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Accumulation of $p21^{ras}$·GTP in response to stimulation with epidermal growth factor and oncogene products with tyrosine kinase activity

(GTP-binding protein/signal transduction)

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ABSTRACT The *ras* gene product (p21) is a GTP-binding protein and has been thought to transduce signals regulating proliferation or differentiation of cells. Like other GTP-binding proteins, p21·GTP is an active conformation, which can transduce the signals downstream, whereas p21·GDP is an inactive one. Recently, we have shown that p21·GTP levels increased in cells treated with fetal bovine serum or platelet-derived growth factor to initiate DNA synthesis. In this paper, we report that epidermal growth factor can also increase the amounts of p21·GTP in the cells. Effects of epidermal growth factor and platelet-derived growth factor are not additive. In contrast, mutant [Val[12]]p21, which has transforming activity, responded neither to platelet-derived growth factor nor to epidermal growth factor. We also found that the ratio of p21·GTP to p21·GDP increased 3- to 4-fold in transformants carrying activated *erbB-2/neu* or v-*src* oncogenes. These results strongly suggest an important role of p21 in transduction of signals for both normal proliferation and malignant transformation through growth factor receptors with tyrosine kinase activity or related oncogene products.

Mammalian *ras* genes are thought to be involved in signal transduction pathways of proliferation or differentiation in many types of cells (1). The *ras* gene product (p21) is a GTP-binding protein, and it has been predicted that the ligand (GDP or GTP)-induced conformation of the protein determines its activity as proposed in previous studies on elongation factor (EF)-Tu (2) and guanine nucleotide-binding proteins (G proteins) (3). This contention has been supported by the following observations: (*i*) mutated p21 proteins with increased transforming activity preferentially bind GTP rather than GDP both *in vitro* (1) and *in vivo* (4–7), and (*ii*) p21·GTP but not p21·GDP is biologically active when injected into cells (7, 8). Recently, we have detected more p21·GTP in vigorously growing cells than in quiescent cells, and in addition, it has been revealed that the amount of p21·GTP actually increased when quiescent cells were stimulated to initiate DNA synthesis with fetal bovine serum or platelet-derived growth factor (PDGF) (9). To our knowledge, these results are the first evidence for the formation of an active p21·GTP complex in response to the stimulus of growth factors.

The receptor for PDGF has intrinsic tyrosine kinase activity, which is essential for mitogenesis (10). However, substrates of the receptor-tyrosine kinase that are important for the mitogenic signaling have not yet been fully clarified. Recently, it has been reported that GTPase-activating protein (GAP) (7, 11, 12) is a substrate for various tyrosine kinases including the PDGF receptor, the epidermal growth factor (EGF) receptor, and products of oncogenes such as *src*, *fms*, *fps*, and *abl* (13–15). This suggests the involvement of GAP in signal transduction pathways from these kinases, although alteration of GAP activity by phosphorylation of tyrosine residues has not been observed. Since GAP may be a regulator of p21, it is possible that the tyrosine kinases may modulate *ras* function through GAP.

In the present report, we describe that the EGF receptor causes an increase in the p21·GTP complex in a similar manner as the PDGF receptor and that the effect of EGF is not additive to that of PDGF. We detected no increase in the ratio of the GTP-bound form of the transforming [Val[12]]p21 in response to these growth factors, suggesting insensitivity of the mutated protein to upstream signals. We also report here that p21·GTP is increased 3- to 4-fold in transformed cells carrying oncogene products with tyrosine kinase activity.

MATERIALS AND METHODS

Materials. EGF and PDGF were purchased from Takara Shuzo (Kyoto, Japan).

Cell Culture. G54 cells (9) and V59 cells (a transformant of Swiss 3T3 cells that conditionally produce [Val[12]]p21; M.E., S.N., and Y.K., unpublished results) were cultured in Dulbecco's modified Eagle's medium (DMEM) (GIBCO) supplemented with 10% (G54 cells) or 4% (V59 cells) (vol/vol) fetal bovine serum (M.A. Bioproducts) in 5% CO_2 at 37°C. G54 and V59 cells were arrested at the quiescent state as follows. The cells were seeded at a density of 5.4×10^3 per cm^2 (G54 cells) or 2.8×10^3 per cm^2 (V59 cells), and the medium was replaced with DMEM containing 6% (G54 cells) or 4% (V59 cells) fetal bovine serum on the next day. After 5 days, the cells were arrested at the quiescent state. NIH 3T3, A4, and CC104 cells were cultured in DMEM supplemented with 6% fetal bovine serum in 5% CO_2 at 37°C.

Analysis of p21-Bound GDP/GTP. The quiescent G54 or V59 cells were labeled for 18 hr with 0.5 mCi (1 Ci = 37 GBq) of $^{32}P_i$ (NEX-053; NEN) per ml in phosphate-free DMEM supplemented with 1 μM $CdCl_2$. Growth factors were added to this culture medium. After treatment for the specified

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Abbreviations: PDGF, platelet-derived growth factor; EGF, epidermal growth factor; GAP, GTPase-activating protein; G protein, guanine nucleotide-binding protein.

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periods, the cells were disrupted, and the ratio of GDP to GTP bound to p21 was analyzed as described (9).

NIH 3T3, A4, and CC104 cells were seeded at a density of 2.4×10^4 per cm^2, and the medium was replaced with phosphate-free DMEM containing 1.5 mCi of $^{32}P_i$ per ml the next day. After labeling for 14 hr, the cells were disrupted, and the ratio of GDP to GTP bound to p21 was analyzed.

Incorporation of [^{3}H]Thymidine. Incorporation of [^{3}H]-thymidine into the trichloroacetic acid-insoluble fraction was measured as described elsewhere (9).

RESULTS

The G54 cell line is a derivative of Swiss 3T3 mouse cells that produces c-Ha-*ras* protooncogene products at ≈30-fold higher levels than the parent line. This cell line was found to display nontransformed phenotypes in regards to morphology at confluent density, contact inhibition of proliferation, and requirement for serum or growth factors for the initiation of DNA synthesis (9). We have demonstrated that the relative amount of p21·GTP in the cells stimulated by PDGF increased 2- to 3-fold, although bombesin plus insulin, which can induce DNA synthesis to a similar extent as PDGF, had no effect on p21 levels (9).

In the present study, we show similar effects of EGF on p21 as in the case of PDGF. First, we measured changes in the ratio of p21-bound GDP/GTP after the addition of EGF. EGF was added to ^{32}P-labeled quiescent G54 cells for 30 min, and then the p21-bound nucleotides were analyzed. As shown in Fig. 1*A*, we could detect more p21·GTP in the EGF-treated cells as compared to the untreated cells. As shown below (see Figs. 2–4), we also quantitated the radioactivity in the spots by using a two-dimensional image analyzer and calculated the molar ratio of p21-bound GTP as follows: GTP (%) = {GTP (cpm)/[GDP (cpm) × 1.5 + GTP (cpm)]} × 100. The time course of response after the addition of EGF is described in Fig. 1*B*. We could detect increased p21·GTP after 1 min as with PDGF. High levels of p21·GTP continued for at least 30 min.

Both the EGF receptor and the PDGF receptor have an intrinsic tyrosine kinase activity, but the relationship of the pathways from these growth factors is still unclear. As shown in Fig. 2*A*, the G54 cells respond to both EGF and PDGF to

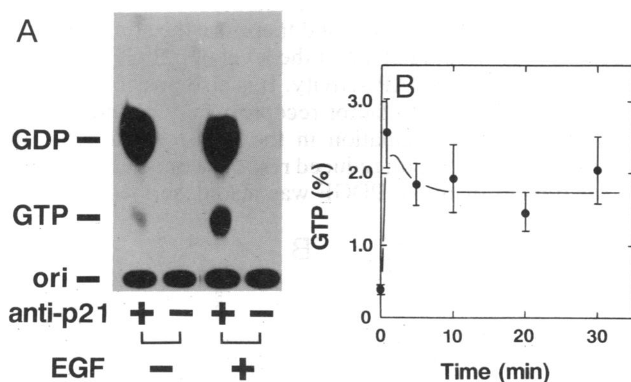

FIG. 1. Increase of p21·GTP in EGF-treated G54 cells. (*A*) Analysis of p21-bound GDP/GTP with thin-layer chromatography. The quiescent ^{32}P-labeled G54 cells were untreated (−) or treated (+) with EGF (50 ng/ml) for 30 min and then were disrupted. The cell lysates were subjected to immunoprecipitation with (+) or without (−) Y13-259 (monoclonal anti-p21 antibody). Immunoprecipitates were developed on thin-layer chromatography. The origin (ori) and the positions of GDP and GTP are indicated. (*B*) Time course of the increase of p21·GTP by EGF. EGF (5 ng/ml) was added to quiescent G54 cells labeled with $^{32}P_i$, and the cells were disrupted at the specified time. The amounts of p21·GTP were measured. Data are shown as the mean ± SEM (n = 3 or 4).

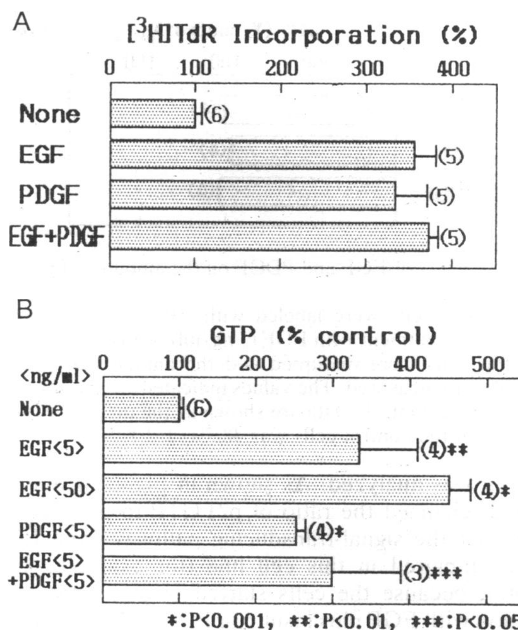

FIG. 2. Comparison of the response to EGF and PDGF. (*A*) Stimulation of DNA synthesis by EGF and PDGF. Quiescent G54 cells were treated with EGF (5 ng/ml) and/or PDGF (5 ng/ml), and the incorporation of [^{3}H]thymidine ([^{3}H]TdR) into the trichloroacetic acid-insoluble fraction during the following 24 hr was measured. Data are shown as the percentages compared to the control (without addition). The bars indicate the mean ± SEM (n = 5 or 6, indicated in parentheses). The control value was 30,037 ± 2605 cpm per dish. (*B*) Effects of EGF and PDGF on the formation of p21·GTP. Quiescent ^{32}P-labeled G54 cells were treated with EGF (5 ng/ml or 50 ng/ml) for 30 min and then were disrupted. The amounts of p21-bound GDP/GTP were measured. Data are shown as the percentages compared to the control. The bars indicate the mean ± SEM (n = 3–6, indicated in parentheses). p21·GTP in the control cells was 0.604% ± 0.0316%.

start DNA synthesis, but the combination of these factors did not have an additive effect. The results suggest the presence of a common pathway downstream of these receptors. Therefore, we compared the effects of these factors on the increase of p21·GTP. Both EGF and PDGF could enhance the formation of p21·GTP to a similar extent at 5 ng/ml as shown in Fig. 2*B*, but again the combination of the two did not affect p21-bound GDP/GTP additively. Either PDGF (9) or EGF (Fig. 2*B*) at 5 ng/ml is sufficient to induce a maximal increase of p21·GTP. Therefore, it is plausible to assume the presence of a common regulator, which may mediate the signals from EGF and PDGF to p21.

Changing the 12th amino acid residue from glycine to valine gives transforming activity to p21. The mutated [Val^{12}]p21 binds more GTP than normal p21 in cells as described in the previous studies (4–7), and the accumulation of the GTP-bound form of p21 is thought to induce uncontrolled cell growth. Thus, we examined whether the ratio of GDP to GTP bound to the transforming [Val^{12}]p21 is altered in response to growth factors. We used V59 cells, which can conditionally produce [Val^{12}]p21 under the control of the metallothionein promoter. We estimated, by immunoblotting, that the amount of [Val^{12}]p21 in the induced cells was ≈4-fold higher than the amount of endogenous p21 (M.E., S.N., and Y.K., unpublished results). When expression of the exogenously introduced [Val^{12}]p21 is not induced, this cell line also exhibits nontransformed phenotypes and can be arrested at the quiescent state. We added $CdCl_2$ to the quiescent V59 cells and simultaneously labeled with $^{32}P_i$ as described in *Materials and Methods*. Then the cells were treated with EGF or PDGF for 30 min, and the p21-bound

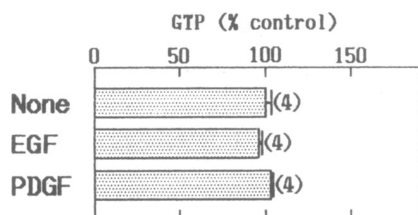

FIG. 3. Effects of EGF and PDGF on the amounts of p21-bound GDP/GTP in V59 cells. The V59 cells were arrested at the quiescent state. Then the cells were labeled with $^{32}P_i$, and [Val^{12}]p21 was induced. After treatment with EGF (5 ng/ml) or PDGF (5 ng/ml) for 30 min, the cells were disrupted and the amounts of p21-bound GDP/GTP were measured. The values indicated are the percentages compared to the control. Data are shown as the mean ± SEM (n = 4). p21·GTP in the control cells was 48.7% ± 1.87%.

GDP/GTP was analyzed. As shown in Fig. 3, neither EGF nor PDGF changed the ratio of p21·GTP to p21·GDP. It is unlikely that the signal-transducing pathway from EGF or PDGF is impaired in this cell line (for example, lack of receptors), because the cells started DNA synthesis after stimulation with EGF plus insulin or PDGF plus insulin to the same extent as with 10% fetal bovine serum, though insulin alone did not have any effect (data not shown).

We further investigated the effects of oncogene products with tyrosine kinase activity on p21-bound GDP/GTP. In the experiments shown in Fig. 4, we compared the ratio of p21·GTP to p21·GDP in three NIH 3T3-derived cell lines with transforming phenotypes and with that of the parental cells. Both A4 and CC104 cells are transformants of activated *erbB-2/neu* oncogenes (T.A., S. Matsuda, Y. Namba, T. Saitoh, K. Toyoshima, and T.Y., unpublished results). In the case of A4 cells, the transforming activity of the *erbB-2/neu*-encoded protein is due to substitution of an amino acid residue positioned in the putative transmembrane domain. In CC104 cells, the activation is caused by both the substitution in the transmembrane domain and deletion of the C-terminal domain composed of 230 amino acid residues. We detected a 3- to 4-fold increase in the level of p21·GTP in both transformed cell lines with activated *erbB-2/neu*. Furthermore, we were able to detect a similar increase of p21·GTP in the transformed cells with v-*src* oncogenes (Fig. 4). The results strongly suggest the involvement of p21 in pathways from these oncogene products with tyrosine kinase activity.

DISCUSSION

In the present report, we have shown the activation of $p21^{ras}$ by growth factors (EGF and PDGF) or oncogene (*erbB-2/neu* and v-*src*) products with intrinsic tyrosine kinase activity. As shown in Fig. 1, EGF rapidly increased the level of the active p21·GTP complex as reported elsewhere for PDGF (9). The observation strongly suggests the involvement of *ras* in transduction of the signals from these growth factors. The essential role of *ras* in these pathways has also been suggested in a previous report (16). Both the EGF receptor and the PDGF receptor have tyrosine kinase activity, and the phosphorylation of tyrosine residues of putative key substrates is thought to be an initial event in signal transduction. A number of proteins phosphorylated by these growth factor receptors in a ligand-dependent manner have been identified, including phospholipase C type γ (17–19), *raf* oncogene products (20), phosphatidylinositol kinase (21), and GAP (13–15), suggesting the existence of multiple pathways. It is not known at present which components are directly linked to p21 and how p21 is activated. Since EGF and PDGF had no additive effects (Fig. 2), a common component may mediate the signals from these factors to p21.

From the general scheme of the mechanisms of *ras* function, we can consider two possibilities for the activation of p21: first, repression of GTPase and second, enhancement of the GDP/GTP exchange. In the former case, GAP can be a candidate for the mediator. GAP stimulates GTPase of normal p21 but not [Val^{12}]p21, resulting in the constitutive activation of the latter (7, 11, 12). Assuming that the activity of GAP is inhibited when the growth signal is turned on, normal p21 may be activated like the transforming counterpart. In *Saccharomyces cerevisiae*, two homologues of mammalian GAP, *IRA1* and *IRA2* gene products, may function as regulators of RAS (22, 23). On the other hand, it has also been proposed that GAP may be the target of p21, because GAP acts on the putative effector domain of p21 (12). The enhancement of GDP/GTP exchange is based on the analogy to the G protein-mediated signaling systems, where the binding of agonists to receptors stimulates the exchange of GDP/GTP bound to G protein α subunits (3). Although direct interaction between growth factor receptors and p21 has not yet been demonstrated, there may be a mechanism regulating the exchange reaction as proposed for p21 (24–26) and other low molecular weight GTP-binding proteins (27).

The level of p21·GTP in the cells carrying transforming [Val^{12}]p21, which was much increased (49%) as compared to the parental cells (0.6%), was not altered by stimulation with either EGF or PDGF (Fig. 3). This is reasonable since the impaired GTPase activity of the mutated [Val^{12}]p21 is not activated by GAP (7, 11, 12), and therefore the stimulus from EGF or PDGF would not affect the level of p21·GTP through the regulation of the GAP activity. It is also possible that the signals from the growth factor receptors may be interrupted by some feedback regulation in the transformed cells. In previous reports (28–30), reduced response or insensitivity of EJ-*ras* transformants to PDGF was also described.

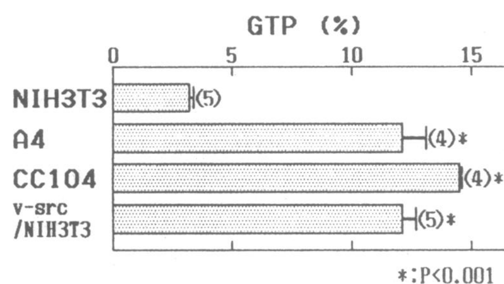

FIG. 4. Increase in the amounts of p21·GTP in NIH 3T3 cells transformed with activated *erbB-2/neu* or v-*src* oncogenes. The amounts of p21-bound GDP/GTP in each cell line were measured. Data are shown as the mean ± SEM (n = 4 or 5, indicated in parentheses).

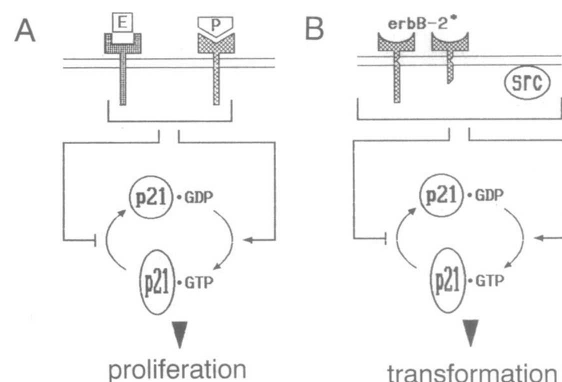

FIG. 5. Schematic representation of the relationship between tyrosine kinases and $p21^{ras}$ in normal cell growth (*A*) or transformation (*B*). E, EGF; P, PDGF.

Malignant transformation by oncogenes coding tyrosine kinases is thought to be due to the constitutively enhanced activity of the oncogene products to phosphorylate tyrosine residues in target molecules. Since p21 is activated by ligand-bound receptor-tyrosine kinases as described above, it is likely that p21 is activated in a manner independent of the extracellular stimuli in transformed cells carrying oncogene products with constitutively activated tyrosine kinase activity. Therefore, we examined the effects of the oncogenes of p21-bound GDP/GTP. Both receptor-type (*erbB-2/neu*) and non-receptor-type (*src*) tyrosine kinases actually increased the ratio of p21·GTP to p21·GDP 3- to 4-fold without exogenous stimuli (Fig. 4). From this observation, we proposed the involvement of p21 not only in normal growth but also in malignant transformation induced by tyrosine kinases (Fig. 5). The observation by Smith *et al.* (31) that microinjection of anti-p21 inhibited the transformation by *src* or *fms* strongly supports this idea. Other investigators have also described the important role of inositol phospholipid metabolism in transformation by *ras* and other oncogenes including *src* (32, 33).

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