml), and somatostatin (10 ng/ml) (19). This medium is referred to as 6H medium. For all experiments, cells were initially cultivated in 6H medium for at least 3 days. As appropriate to individual experiments, cells were then shifted to medium containing no thyrotropin, no insulin, and only 0.2% calf serum, which is referred to as 4H medium, for at least 5 days before use in individual experiments. Fluorescence-activated cell sorter analysis revealed that the percentage of cells in G1/G0 at that point is over 95% (18). These cells are referred to as quiescent cells; the quiescent cells rechallenged with 6H medium are referred to as growth-stimulated cells.

Whole cell lysates were prepared, Cdk2 activity was measured, and [3H]thymidine incorporation into DNA was determined as previously reported (19). HMCoA reductase activity was determined as reported by Grieco et al. (14). The cell cycle profiles of samples were analyzed, and crude membrane- or cytosol-containing fractions were prepared as previously reported (18, 19). Pravastatin and GGPP liposomes. The membrane fraction was recovered at 30 h and solubilized. Rho A was measured by fluorography after gel electrophoresis. In a separate experiment, growth-stimulated cells treated with pravastatin after the addition of 6H medium were incubated with liposomes containing HMG-CoA reductase inhibitor (20) was kindly provided by Dr. S. Kurakata (Sankyo Pharmaceutical Co., Ltd. Tokyo, Japan).

Growth-stimulated FRTL-5 cells that had been treated with pravastatin and incubated with GGPP liposomes (10 μg) were pulse labeled with [3H]methionine and [3H]S-cysteine for a 6-h period, starting 18 h after the addition of 6H medium and pravastatin and 2 h after adding the GGPP-containing liposomes after the 6-h labeling period. Cells were washed and the incubation continued in unlabeled 6H medium plus pravastatin and GGPP liposomes. The membrane fraction was recovered at 30 h and solubilized. Rho A was measured by fluorography after gel electrophoresis. In a separate experiment, growth-stimulated cells treated with pravastatin after the addition of 6H medium were incubated with liposomes containing [3H]GGPP (18.5 MBq/μmol) starting at 16 h, and membranes were recovered and processed in the same way to detect labeled geranylgeranyl in the Rho A.

Preynl group identification of Rho A was performed by the method of Farrel et al. (21). In brief, labeled Rho A in the membrane fraction was delipidated, and the prenyl groups were removed by methyl iodide cleavage. The labeled lipids were separated by C18 reverse-phase HPLC as reported by Farrel et al. (21).

Preparation of Human Rho A cDNA—Full-length human Rho A cDNA encoding nucleotides 1–613, the entire coding region as reported by Fagan et al., was obtained using polymerase chain reaction, using the following oligonucleotides as primers, 5'-TGGTGGCTGAGGACATG-GCTGCCA-3' and 5'-GCAAGTCTTTCAAGACAGCC-3', and using cDNA prepared from human kidney poly(A)⁺ RNA using a cDNA synthesis kit. Northern analysis of Rho A was performed as described previously (24).

RESULTS

Time Course of HMG-CoA Reductase Activation and DNA Synthesis in Growth-stimulated FRTL-5 Cells—Challenging quiescent FRTL-5 cells with thyrotropin, insulin, and 5% calf serum resulted in the increased of HMG-CoA reductase activity and mevalonate synthesis after about 10 h (Fig. 1A). Activity reached a maximum at or near the mid-G1 phase of the cell cycle (22 h) and returned to prestimulation levels when these cells entered the S phase (after 30 h) (Fig. 1A). Thymidine incorporation into DNA increased after 20 h and was maximal at 36 h. Thus, growth stimulation-induced activation of HMG-CoA reductase preceded the onset of DNA synthesis.

Time Course Study for p27 Elimination and Cdk2 Activation in Growth-stimulated FRTL-5 Cells—Significant elimination of p27 begins 24 h after exposure to growth stimulation, proceeds at a relatively slow rate for the next 6 h, and exponentially decreases thereafter until disappearance after 36 h (Fig. 1B). Immunoblotting reveals a coincident increase of the rapidly migrating, phosphorylated form of Cdk2 as a single major 33-kDa band (Fig. 1B), when p27 decreases toward nonmeasurable levels. The kinase activity associated with Cdk2 was increased at 24 h and maximal at 36 h (Fig. 1B). Thus, growth stimulation-induced activation of HMG-CoA reductase coincides with the onset of p27 elimination and Cdk2 activation.

Time Course Study for Membrane Association of Ras and Rho A during G1 Progression—Immunoblot analysis of membrane fractions for Ras and Rho A reveals that Rho A begins to disappear from the membrane 12 h after exposure to the

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Fig. 1. Time course of HMG-CoA reductase activation and DNA synthesis (A), the elimination of p27 (B), Cdk2 activation (B), and membrane association of Ras and Rho A (C) in growth-stimulated FRTL-5 cells. Quiescent FRTL-5 cells were incubated with 6H medium for the indicated times. In A, HMG-CoA reductase activity assays were performed as described under “Experimental Procedures,” and thymidine incorporation into DNA was measured during the last 2 h of each noted incubation period. Data in both cases are the means ± S.D. of three independent experiments. To measure Cdk2 activity and the elimination of p27, cells were harvested by trypsinization. Cell lysates (30 μg) were analyzed by immunoblotting with antibodies against Cdk2 and p27 as described under “Experimental Procedures.” In C, membrane-containing fractions were prepared at the indicated times (“Experimental Procedures”), and 30 μg of each sample was analyzed by immunoblotting with antibodies against Ras and Rho A.
TABLE I

| Thymidine incorporation into DNA and G1/S transition in FRTL-5 cells | \( \text{dpm} \times 10^5 \) | \% S phase |
|---|---|---|
| 4H | 805 ± 148 | 3.5 ± 0.4 |
| 6H | 37556 ± 1844 | 29.5 ± 1.3 |
| 6H + Pra | 1369 ± 209 | 4.8 ± 0.6 |
| 6H + Pra + GGPP (0–16 h) | 1838 ± 305 | 5.2 ± 0.9 |
| 6H + Pra + GGPP (16–36 h) | 35823 ± 2065 | 30.5 ± 1.2 |
| 6H + Pra + FPP (16–36 h) | 2251 ± 378 | 5.1 ± 0.7 |

**Requirement of GGPP, not FPP, for thymidine incorporation into DNA and G1/S transition in FRTL-5 cells**

Quiescent FRTL-5 cells were incubated with 6H medium for 36 h with or without the noted additions. \(^{3}H\)/Thymidine incorporation into DNA and the analysis of cell cycle progression were performed as described in the text. Data represent the means ± S.D. of three independent experiments. Pra, pravastatin.

**DISCUSSION**

Pravastatin (1200 \( \mu \)g/ml) inhibited growth-stimulated DNA synthesis and induced G1 arrest in FRTL-5 cells (Table I). All of the effects of pravastatin on DNA synthesis and cell cycle progression were wholly reversed by the addition of GGPP (0–16 h) but not FPP (16–36 h). Thus, pravastatin-induced cell cycle arrest in the presence of pravastatin and 10 \( \mu \)M cold GGPP was added to [\(^{3}H\)]GGPP (data not shown). HPLC analysis of the lipids associated with the protein showed that the protein on the membrane in this experiment was geranylgeranylated (Fig. 3C). This indicates that newly synthesized Rho A was geranylgeranylated and translated to membranes in the presence of GGPP.

**Growth-stimulated FRTL-5 Cells**—Northern analysis showed that Rho A mRNA was present as a doublet as reported previously (25) and that Rho A mRNA levels did not change during G1 progression (Fig. 3A). This indicates that the growth stimulation of FRTL-5 cells had no effect on Rho A gene expression.

**Pulse Labeling of Rho A with [\(^{35}S\)]Methionine** and \([\(^{14}H\)]GGPP**—** Immunoprecipitation and fluorography of Rho A in the membrane fraction of cells pulsed with \([\(^{35}S\)]\)methionine and \([\(^{14}H\)]GGPP** showed** that Rho A mRNA levels did not change during G1 progression (Fig. 3A). This indicates that the growth stimulation of FRTL-5 cells had no effect on Rho A gene expression.

**Time Course Study for the Expression of Rho A mRNA** in the presence of pravastatin and 10 \( \mu \)M GGPP revealed that the newly synthesized Rho A (Fig. 3B). In a separate experiment wherein the protein was labeled by supplementation with \([\(^{35}S\)]\)methionine and \([\(^{14}H\)]GGPP** showed** that Rho A mRNA levels did not change during G1 progression (Fig. 3A). This indicates that the growth stimulation of FRTL-5 cells had no effect on Rho A gene expression.

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Growth stimulation induces expression of the HMG-CoA reductase activity in various cells (13, 14), thereby allowing Ras and Rho A to be activated after isoprenylation with FPP or GGPP, which are derived from mevalonate (1, 8, 7). To see which of these small GTPases were playing a critical role in cell cycle progression during the growth of rat FRTL-5 cells, we compared time-dependent changes of Ras and Rho A in the membrane fraction with time dependent changes in HMG-CoA reductase activity, DNA synthesis, and the cell cycle regulating molecules, p27 and Cdk2, which control G1/S transition in growth-stimulated FRTL-5 cells.

In this study, both Ras and Rho A were detected in membrane fraction at G0. Growth stimulation of FRTL-5 cells induced the elimination of Rho A from membranes at mid-G1, but the level of Rho A in membranes returned to prestimulation levels at late G1. In contrast, Ras remained in membrane fraction throughout the cell cycle progression from G1 to S phase. The elimination of Rho A from membranes precedes the activation of HMG-CoA reductase and is already significant at 12 h when the reductase activation is just starting. Thus, we demonstrate that growth stimulation of FRTL-5 cells causes an elimination of Rho A from membranes, not Ras, in early G1 and that Rho A re-associates with the membrane in association with the activation of HMG-CoA reductase at mid-G1. Ras does not show such changes.

Pravastatin inhibition of HMG-CoA reductase activity in growth-stimulated FRTL-5 cells blocks DNA synthesis and induces G1 arrest. The mevalonate metabolite, FPP, does not reverse this inhibitory effect of pravastatin, despite the fact that PPP is incorporated into cells as well as GGPP, as measured in cells exposed to liposomes containing either [3H]FPP or [3H]GGPP. This demonstrates that FPP is not required for G1/S transition of FRTL-5 cells. Vogt et al. also reported that protein farnesylation is not required for the G1 to S phase transition of the cell cycle in mouse fibroblasts (15).

In this study, Ras was detected in the membrane fraction at G0, and the level of Ras in the membrane fraction did not change during G1/S progression. Furthermore, Ras in membranes was not affected by the treatment with pravastatin and supplementation with PPP. These results suggest that the farnesylation of Ras and its translocation into membranes does not occur during G1/S progression in FRTL-5 cells. These experiments may therefore explain why FPP is not required for G1/S transition in FRTL-5 cells; however, it remains unclear when Ras is farnesylated and translocated to the membrane during cell cycle progression.

In contrast to Ras, Rho A diminishes in the membrane fraction at the near mid-G1 phase of the cell cycle (18 h). It is not clear whether Rho A lost from membranes during this period is further processed or not; the degradation pathway of Rho A has not yet been determined in detail. We have, however, examined the effects of several protease inhibitors on Rho A elimination in growth-stimulated FRTL-5 cells. MG-115, an inhibitor of the ubiquitin-proteasome pathway, had no effect on growth-stimulated elimination of Rho A from membranes (data not shown). It therefore seems unlikely that the ubiquitin-proteasome pathway is involved in growth-stimulated elimination of Rho A from membranes in FRTL-5 cells.

After its elimination from membranes at the near mid-G1 phase of the cell cycle, the level of Rho A in the membranes returns to prestimulation levels at late G1. Pravastatin completely blocked the reappearance of Rho A in the membrane fraction at late G1, and Rho A was detected only in the cytosolic fraction. This inhibitory effect of pravastatin on the reappearance of Rho A in the membrane was completely reversed by GGPP, when GGPP was added 16 h after stimulation (at the onset of HMG-CoA reductase activation). On the other hand, the presence of GGPP during the first 16 h of culture did not reverse the inhibitory effect of pravastatin; the requirement of GGPP for G1/S transition and DNA synthesis had the same time course dependence. Thus, the inhibitory effect of pravastatin on G1/S transition and DNA synthesis had the same time course dependence. Thus, the inhibitory effect of pravastatin on G1/S transition and DNA synthesis was not reversed by GGPP during the first 16 h of culture but rather by its addition and continued presence beyond 16 h. These data indicate that growth-stimulated activation of HMG-CoA reductase is closely linked to the translocation of Rho A to membranes, not Ras, and to G1/S transition in FRTL-5 cells.

The membrane association of prenylated proteins has been reported to be cyclical (26). Such cycling of prenylated proteins...
could be caused by proteins such as the guanine nucleotide dissociation inhibitor (GDI) that appears to extract prenylated small GTPases out of the membrane and into the cytoplasm (27, 28). Rho GDI makes a complex with the GDP-binding form of the Rho proteins and thereby inhibits the dissociation of GDP from and the subsequent binding of GTP to the Rho gene products (28). In addition to this regulatory function of Rho GDI, Rho GDI regulates the binding of Rho A in the membranes (29). It remains unclear whether GDI might be involved in growth-stimulated elimination of Rho A from membranes in FRTL-5 cells.

In this report, we performed pulse label studies using [35S]methionine and [35S]cysteine in the presence of transport inhibitors and in cells supplemented with 10 μM GGPP. Pulse label experiments revealed that newly synthesized Rho A was translocated to membranes in the presence of GGPP. A separate labeling experiment using [3H]GGPP-containing liposomes showed that the protein was also labeled with [3H]GGPP; and HPLC analysis of the lipids associated with the protein showed that the protein was geranylgeranylated (data not shown).

These results indicate that GGPP is required for geranylgeranylation of newly synthesized Rho A and its translocation to membranes. When pulse labeling experiments were performed, wherein the labeling preceded the initiation of the growth cycle, we could not detect significant mature Rho A (i.e. old geranylgeranylated Rho A) recycling to the membrane. Nevertheless, it cannot be concluded that some mature Rho A does recycle and that membrane association of such recycled mature Rho A is regulated by a process that requires GGPP through another mechanism. We can conclude that newly synthesized Rho A appears on the membrane but not that only newly synthesized Rho A appears on the membrane.

The possibility of re-geranylgeranylation of Rho A extracted from membranes seems to be unlikely. Because geranylgeranylation by geranylgeranyltransferase I has been demonstrated to require the carboxyl-terminal CAAX box of Rho proteins, (C, cysteine, A, aliphatic amino acid; X, leucine) (6). After prenylation by geranylgeranyltransferase I, the three COOH-terminal amino acids (the AAX residues) are removed from Rho A (30, 31). Subsequent to proteolysis, the prenylated cysteine residue is carboxymethylated (23).

This study shows that growth stimulation eliminates Rho A from membranes just before the activation of HMG-CoA reductase in mid-G1 phase and that the reappearance of the protein in membranes in late G1 phase is associated with p27 activation and Cdk2 activation. GGPP promotes the geranylgeranylation and the translocation of newly synthesized Rho A to membranes. In contrast, Ras in membrane fraction does not change through cell cycle progression from G1 to S phase. The difference in processing of these proteins during G1/S progression might explain the reason why GGPP, not FPP, is required for G1/S transition in FRTL-5 cells. The mechanisms underlying Rho A processing during G1/S progression, particularly the elimination of Rho A from membranes, require further investigation.

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