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Platelet-derived growth factor stimulates formation of active p21^{ras}·GTP complex in Swiss mouse 3T3 cells

(GTP-binding protein/signal transduction)

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ABSTRACT **The *ras* gene product (p21) is a GTP-binding protein and is thought to play an important role in signal transduction of growth and differentiation in many types of mammalian cells. The p21·GTP complex is an active conformation, as described previously for polypeptide chain elongation factors (EF-Tu and EF-G) and heterotrimeric GTP-binding proteins (G proteins). In the study reported here, we measured the amounts of p21-bound guanine nucleotides under various conditions in the G54 cell line, a derivative of Swiss 3T3 cells that overexpresses normal c-Ha-*ras*. More p21·GTP complexes were present in growing cells than in quiescent cells. When quiescent cells were stimulated with fetal bovine serum to promote DNA synthesis, p21·GTP increased ≈2-fold. Among a number of purified growth factors, platelet-derived growth factor enhanced the formation of p21·GTP, whereas the combination of bombesin and insulin, which also induces DNA synthesis, did not. These results strongly suggest that p21 is a transducer of the growth signal from the platelet-derived growth factor receptor in Swiss 3T3 cells and that the signal is transmitted through a p21·GTP complex.**

The *ras* protooncogene product (p21) is a GTP-binding protein involved in the transduction of signals controlling cellular proliferation and differentiation (1). The activity of a GTP-binding protein is regulated by the bound guanine nucleotide, GDP or GTP. The protein–GDP complex is an inactive conformation and nucleotide exchange from GDP to GTP activates the protein. The active protein–GTP complex specifically interacts with an effector molecule, which transduces the signal downstream. Hydrolysis of the bound GTP to GDP and P_i converts the protein–guanine nucleotide complex to an inactive form. Thus, the activity of a GTP-binding protein is regulated at two steps—i.e., the GDP/GTP exchange reaction ("on" step) and the GTPase reaction ("off" step). Such a regulatory mechanism of a GTP-binding protein was originally proposed in studies of polypeptide chain elongation factors (EF-Tu and EF-G) and was extended to the heterotrimeric GTP-binding proteins (G proteins), *ras* proteins, and some ATP-binding proteins (2, 3).

In the case of *ras* proteins, a number of studies have suggested a significant role for GTP. Mutations, either natural or artificially introduced, that cause the protein to preferentially bind GTP (decreased GTPase and/or increased dissociation rate of GDP) *in vitro* always enhance transforming activity (1). On the other hand, a mutant that preferentially binds GDP has a dominant negative effect on growth of mammalian cells or *Saccharomyces cerevisiae* (4, 5). Direct comparison of the biological activities of recombinant p21s complexed with GDP, GTP, or nonhydrolyzable analogues of GTP has been carried out. Microinjection of the protein into mammalian cells or *Xenopus* oocytes revealed that only the p21·GTP complex is active (6, 7). In various types of cells, including PC12 rat pheochromocytoma cells (8), NIH 3T3 mouse fibroblasts (9), *Xenopus* oocytes (7), and *S. cerevisiae* (10), it was found that mutant p21s with an increased transforming activity bound more GTP than the normal p21. However, there was no direct evidence that signals from outside actually regulate the exchange and/or hydrolysis of p21-bound guanine nucleotides in living cells. A protein that stimulates the GTPase activity of normal p21 but not of mutated p21s, designated GAP (GTPase-activating protein), has been reported (7, 11). Since this protein acts on the effector domain of p21, it has been suggested that GAP may be the target molecule of p21 (11). Alternatively, GAP may be a modulator of p21 that regulates the ratio of p21·GTP to p21·GDP in response to upstream signals (12).

A number of investigations have been carried out to elucidate the role of *ras* genes in the signal-transduction pathway of mammalian cells. One useful approach is the microinjection of anti-p21 antibody into the cells. The observation that NIH 3T3 cells injected with the antibody failed to initiate DNA synthesis in response to serum or some growth factors (13) suggests the essential role of p21. Smith *et al.* (14) proposed that the transforming potential of an oncogene encoding a tyrosine kinase, such as *fes*, *fms*, or *src*, depends on *ras* gene function, since transformation by the former was interrupted by anti-p21. Another approach is measurement of the metabolic change caused by growth factors in normal and *ras*-transformed fibroblast cell lines. It has been reported that platelet-derived growth factor (PDGF) and some other growth factors enhance the breakdown of phosphatidylinositol biphosphates (15). Increased phosphatidylinositol turnover in *ras*-transformed cell lines has been reported by many investigators (16–21), suggesting that p21 may be a transducer of the signal from growth factor receptors. However, it is still obscure which growth factors activate p21 function, because the effector of p21 has not been identified, and therefore it has not been possible to quantitate the activity of p21.

In the present work, we compared, under various conditions, the relative amounts of p21·GTP in a Swiss mouse 3T3-derived cell line (G54) that overexpresses the c-Ha-*ras* gene. We found that the p21·GTP/p21·GDP ratio actually increased when the cells were stimulated to initiate DNA synthesis with fetal bovine serum. Furthermore, we observed an increase of the p21·GTP complex in cells stimulated by PDGF, but not by bombesin plus insulin. These results demonstrate an alteration of p21-bound nucleotide induced by a mitogenic signal in living cells and strongly suggest that

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Abbreviations: GAP, GTPase-activating protein; G protein, heterotrimeric GTP-binding protein; PDGF, platelet-derived growth factor.

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the p21·GTP complex transduces the growth signal from the PDGF receptor.

MATERIALS AND METHODS

Materials. Human PDGF was purchased from R & D Systems (Minneapolis) and Takara Shuzo (Kyoto, Japan). Affinity-purified anti-recombinant PDGF-BB antibody (which also reacts with the PDGF-AB heterodimer) was from Genzyme. Bombesin and insulin were from Sigma.

Cell Culture. The derivation of the G54 cell line will be described elsewhere (M.E., S.N., and Y.K., unpublished results). In brief, Swiss 3T3 cells were transfected with human c-Ha-*ras* cDNA designed to be under the control of a metallothionein promoter. Several stable transfectant clones were isolated. G54 is one of the transfectants that showed an increased level of p21 but normal phenotypes.

Swiss 3T3 and G54 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (GIBCO) supplemented with 10% fetal bovine serum (M.A. Bioproducts) under 5% CO_2 at 37°C. Quiescent cells were obtained as follows. The cells were seeded at a density of 5.4×10^3 per cm^2 and the medium was replaced with DMEM containing 6% fetal bovine serum on the next day. After 5 days, the cells were arrested at the quiescent state.

Analysis of GDP and GTP Bound to p21. Quiescent G54 cells were labeled for 18 hr with $[^{32}P]P_i$ (NEN; catalogue no. NEX-053) at 0.5 mCi/ml (1 mCi = 37 MBq) in phosphate-free DMEM supplemented with 1 μM $CdCl_2$. Fetal bovine serum or growth factors were added to this culture medium. After treatment for a specified period, the cells were washed twice with phosphate-free medium and twice with Tris-buffered saline (50 mM Tris·HCl, pH 7.5/150 mM NaCl). Then the cells were scraped off with a rubber policeman and harvested by centrifugation in a 1.5-ml plastic tube. Thereafter, all procedures were carried out at 0–4°C. Six hundred microliters of immunoprecipitation buffer (50 mM Tris·HCl, pH 7.5/20 mM $MgCl_2$/150 mM NaCl/0.5% Nonidet P-40/1% aprotinin) was added to the cell pellet. The suspension was gently mixed for 10 min and was centrifuged at 750 × *g* for 5 min. Charcoal precoated with bovine serum albumin and suspended in immunoprecipitation buffer was added to the supernatant. After vigorous mixing, the charcoal was removed by centrifugation, and the lysate was stored at −80°C until use.

Anti-p21 monoclonal antibody Y13-259 was mixed with rabbit anti-rat IgG antiserum (Cappel Laboratories) and protein A-Sepharose CL-4B (Sigma) and was incubated at 0°C for 1 hr. The cell lysate was added to the antibody–protein A-Sepharose complex and mixed gently for 1 hr. The precipitate was washed twice with immunoprecipitation buffer and twice with washing buffer (50 mM Tris·HCl, pH 7.5/20 mM $MgCl_2$/150 mM NaCl) and was suspended in 20 μl of 20 mM Tris·HCl, pH 7.5/20 mM EDTA/2% SDS/0.5 mM GDP/0.5 mM GTP. The suspension was heated at 65°C for 5 min and centrifuged. The supernatant was spotted onto a polyethyleneimine-cellulose thin-layer plate and developed with 0.75 M KH_2PO_4 (pH 3.4). The radioactivity was quantitated with an AMBIS radioanalytic image system (AMBIS, San Diego, CA).

Incorporation of [^{3}H]Thymidine. G54 cells were seeded on duplicate 35-mm dishes at a density of 5.4×10^3 per cm^2. The medium was changed the next day to lower the concentration of fetal bovine serum to 6%. After 5 days of culture, the medium was replaced with serum-free DMEM. Fetal bovine serum or various growth factors and [*methyl*-^{3}H]thymidine (Amersham; TRK.686, 0.5 μCi/ml, 1 μM) were added to the culture and after 24 hr of incubation the incorporation of thymidine into the trichloroacetic acid-insoluble fraction was measured as follows. After the removal of the medium, the cells were washed twice with phosphate-buffered saline and 5% (wt/vol) trichloroacetic acid was added to the cells. The cells were incubated at 4°C for 30 min and washed three times with 5% trichloroacetic acid. Then the cells were disrupted with 1 ml of lysis buffer (10 mM Tris·HCl, pH 7.5/1 mM EDTA/0.6% SDS). The radioactivity was measured with a liquid scintillation spectrometer.

RESULTS

Characterization of G54 Cells. Because of the low levels of endogenous p21 we first constructed a Swiss 3T3-derived cell line (G54) that expressed a high amount of normal p21 encoded by c-Ha-*ras* cDNA. The amount of p21 in G54 cells was estimated as about 30-fold greater than in Swiss 3T3 cells by immunoblotting. Although we had expected inducible expression from the metallothionein promoter, the expression of the *ras* cDNA was constitutive in almost all the clones we tested, including G54 cells (data not shown). The G54 cells were morphologically similar to the parental Swiss 3T3 line and did not display transformed phenotypes in spite of the high level of p21. Growth of the cells plateaued at a density of 6.5×10^4 per cm^2, which is equivalent to the confluent density of the parental Swiss 3T3 cells. The amount of p21 needed to transform the cells may be different for each cell line. We selected the G54 cells, since they produce plenty of p21 but do not display transformed phenotypes.

Then we examined the requirement for growth factors for DNA synthesis in G54 cells. Quiescent G54 cells were treated with fetal bovine serum or growth factors, and the incorporation of [^{3}H]thymidine into the trichloroacetic acid-insoluble fraction during the following 24 hr was measured (Fig. 1). We detected a 6- to 7-fold increase in the rate of DNA synthesis in cells treated with 10% fetal bovine serum or the combination of bombesin and insulin. PDGF plus insulin also induced a significant increase in the rate of DNA synthesis, though the effect was somewhat lower.

More p21 Binds GTP in Growing Cells Than in Quiescent Cells. First, we compared the relative amounts of p21·GTP in growing and quiescent cells. The cells were labeled with $[^{32}P]P_i$ for 18 hr and harvested. Since the specific activities of GDP and GTP were thought to be saturated at this time, as previously described (9), we determined the molar ratio of GDP to GTP by multiplying the ratio of their radioactivities times 1.5. Cell lysates were prepared under mild conditions and the p21s were recovered from the lysates with anti-p21 monoclonal antibody Y13-259. Then the p21-bound nucleotides were extracted from the immunoprecipitates, and ap-

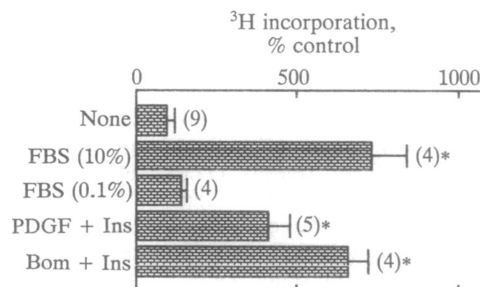

FIG. 1. Stimulation of DNA synthesis in G54 cells. Quiescent G54 cells were stimulated with fetal bovine serum (FBS) or various growth factors and the incorporation of [^{3}H]thymidine into the trichloroacetic acid-insoluble fraction during the following 24 hr was measured. The concentrations of PDGF, bombesin (Bom), and insulin (Ins) were 5 ng/ml, 30 nM, and 5 μg/ml, respectively. Data are shown as percentages compared to controls without addition. Control value was 22,473 ± 5323 cpm per dish. Bars indicate mean ± SEM (n = 4–9, indicated in parentheses). Asterisks indicate $P < 0.001$ as compared with the control value.

plied to a polyethyleneimine-cellulose thin-layer plate. The ratio of p21·GTP to p21·GDP was higher in growing cells than in quiescent cells (Fig. 2A). Quantitative analyses showed that the proportion of p21·GTP relative to total p21 was ≈0.5% at the confluent density, whereas the amount was ≈1% in the proliferating cells (Fig. 2B). The result suggests that p21 is a transducer of the mitogenic signal and that the active p21·GTP complex is formed when the cells are growing exponentially.

Serum Stimulation of DNA Synthesis in Quiescent Cells Increases the p21·GTP Complex. When the quiescent cells were treated with 10% fetal bovine serum to start DNA synthesis, the percentage of p21·GTP doubled within 10 min and higher amounts were maintained at least for 2 hr (Fig. 3). After 16 hr, the proportion of p21·GTP returned to the basal level. In contrast, a lower concentration of serum (0.1%), which could not induce proliferation (see Fig. 1), had no effect on the ratio of p21-bound GDP and GTP.

PDGF, But Not Bombesin plus Insulin, Enhances p21·GTP Formation. When PDGF, bombesin, or insulin was added to the quiescent G54 cells, only PDGF induced an increase in p21·GTP (Fig. 4A). This increase was equal to or greater than that obtained with 10% fetal bovine serum. This effect of PDGF was completely inhibited by anti-PDGF antibody. The combination of PDGF and insulin also induced the formation of p21·GTP, although insulin had no additional effect. In contrast, the combination of bombesin and insulin had no effect (Fig. 4A), although it was able to induce DNA synthesis (see Fig. 1).

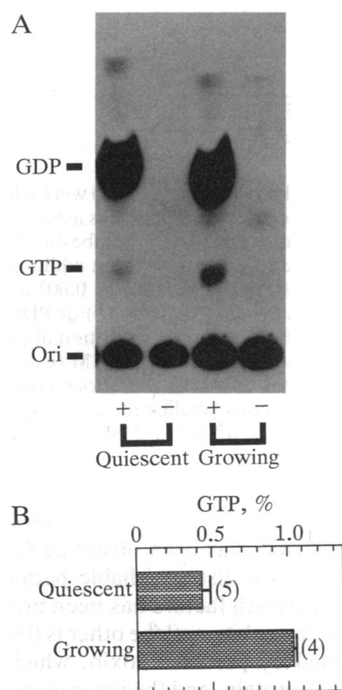

FIG. 2. Increased p21·GTP in proliferating G54 cells. (A) Thin-layer chromatographic analysis of the guanine nucleotides bound to p21. For growing cells, G54 cells cultured in medium containing 10% fetal bovine serum were labeled with [^{32}P]P_i and harvested at a density of 3×10^4 per cm^2. For quiescent cells, G54 cells arrested at the quiescent state were labeled with [^{32}P]P_i and then harvested (the cell density was 6.5×10^4 per cm^2). Cell lysates were prepared and immunoprecipitates with (+) or without (−) anti-p21 antibody Y13-259 were subjected to chromatography. Positions of the origin (Ori), GDP, and GTP are indicated. (B) Quantitation of GTP as a percentage of p21-associated guanine nucleotides: 100 (GTP cpm)/[1.5 (GDP cpm) + (GTP cpm)]. Bars show mean ± SEM (n = 4 or 5, indicated in parentheses).

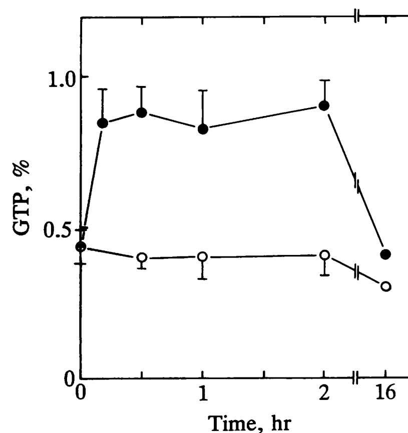

FIG. 3. Time course of the formation of p21·GTP induced by fetal bovine serum. G54 cells were arrested at the quiescent state and labeled with [^{32}P]P_i. Fetal bovine serum [0.1% (○) or 10% (●)] was added to the culture and the cells were harvested at the specified time. Amounts of p21-bound nucleotides were measured as for Fig. 2B. Data are shown as mean ± SEM (n = 3).

The formation of p21·GTP induced by PDGF is a rapid response (Fig. 4B). The initial increase in p21·GTP could be detected in 1 min and the maximal level of p21·GTP was achieved within 5 min. In contrast, no increase in p21·GTP was observed in cells stimulated with bombesin and insulin for 15 sec to 30 min. These results suggest the involvement of p21 in the initial cellular responses to PDGF. Dose–response curves showed that the PDGF concentrations required for the formation of p21·GTP and the induction of DNA synthesis corresponded well (Fig. 4C).

DISCUSSION

In spite of an intensive effort, little is known about the role of *ras* genes in the signal-transduction pathway controlling proliferation of mammalian cells. Participation of *ras* genes in cellular responses to some specific growth factors has been suggested (14, 17, 21–25). Wakelam *et al.* (17) reported that N-*ras* p21 mediates the mitogenic signals from bombesin, and Burgering *et al.* (25) suggested the involvement of c-Ha-*ras* p21 in signal transduction from insulin and insulin-like growth factor I. However, since the effector of p21 was not known, it was impossible to analyze the activity of p21 itself, and direct evidence for coupling of specific growth factors and p21 has not been shown.

In this study, we tried to solve this problem by comparing the amounts of p21-bound GDP and GTP in cultured 3T3 cells under various conditions. In previous work, we and others have shown that (*i*) p21·GTP is biologically active, in contrast to p21·GDP, in several types of cells and (*ii*) mutated p21s with increased transforming activity preferentially bind GTP compared to GDP *in vitro* and *in vivo*. From these observations we have proposed that the activity of p21 is regulated in a manner similar to other GTP-binding proteins, such as elongation factors or G proteins. According to this concept, we considered that the upstream signal must alter the steady-state concentrations of p21·GDP and p21·GTP, so that analyses of p21-bound nucleotides under various conditions should help identify the upstream signal of p21.

In the present experiments, we have used the G54 cell line, in which the normal p21 is overproduced about 30-fold. The use of cells expressing high levels of p21 was necessary because we immunoprecipitated cellular p21 and quantitated the bound guanine nucleotides. In previous reports with mammalian (8, 9) as well as yeast (10, 12) cells, similar quantitation experiments have been carried out with p21-

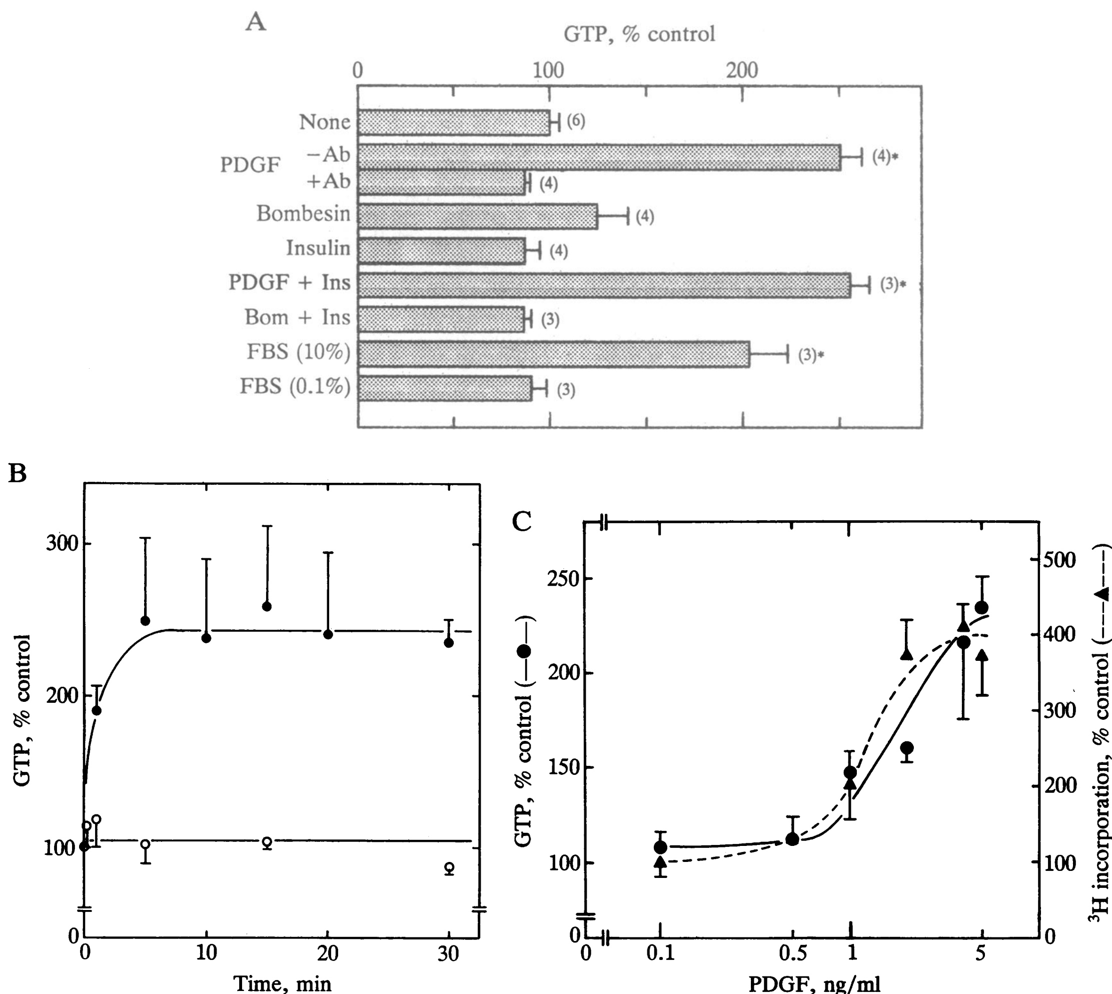

FIG. 4. (A) Enhancement of the formation of p21·GTP by PDGF. Various growth factors or fetal bovine serum (FBS) were added to quiescent G54 cells labeled with [^{32}P]P$_i$. After 30 min the amounts of p21·GTP in the cells were measured. The effect of PDGF was assayed in the presence (+Ab) or absence (−Ab) of the affinity-purified anti-recombinant PDGF antibody. The concentrations of PDGF, bombesin (Bom), and insulin (Ins) were 5 ng/ml, 30 nM, and 5 μg/ml, respectively. Data are shown as percentages compared to controls without addition. Control value was 0.602 ± 0.0350 (% GTP). Bars indicate mean ± SEM (n = 3–6, indicated in parentheses). Asterisks indicate $P < 0.001$ as compared with the control value. (B) Time course of the increase of p21·GTP by PDGF. Bombesin (30 nM) plus insulin (5 μg/ml) (○) or PDGF (5 ng/ml) (●) was added to quiescent G54 cells labeled with [^{32}P]P$_i$, and the cells were harvested at the specified times for determination of p21·GTP. Percent values compared to control are indicated. Control value was 0.564 ± 0.0148 (% GTP). Data are shown as mean ± SEM (n = 3–5). (C) PDGF dose–response curves for p21·GTP formation and DNA synthesis. PDGF was added to quiescent G54 cells at various concentrations. The amounts of p21·GTP (after 30 min of treatment, ●) and the incorporation of [^{3}H]thymidine (continuous labeling for 24 hr, ▲) were measured. The percentages compared to control are indicated. Control values were 0.584 ± 0.0340 (% GTP) and 12,587 ± 2388 (cpm per dish). Data are shown as mean ± SEM (n = 3 or 4).

amplified cell lines. Phenotypes of G54 cells overproducing normal p21 were not very different from the parental cells in terms of growth rate and morphology (data not shown). Also, the effects of PDGF and of bombesin plus insulin on DNA synthesis were similar in Swiss 3T3 and G54 cells (Fig. 1).

First, we compared the amounts of p21·GTP in growing and quiescent cells. The observation that p21·GTP was much more abundant in growing cells than in quiescent cells (Fig. 2) strongly suggested that an active p21·GTP complex was functioning when the cells were exponentially growing. Then we found that p21·GTP increased about 2-fold when the quiescent cells were treated with serum to initiate DNA synthesis. The p21·GTP/p21·GDP ratio increased within a few minutes after the addition of growth factors (Figs. 3 and 4B), indicating that the formation of the active form of p21 was relatively rapid. The higher level of p21·GTP was retained for at least 2 hr, but after 16 hr it decreased to the initial level. What this reduction means is not clear. It is possible that the signal from growth factors may be interrupted by some feedback regulation, since reduced response of EJ-*ras* transformants to PDGF has been observed (22–24).

The existence of two distinguishable pathways of signal transduction from growth factors has been proposed. One is from bombesin or thrombin, and the other is from PDGF. The former is disrupted by pertussis toxin, which suggests the involvement of a G protein, and the latter is insensitive to the toxin (26–31). Our results presented in Fig. 4 clearly indicate that c-Ha-*ras* is involved in the pathway from PDGF, but not from bombesin or insulin. These results agree with a previous report (13). We have also observed that high expression of [Val^{12}]p21 in Swiss 3T3 cells abrogates the requirement for PDGF for initiation of DNA synthesis (M.E., S.N., and Y.K., unpublished results).

PDGF is one of the best-characterized growth factors. The receptor for PDGF has intrinsic tyrosine kinase activity, and when PDGF binds to its receptor it elicits a series of growth-associated events in fibroblasts (32). How does the PDGF receptor increase the amount of p21·GTP? There are two

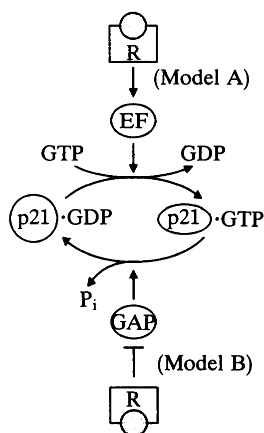

FIG. 5. Working hypotheses for the regulation of p21. For details, see text. EF, a putative GDP/GTP exchange factor of p21; R, PDGF receptor.

possibilities (Fig. 5). One is enhancement of GDP/GTP exchange (model A) and the other is suppression of GTPase (model B). Model A arose from the analogy with the reaction mechanism of G proteins. When agonists bind to receptors coupled to G proteins, they stimulate dissociation of GDP from the α subunits. Therefore, we can suppose that the PDGF receptor itself or some associated exchange factor may enhance the dissociation of p21·GDP when PDGF binds to its receptor. In model B, GAP is a candidate for the regulator of p21. GAP has putative phosphorylation sites and regions of amino acid sequence homology to *src*-encoded kinase or phospholipase C-γ (33). Although the functional significance of these structures is not clear, the idea that GAP activity is modulated by some upstream signals is attractive. Hoshino *et al.* (9) reported that GAP activity changed in accordance with cell density. Therefore, it is likely that PDGF treatment may decrease GAP activity and consequently increase p21·GTP in the cells. Recently, it has been reported that PDGF and epidermal growth factor, but not fibroblast growth factor or insulin, rapidly induce tyrosine phosphorylation of GAP (34–36). Although it has not been shown how the activity of GAP is altered by phosphorylation, these observations support the possibility that GAP may play a role as a regulator in transducing the signal from the PDGF receptor to p21.

In experiments to be published elsewhere, we have found that epidermal growth factor also increases p21·GTP. This gives further support to the idea that the increase in p21·GTP is due to the modulation of GAP activity by tyrosine phosphorylation.

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