Geranylgeranylated Rho Small GTPase(s) Are Essential for the Degradation of p27\(^\text{Kip}^1\) and Facilitate the Progression from G\(_1\) to S Phase in Growth-stimulated Rat FRTL-5 Cells*

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Cyclin-dependent kinase (Cdk) enzymes are activated for entry into the S phase of the cell cycle. Elimination of Cdk inhibitor protein p27\(^\text{Kip}^1\) during the G\(_1\) to S phase is required for the activation process. An inhibitor of 3-hydroxy-3-methylglutaryl-CoA reductase prevents its elimination and leads to G\(_1\) arrest. Mevalonate and its metabolite, geranylgeranyl pyrophosphate, but not farnesyl pyrophosphate, restore the inhibitory effect of pravastatin on the degradation of p27 and allow Cdk2 activation. By the addition of geranylgeranyl pyrophosphate, Rho small GTPase(s) are geranylgeranylated and translocated to membranes during G\(_1\)/S progression. The restoring effect of geranylgeranyl pyrophosphate is abolished with botulinum C3 exoenzyme, which specifically inactivates Rho. These results indicate (i) among mevalonate metabolites, geranylgeranyl pyrophosphate is absolutely required for the elimination of p27 followed by Cdk2 activation; (ii) geranylgeranylated Rho small GTPase(s) promote the degradation of p27 during G\(_1\)/S transition in FRTL-5 cells.

Transition from G\(_1\) to S phase in mammalian cells is regulated by cyclin-dependent kinase 2 (Cdk2) and the cyclin E complex (1, 2). p27\(^\text{Kip}^1\), one of the Cdk inhibitors, governs Cdk2 activity during the transition from G\(_1\) to S phase. The amount of p27 decreases as cells are induced to enter the cell cycle (3, 4); it has been demonstrated that the abundance of p27 in cells is regulated by degradation by the ubiquitin-proteasome pathway (5); overexpression of p27 can block cell cycle progression in G\(_1\) phase (6, 7); and p27 appears to be involved in G\(_1\) arrest caused by transforming growth factor-\(\beta\), cyclic AMP, and cell-cell contact (6–8); conversely antisense vectors targeted to p27 mRNA increase the fraction of cells in S phase (9). In recent papers, it was demonstrated that targeted disruption of the murine p27 gene enhances growth of mice without an increase in the amounts of either growth hormone or insulin-like growth factor-1 and that disruption of p27 function leads to striking enlargement of thymus, pituitary, and adrenal glands and gonadal organs (10–12). These evidences suggest that p27 might have a pivotal role in the control of cell proliferation.

p27 has been implicated in G\(_1\) arrest induced by an inhibitor of 3-hydroxy-3-methylglutaryl-CoA reductase (13, 14). Recent studies have also demonstrated that the ability of an HMG-CoA reductase inhibitor to interfere with cell cycle progression could be attributed to its ability to suppress the isoprenylation of proteins, rather than its ability to interrupt cholesterol synthesis (15, 16). The requirements of mevalonate and its metabolites for the elimination of p27 and the activation of Cdk2 during the transition from G\(_1\) to S phase have not yet been examined.

The present investigation was performed in order to develop insights into the regulation of p27 by mevalonate metabolite(s) during the transition from G\(_1\) to S phase. For this purpose, we studied the following questions: 1) which metabolite(s) of mevalonate are active in promoting the elimination of p27? 2) Is protein isoprenylation involved in the elimination of p27? Rat thyroid FRTL-5 cells provide a suitable model for these studies, since their progression from quiescence into the cell cycle is associated with a burst of mevalonate synthesis caused by a large transcriptional activation of the HMG-CoA reductase gene by thyrotropin (TSH) (17).

**EXPERIMENTAL PROCEDURES**

**Preparation of Liposomes of Isoprenoids**—[\(^3\)H]Geranylgeranylated protein phosphatase ([\(^3\)H]GGPP) and [\(^3\)H]farnesyl protein phosphatase ([\(^3\)H]FPP) were purchased from DuPont NEN. GGPP and FPP were purchased from Sigma. To make liposomes containing each, an aliquot of a mixture of dipalmitoylphosphatidylcholine (5 \(\mu\)mol) and GGPP or FPP (200 \(\mu\)g) was added to a shaped flask, and the solvent was removed by rotary evaporation and then a vacuum pump. The dried lipid film was then dispersed in 0.5 ml of phosphate-buffered saline. Warming the flask to 50 °C facilitates smooth dispersion. The liposomes were sonicated and stored at 4 °C.

**Cell Culture, Fractionation, and Assays**—FRTL-5 cells (ATCC CRL 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 of TSH (1 \(\times\) 10\(^{-11}\) M), insulin (10 \(\mu\)g/ml), hydrocortisone (0.4 ng/ml), human transferrin (5 \(\mu\)g/ml), glycyrrhetinic acid (10 ng/ml), and somatostatin (10 ng/ml) (18). This medium is referred to as 6H medium. For all experiments, cells were initially cultured in 6H medium for at least 3 days. As appropriate to

*The abbreviations used are as: Cdk2, cyclin-dependent kinase 2; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; TSH, thyrotropin; GGPP, geranylgeranyl pyrophosphate; FPP, farnesyl pyrophosphate; IPP, isopentenyl pyrophosphate; GST, glutathione S-transferase.

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individual experiments, cells were then shifted to medium containing no TSH, 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 G0/G1 at that point is over 95%. These cells are referred to as quiescent cells; the quiescent cells challenged with 6H medium are referred to as growth-stimulated cells. For whole cell lysates, cells were collected and resuspended in cold lysis buffer consisting of 50 mM HEPES at pH 7.0, 2 mM MgCl2, 2 mM EGTA, 1 mM MgCl2, 2 mM dithiothreitol, 10 mM Na3VO4, 10 mM Na4P2O7, 10 mM NaF, 0.1% Nonidet P-40, and a protease inhibitor mixture (10 μg/ml antipain, 10 μg/ml aprotinin, 30 μg/ml pepstatin A, 10 μg/ml antipain, 10 μg/ml chymostatin, 10 μg/ml leupeptin, 10 μg/ml antipain). The cells were allowed to lyse on ice for 60 min. The homogenate was centrifuged for 5 min in a Microfuge at 4 °C to obtain supernatants. For subcellular fractionation, cells were disrupted by sonication in hypotonic buffer (5 mM Tris-HCl, pH 7.0, 5 mM NaCl, 1 mM EGTA, 1 mM dithiothreitol, 2 mM Na3VO4, 10 mM Na3P2O7, 10 mM NaF, 0.1% Nonidet P-40, and a protease inhibitor mixture) containing the protease inhibitor mixture and separated into crude membrane- and cytosol-containing fractions by centrifugation (100,000 × g, 30 min). For pulse-chase analysis, cells were labeled with [35S]methionine and [35S]cysteine for 2 h, 18–20 h after growth stimulation, and then chased for 20–90 min. For labeling cells with [3H]GGPP, cells were incubated for 36 h with 6H medium in the presence of pravastatin, an HMG-CoA reductase inhibitor (19) with supplementation of [3H]GGPP (18.5 MBq/μmol) in liposomes. Pravastatin was kindly provided by Dr. S. Kurakata (Sankyo Pharmaceutical Co., Ltd., Tokyo, Japan). Cdk2 activity was determined as described previously (18).

The stimulation of quiescent FRTL-5 cells with TSH, insulin, and 5% calf serum resulted in the increase of thymidine incorporation into DNA (Fig. 1A, lane 2 versus 1). Immunoblotting revealed a coincident increase of the rapidly migrating, phosphorylated form of Cdk2 as a single major 33-kDa band (Fig. 1B, lane 2 versus 1). The kinase activity associated with Cdk2...
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Fig. 2. Regulation of p27 metabolism by geranylgeranylated pyrophosphate in FRTL-5 cells. A, effect of pravastatin, mevalonate, and its metabolites on mRNA of p27 in growth-stimulated FRTL-5 cells. Quiescent FRTL-5 cells were incubated in 6H medium for 18 h. Northern blot analysis was performed as described in the text: lane 1, 6H; lane 2, 6H + pravastatin; lane 3, 6H + pravastatin + mevalonate; lane 4, 6H + pravastatin with control liposomes; lane 5, 6H + pravastatin + FPP; lane 6, 6H + pravastatin + GGPP. B, pulse-chase analysis of p27 in growth-stimulated FRTL-5 cells was performed as described in the text: lanes 1–3, 6H; lanes 4–8, 6H + pravastatin (6H + Pra); lanes 9–11, 6H + pravastatin + GGPP (6H + Pra + GGPP). C, in vitro ubiquitination and proteasome-mediated degradation of p27 in cell extracts. Quiescent FRTL-5 cells were incubated in 6H medium for 22 h and cell extracts were prepared. The degradation of p27-GST fusion protein in cell extracts was performed as described in the text: lanes 1–3, 6H; lanes 4–6, 6H + pravastatin (6H + Pra); lanes 7–9, 6H + pravastatin + GGPP (6H + Pra + GGPP).

was also stimulated (Fig. 1C, lane 2 versus 1). In contrast, the level of p27 in quiescent FRTL-5 cells was high and diminished after growth stimulation (Fig. 1D, lane 2 versus 1).

Pravastatin (1200 μM) inhibited growth-stimulated DNA synthesis and induced G1 arrest in FRTL-5 cells (Fig. 1A, lane 3 versus 2 and Fig. 1E). Both the activation of Cdk2 and the elimination of p27 during the transition from G1 to S phase were also inhibited by pravastatin (Fig. 1, B–D, lane 3 versus 2). The presence of pravastatin during the first 16 h of culture did not affect the activation of Cdk2 nor the elimination of p27 in growth-stimulated FRTL-5 cells; however, the continued presence of pravastatin beyond 16 h inhibited both the activation of Cdk2 and the elimination of p27 (Fig. 1, B and D, lanes 3–5 versus 2). The kinase activity associated with Cdk2 had the same time course-dependence of down-regulation in pravastatin-treated cells (Fig. 1C, lanes 3–5 versus 2).

Pravastatin did not increase p27 mRNA levels (Fig. 2A, lane 1 versus 2); instead, pulse-chase analysis demonstrated that the half-life of p27 in pravastatin-treated cells was nearly three times as long as its half-life in non-treated cells (Fig. 2B, lanes 1–3 versus lanes 4–8). Since the rate of p27 degradation was markedly slowed by the treatment with pravastatin in the presence of constant amount of mRNA, the accumulation of p27 could be attributed to reduced protein degradation rather than to increased protein synthesis. We used extracts from pravastatin-treated and nontreated cells as a source of ubiquitinating enzymes and proteasomes. Purified recombinant mouse p27-GST fusion protein was used as a substrate. We found that pravastatin treatment had no significant effect on the degradation of p27 in cell extracts (Fig. 2C, lanes 1–3 versus lanes 4–6). This indicates that pravastatin may impair the degradation of p27 at the intracellular processing step(s) proximal to the ubiquitination-proteasome pathway.

Mevalonolactone (0.5 mg/ml) reversed pravastatin-induced inhibition of DNA synthesis (Fig. 1A, lane 6 versus 2). Pravastatin-induced inhibition of Cdk2 activation and p27 elimination were reversed by the addition of mevalonolactone (Fig. 1, B–D, lane 6 versus 2). Mevalonolactone only overcame the pravastatin effect when added within the first 24 h following growth stimulation (data not shown). In contrast, mevalonolactone did not reverse pravastatin action at later times. Mevalonolactone did not decrease p27 mRNA levels (Fig. 2A, lane 3).

Mevalonate acts as an isoprenyl precursor for farnesyl or geranylgeranyl molecules which have an important signaling function (22). Exogenous FPP and/or GGPP might be expected to counteract the effect of pravastatin. Unfortunately, the experimental use of these compounds is limited by their membrane impermeability and sensitivity to thiol reagents present in the culture medium. Therefore, we introduced a novel approach to evaluate the role of isoprenoids and protein isoprenylation in the elimination of p27: the preparation of liposomes of these isoprenoids.

All of the effects of pravastatin on DNA synthesis, Cdk2 activation, the elimination of p27, and cell cycle progression were wholly reversed by the addition of liposomes containing GGPP at the concentration of 10 μM (Fig. 1, A–D, lane 9 versus lane 7 or 3, and Fig. 1E). p27 mRNA levels were not affected by GGPP (Fig. 2A, lane 6). Pulse-chase analysis of the turnover rate of p27 demonstrated that the elongated half-life of p27 in pravastatin-treated cells is shortened to the level of nontreated cells by the addition of GGPP (Fig. 2B, lanes 4–8 versus 9–11). In contrast, the degradation of p27 in cell extracts containing ubiquitinating enzymes and proteasomes was not affected by pravastatin treatment and the supplementation with GGPP (Fig. 2C, lanes 4–6 versus lanes 7–9). These data indicate that GGPP is required for growth stimulation-induced p27 degradation and does affect the availability of p27 to the ubiquitin-proteasome pathway. The addition of FPP had no effect on the inhibitory action of pravastatin (Fig. 1, A–D, lane 8 versus lane 7 or 3), although incorporation of [3H]FPP in liposomes into FRTL-5 cells was almost equal to that of [3H]GGPP (data not shown).

GGPP is biosynthetically derived from the single condensation of FPP and IPP. Since IPP could not be synthesized in pravastatin-treated cells, FPP could not be converted to GGPP. These data indicate that GGPP can rescue the pravastatin-induced G1 arrest in the absence of upstream intermediates of cholesterol biosynthesis and that geranylgeranylated rather than farnesylated proteins are responsible for inducing p27 elimination in FRTL-5 cells.

A class of geranylgeranylated small GTP-binding proteins, termed Rho small GTPases, are proposed to be involved in the transition from G1 to S phase (23, 24). Carboxyl-terminal lipids form part of the hydrophobic signal that targets proteins to various membranes within the cell. We, therefore, determined the subcellular distribution of Rho small GTPases in pravastatin-treated cells with or without GGPP supplementation. Immunoblot analysis of membrane and cytosolic fractions for Rho A and Rho B revealed that GGPP promotes the translocation of Rho A and Rho B from the cytoplasm to membranes during the transition from G1 to S phase (Fig. 3A). In contrast, these proteins were detected only in the cytosolic fraction in the absence of GGPP. The immunoprecipitation of Rho A and Rho B in membrane fraction demonstrated that these proteins were labeled by the supplementation with [3H]GGPP in liposomes (Fig. 3B). High performance liquid chromatography analysis of the lipids associated with these proteins showed that these...
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FIG. 3. Geranylgeranylation of Rho proteins during G1/S transition. A, immunoblot analysis of Rho proteins in membrane and cytosol fractions in growth-stimulated FRTL-5 cells. Quiescent FRTL-5 cells were incubated in 6H medium for 36 h and crude membrane- and cytosol-containing fractions were prepared as described in the text. Each sample (30 μg) was analyzed by immunoblotting with antibodies against Rho A and Rho B: lane 1, 6H; lane 2, 6H + pravastatin; lane 3, 6H + pravastatin + mevalonate; lane 4, 6H + pravastatin + GGPP. B, fluorography of immunoprecipitated [3H]geranylgeranylated Rho proteins. lane 1, 6H; lane 2, 6H + pravastatin; lane 3, 6H + pravastatin + GGPP; lane 4, 6H + pravastatin + GGPP.

FIG. 4. Effect of botulinum C3 exoenzyme on geranylgeranyl pyrophosphate-dependent Cdk2 activation and p27 degradation. Quiescent FRTL-5 cells were incubated in 6H medium for 36 h with or without 60 μg/ml of C3 exoenzyme plus the other noted additions. Cell lysates (30 μg) were analyzed by immunoblotting with antibodies against Cdk2 and p27 as described in the text: lane 1, 4H; lane 2, 6H; lane 3, 6H + pravastatin; lane 4, 6H + pravastatin + GGPP; lane 5, C3 exoenzyme with 6H + pravastatin + GGPP.

Our findings indicate that among mevalonate metabolites, GGPP, not FPP, reversed the reduced p27 degradation induced by pravastatin. In the presence of GGPP, Rho proteins necessary for S phase development are also geranylgeranylated and translocated to membranes during G1/S progression. The restoring effect of GGPP was abolished with botulinum C3 exoenzyme, which specifically inactivates Rho proteins. Thus, geranylgeranylated protein(s), most likely Rho, appear to play a critical role in regulating p27 degradation and Cdk2 activation during G1/S progression. Recently, a family of target proteins of Rho small GTPases has been identified (31, 32). The mechanisms underlying Rho-regulated p27 degradation require further investigation.

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