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Biochemistry

Mitogenic activation of the Ras guanine nucleotide exchange factor in NIH 3T3 cells involves protein tyrosine phosphorylation

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ABSTRACT **We report biochemical evidence that epidermal growth factor and platelet-derived growth factor stimulate the Ras guanine nucleotide exchange factor activity in quiescent NIH 3T3 cells. Moreover, the exchange activity is constitutively enhanced in NIH 3T3 cells transformed by Src and ErbB2 oncogenic tyrosine protein kinases (TPKs), whereas transformation by oncogenic Mos and Raf does not alter the activity. GTPase-activating protein activity was not affected under these conditions. Overexpression of $pp60^{c\text{-}Src}$ mutants containing activated and suppressor TPK mutations resulted in stimulation and inhibition of the exchange factor activity, respectively. A TPK inhibitor, genistein, prevented the activation of the exchange factor in epidermal growth factor/platelet-derived growth factor-treated cells and *src*-transformed cells. Furthermore, the exchange factor activity bound to an anti-phosphotyrosine antibody immunoaffinity column. These findings suggest that the guanine nucleotide exchange factor, but not GTPase-activating protein, plays a major role in the Ras activation in cell proliferation initiated by growth factor receptor TPKs and malignant transformation by oncogenic TPKs and that tyrosine phosphorylation of either the exchange factor or a tightly bound protein(s) may mediate the activation of the exchange factor by these TPKs.**

It is generally accepted that Ras activity is controlled by two regulatory proteins (1). The guanine nucleotide exchange factor catalyzes the exchange of bound GDP for external GTP and thus leads to an active GTP-bound form of Ras that transmits signals to the downstream Ras effector. In contrast, Ras–GTPase-activating protein (GAP) (2) and neurofibromatosis type 1 protein (3) generate an inactive GDP-bound form of Ras by promoting hydrolysis of GTP bound to Ras. Either the activation of the exchange factor or the suppression of GAP would be expected to result in the accumulation of Ras in an active GTP-bound state. Recently, the exchange activity of guanine nucleotides bound to Ras has been detected in various mammalian cell extracts (4–7). The gene encoding Ras-specific guanine nucleotide exchange (releasing) factor has been isolated from rat (8), mouse (9, 10), and human (11) tissues and shares extensive homology in the catalytic domain with yeast *Saccharomyces cerevisiae CDC25* (12, 13). Mammalian cells also contain genes (*mSOS-1* and *-2*; ref. 14) related to *Drosophila SOS* (15), which is homologous to *CDC25* and *Saccharomyces pombe STE6* (16). Despite their structural similarity, these mammalian gene products differ in molecular mass and in tissue distribution, suggesting the existence of a family of the exchange factors.

While some structural and functional aspects of the exchange factor have been revealed, it is not clear how the exchange factor is physiologically involved in the signal transduction pathway activated by growth signals. A number of studies have shown an increase in the amount of active Ras–GTP complex in cells induced to proliferate with epidermal growth factor (EGF) (17, 18) and platelet-derived growth factor (PDGF) (19), in cells transformed by oncogenes such as *src*, *erbB2*, *abl*, and *fms* (18, 20), and in hemopoietic cells stimulated by T-cell receptor antibodies (21), interleukin 2, 3, and 5, and granulocyte–macrophage colony-stimulating factor (22, 23). These observations implicate the involvement of Ras–GTP formation in growth signaling mechanisms. We have recently found that nerve growth factor increased the amount of Ras–GTP and stimulated the activities of the exchange factor and GAP in PC12 cells leading to the neuronal differentiation of cells (24). In this case, the activation of Ras might result from the dominance of the exchange factor activity over GAP activity. Some molecular genetic studies showed that PDGF, EGF, and insulin increased the rate of guanine nucleotide binding to Ras and that this increase was associated with elevations in Ras–GTP levels (25, 26).

Involvement of tyrosine kinases in the Ras activation has been suggested by the observations that EGF, PDGF, and nerve growth factor receptor tyrosine protein kinases (TPKs) and oncogenic TPKs increased the level of active Ras–GTP in the cells (18–20). However, the mode of regulation of Ras activity by these TPKs is unknown. Recent studies do not seem to support the hypothesis that tyrosine phosphorylation of GAP regulates Ras by modulating GAP activity (20, 21). Genetic analysis showed that *Drosophila* SOS acts downstream of the TPK receptor Sevenless (27), but to our knowledge, there is no direct biochemical demonstration of the functional involvement of Sevenless in SOS activity as yet.

In the present biochemical studies, we addressed the questions whether the guanine nucleotide exchange factor is modulated during the transmission of upstream growth signals to Ras and how the exchange factor activity is regulated by EGF/PDGF receptor TPKs and Src/ErbB2 oncogenic TPKs.

MATERIALS AND METHODS

Materials. EGF and PDGF were purchased from Collaborative Research, genistein was from Calbiochem, and anti-phosphotyrosine antibody 4G10 was from Upstate Biotechnology (Lake Placid, NY). [^{3}H]GDP, [α-^{32}P]GTP, and [γ-^{32}P]ATP were from Amersham.

Cell Culture. NIH 3T3/v-Src, NIH 3T3/ErbB2 [P. Difroeg, National Cancer Institute (NCI)], NIH 3T3/v-Mos [G. Vande Woode, NCI–Frederick Cancer Research and Development

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Abbreviations: EGF, epidermal growth factor; PDGF, platelet-derived growth factor; TPK, tyrosine-protein kinase; GAP, GTPase-activating protein.

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Center (NCI-FCRDC)], and NIH 3T3/v-Raf (U. Rapp, NCI-FCRDC) were cultured in Dulbecco's modified Eagle's medium (DMEM) (GIBCO) supplemented with 10% (vol/vol) fetal bovine serum in 5% CO_2/95% air at 37°C. NIH (pMC-Src/foc) cells (wild-type c-Src-overexpressing cells), NIH (pcSrc 527/foc/Ep) cells (Tyr-527 → Phe, activated Src-overexpressing cells), and NIH (pcR295/psV2neo/MC) cells (Tyr-416→ Phe, inactive Src-overexpressing cells) have been described (28) and were cultured in DMEM containing 10% (vol/vol) calf serum.

Assay of the Guanine Nucleotide Exchange Factor Activity. NIH 3T3 cells, or transformed NIH 3T3 cells, were seeded in 150-mm diameter dishes and grown to confluence. The cells were refed in 0.2% fetal bovine serum for 18–24 hr. For experiments with growth factor effects, 5×10^7 quiescent NIH 3T3 cells were treated with DMEM containing EGF (200 ng/ml) or PDGF (50 ng/ml) for 10 min. The cells were washed once with ice-cold phosphate-buffered saline and suspended in 300 μl of buffer A [20 mM sodium phosphate, pH 7.5/1 mM $MgCl_2$/0.1 mM EDTA/1 mM dithiothreitol/200 mM sucrose/10 μM phenylmethylsulfonyl fluoride/leupeptin (10 μg/ml)/pepstatin (1 μg/ml)/0.5 mM sodium orthovanadate/10 mM NaF/0.5 mM phosphoserine/0.5 mM phosphotyrosine/1 mM phosphothreonine]. Cell lysates were prepared as described (24). Bacterially made human normal Ha-Ras proteins (0.4 μg) were incubated with 4 μCi of [^{3}H]GDP (10–15 Ci/mmol; 1 Ci = 37 GBq) in 50 μl of the reaction buffer [25 mM Tris·HCl, pH 7.5/5 mM EDTA/1 mM dithiothreitol/bovine serum albumin (200 μg/ml)] for 30 min at 37°C and then incubated with cell lysates (25 μg) in the presence of 18 mM $MgCl_2$ and 2 mM unlabeled GTP for 10 min at 37°C. Ras was immunoprecipitated with anti-Ras antibody (Y13-259) for 1 hr and eluted nucleotides were analyzed by scintillation counting as described (24).

Assay of GAP Activity. Cells were disrupted as described above. Ras proteins (0.4 μg) were incubated with 0.2 μCi of [α-^{32}P]GTP (3000 Ci/mmol) in 50 μl of the reaction buffer at 37°C for 3 min, the binary complexes were incubated with the indicated amounts of cell extracts, and GDP and GTP eluted from immunoprecipitates were analyzed by thin layer chromatography (TLC) as described (24).

Chromatography. Cells were disrupted in 20 mM sodium phosphate, pH 7.5/1 mM EGTA/1 mM dithiothreitol/0.5% Nonidet P-40/aprotinin (10 μg/ml)/0.5 mM phenylmethylsulfonyl fluoride/1 mM sodium orthovanadate/10 mM NaF as described (24). Cell lysates were fractionated by a FPLC Mono Q HR5/5 column (Pharmacia) chromatography using a linear gradient of 0–0.3 M NaCl as described (24).

For immunoaffinity fractionation with anti-phosphotyrosine antibody, 10^8 disrupted cells were solubilized in buffer A containing 0.2% sodium cholate. Lysates were centrifuged at 100,000 × g for 15 min, and the supernatants were mixed with 100 μl of anti-phosphotyrosine antibody 4G10-agarose prepared by the published method (29) for 60 min at 4°C. The resins were washed three times with washing buffer (10 mM Tris·HCl, pH 7.6/5 mM EDTA/50 mM NaCl/50 mM NaF/100 μM sodium orthovanadate) containing 0.1% sodium cholate and were mixed with 10 mM phenyl phosphate in 200 μl of washing buffer for 30 min at 4°C. Twenty microliters of eluted samples was assayed for the exchange factor activity in 300 μl of reaction buffer.

RESULTS

Effects of EGF or PDGF on the Activities of the Exchange Factor and GAP. EGF and PDGF induce cellular DNA synthesis in NIH 3T3 cells, at least in part through Ras protein activity (30), and one of the earliest responses of cells to these growth factors is an increase in the amount of active Ras–GTP complex (17–19). To investigate whether the exchange factor or GAP activity is altered in response to the growth factors, we first examined the effects of both EGF and PDGF on the activity of the exchange factor in NIH 3T3 cells. After the serum starvation of confluent cells, quiescent cells were treated with EGF (200 ng/ml) or PDGF (50 ng/ml) for 10 min, and cell lysates were prepared. [^{3}H]GDP–Ha-Ras complexes were incubated with the extracts in the presence of excess unlabeled GTP for 10 min. Ras proteins were immunoprecipitated and the amount of GDP released from Ras proteins was measured. The amount of [^{3}H]GDP released in the presence of EGF (51.0 ± 14.0%)- or PDGF (49.5 ± 6.2%)-treated cell extracts increased 2- to 2.5-fold compared to that in the presence of extracts from untreated cells (19.0 ± 2.0%). The stimulatory effect of EGF or PDGF on GDP-releasing activity was maximal 10 min after treatment of cells with EGF or PDGF and, thereafter, decreased (B.-Q.L., M.S., H.-f.K., and T.K., unpublished data). There was a correlation with the rapid and transient elevation in the exchange activity and the levels of Ras–GTP complexes, and the subsequent reduction in the Ras–GTP levels was associated with the further decrease in the exchange activity. To further analyze the effect of the growth factors on the exchange factor activity, cell extracts were partially purified by Mono Q anion-exchange chromatography and each fraction was assayed for [^{3}H]GDP dissociation activity. The relative GDP release promoting activity (eluted at ≈0.12 M NaCl) increased 2- to 3-fold after stimulation by EGF or PDGF (Fig. 1). To determine whether GAP activity was also affected by EGF and PDGF, [α-^{32}P]GTP–Ras complexes were incubated with cell lysates and guanine nucleotides bound to Ras were analyzed by TLC after immunoprecipitation. Essentially, there was no difference in the level of GAP activity between EGF- or PDGF-treated cells and untreated cells over the course of a 30-min treatment, indicating that the target of the signals transduced by EGF or PDGF receptor could be the guanine nucleotide

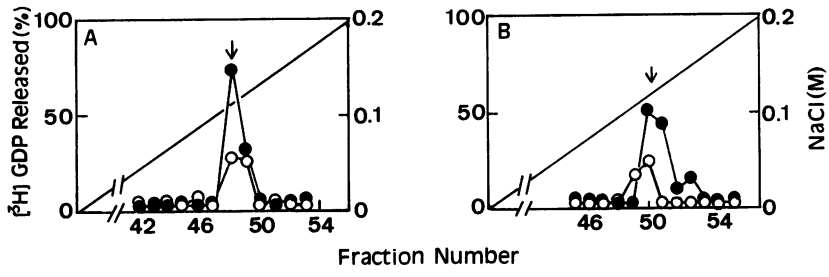

FIG. 1. Anion-exchange chromatography of the GDP-releasing activity. Quiescent NIH 3T3 cells (6×10^7 cells) were untreated (○) or treated (●) with EGF (200 ng/ml) (*A*) or PDGF (50 ng/ml) (*B*). Cell lysates (≈2 mg) were prepared and loaded onto a FPLC Mono Q HR5/5 column (Pharmacia). The proteins were eluted with a linear gradient of 0–0.3 M NaCl. Fractions (0.5 ml) were collected, and 20 μl of a fraction and 0.4 mM GTP were added to [^{3}H]GDP–Ras (0.4 μg) for 10 min at 37°C. The amount of [^{3}H]GDP associated with Ras was determined by nitrocellulose filter binding assay as described (24). Activity is given as the percentage of bound [^{3}H]GDP released. [^{3}H]GDP (30,000 cpm) was bound to Ras proteins without addition. Arrows indicate the positions of peaks.

exchange factor rather than Ras–GAP or neurofibromatosis type 1 protein (Fig. 2).

The Exchange Factor Activity in Cells Transformed by Oncogenic Kinases. Since EGF and PDGF receptors possess TPK activities, this raised the question whether oncogenic TPKs persistently activate the exchange factor without signals from extracellular mitogenic stimuli. When extracts from various transformed cells were tested for the ability to increase the rate of dissociation of [^{3}H]GDP from Ras proteins, [^{3}H]GDP dissociation promoting activity was 2.7- and 3.0-fold enhanced in v-*src* and *erbB2*-transformed cells compared to that seen in untransformed cells, respectively (Fig. 3). In contrast, we did not detect any increase in the activity in cells transformed by *mos* and *raf* oncogenes, suggesting that the activation of the exchange factor was not a consequence of cell transformation. As for EGF- and PDGF-stimulated cells, no significant difference in GAP activity, as well as the amount of Ras–GAP or neurofibromatosis type 1 protein, was detected among NIH 3T3 cells and NIH 3T3 cells transformed by *src*, *erbB2*, *mos*, or *raf* (B.-Q.L., M.S., H.-f.K., and T.K., unpublished data). The *erbB2* gene product is structurally related to the EGF receptor and, like the *src* oncogene product, exhibits persistently activated TPK and transforming activities (31), while *mos* and *raf* oncogenic gene proteins have serine/threonine kinase activities. Our data suggest that these oncogenic TPKs may modulate the activity of the exchange factor. It is unlikely that the stimulation of the exchange factor activity was due to an increase in the amount of the exchange factor, because a 15-min pretreatment of cells with a TPK inhibitor blocked its activation (see below). Our results correlate with the signal-transduction model involving several oncogenes where the function of Src and Abl is mediated by Ras activity and the function of Mos and Raf is Ras-independent (32).

Effects of Overexpression of Mutated c-Src on the Exchange Factor Activity. To further clarify the biochemical links between TPKs and the exchange factor activity, we examined the nucleotide exchange activity in NIH 3T3 cells overexpressing normal pp60$^{c\text{-}Src}$ or pp60$^{c\text{-}Src}$ mutants at major tyrosine phosphorylation sites in which Tyr-527 and Tyr-416 were replaced with Phe. The Tyr-527 → Phe mutation strongly activated pp60$^{c\text{-}Src}$-transforming and TPK activities, whereas the Tyr-416 → Phe mutation suppressed these activities, indicating that phosphorylation states of the two tyrosine residues are crucial for the biological activity of pp60$^{c\text{-}Src}$ (28, 33). Overexpression of normal pp60$^{c\text{-}Src}$ resulted in a moderate increase in [^{3}H]GDP release promoting activity (12% above buffer control), but introduction of the Tyr-527 mutation in pp60$^{c\text{-}Src}$ enhanced the activity by 4-fold compared to normal pp60$^{c\text{-}Src}$ (Table 1). The mutation at Tyr-416 in pp60$^{c\text{-}Src}$ markedly suppressed the exchange activity. The level of exchange activity correlated with both TPK and transforming activities of pp60$^{c\text{-}Src}$ and the results provide evidence that TPK of pp60$^{c\text{-}Src}$ induced the constitutive activation of the exchange factor activity. In contrast, GAP activity was not affected by the mutation at these tyrosine phosphorylation sites in pp60$^{c\text{-}Src}$.

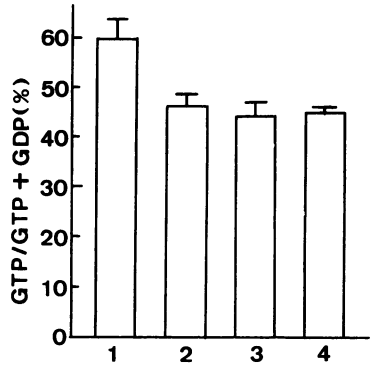

FIG. 2. Effect of EGF or PDGF on GAP activity in NIH 3T3 cells. Quiescent NIH 3T3 cells were untreated (bar 2) or treated with EGF (200 ng/ml, bar 3) or PDGF (50 ng/ml, bar 4) for 10 min at 37°C, and the cells were disrupted in buffer A as described (17). [α-^{32}P]GTP–Ras (0.4 μg) was incubated with 20 μl of the homogenization buffer (bar 1) or cell extracts (25 μg) in the presence of 5 mM $MgCl_2$ and 1 mM unlabeled GTP for 10 min at 37°C and immunoprecipitated with Y13-259 antibody. The eluted guanine nucleotides were analyzed by TLC as described (24). Ratio of GTP to total labeled nucleotides bound to Ras proteins [(GTP/GTP + GDP) × 100] is shown as the mean ± SD (n = 3).

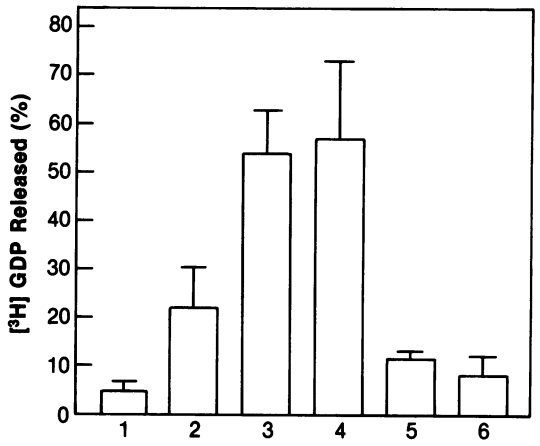

FIG. 3. Comparison of the exchange activity in various transformed cells. Approximately 2 × 10^7 confluent NIH 3T3 (bar 2), NIH 3T3/v-Src (bar 3), NIH 3T3/ErbB2 (bar 4), NIH 3T3/v-Mos (bar 5), and NIH 3T3/v-Raf cells (bar 6) were serum-starved for 24 hr and cell lysates were prepared. [^{3}H]GDP–Ras complexes (0.4 μg) were incubated with cell extracts (25 μg) or the homogenization buffer (bar 1) in the presence of 0.4 mM GTP and the amount of [^{3}H]GDP released was quantitated. Activity [the mean ± SD (n = 3)] is shown as the percentage of bound [^{3}H]GDP released. [^{3}H]GDP (30,000 cpm) was bound to Ras proteins without addition.

A TPK Inhibitor, Genistein, Suppresses the Exchange Factor Activation. In another set of experiments, we tested a tyrosine kinase inhibitor, genistein, for the inhibitory effect on the exchange factor activity. Genistein specifically inhibits TPK activities, such as EGF receptor, pp60$^{c\text{-}Src}$, and pp110$^{gag\text{-}fes}$, but does not inhibit serine and threonine kinases, such as protein kinase C (34). The enhancement of the exchange factor activity in EGF- or PDGF-treated cells was blocked when the cells were pretreated with the drug for 15 min, and the activity in activated Src-transformed cells was similarly inhibited by the drug (Table 2). More than 80% inhibition of the activity was achieved with genistein at 30 μg/ml, which inhibited 90% of total TPK activities in both membrane and cytosolic fractions *in vitro* (M.S., B.-Q.L., H.-f.K., and T.K., unpublished data), whereas an inhibitor of protein kinase C, calphostin C (100 ng/ml) (35), failed to inhibit the activation of the exchange activity (B.-Q.L., M.S., H.-f.K., and T.K., unpublished data). The addition of genistein (30 μg/ml) to the reaction mixtures containing extracts from *src*-transformed cells or EGF/PDGF-treated cells had no significant effect on the exchange factor activity, implying that the suppression of the exchange factor activity in the drug-treated cells was not due to the direct inhibition of the exchange factor activity by genistein.

Affinity Purification of the Exchange Factor Activity by Immobilized Anti-Phosphotyrosine Antibody. Because our data showed that tyrosine kinase activity was required for EGF/PDGF-induced and Src-induced activation of the exchange factor, we speculated that the exchange factor or its associ-

Table 1. Exchange factor and GAP activities in NIH 3T3 cells overexpressing mutant $pp60^{Src}$

Cell type	Src TPK specific activity	Exchange activity, % of [^{3}H]GDP release	GAP activity, %
NIH 3T3	±	8.4 ± 0.5	20.0 ± 1.1
NIH 3T3 (wt Src)	+	16.3 ± 0.02	18.0 ± 1.0
NIH 3T3 (Tyr-527 → Phe, activated Src)	+++	51.1 ± 1.0	17.0 ± 2.0
NIH 3T3 (Tyr-416 → Phe, impaired Src)	±	11.0 ± 0.06	16.0 ± 2.9
Buffer control		4.0 ± 0.1	40.0 ± 5.3

wt, Wild type. Parental NIH 3T3, NIH (pMCSrc/foc) (wild-type c-Src overexpressor), NIH (pcSrc527/foc/Ep) (Tyr-527 → Phe, activated Src overexpressor), and NIH (pcR295/psV2neo/MC) (Tyr-416 → Phe, suppressed Src overexpressor) cells were serum-starved in DMEM containing 0.2% calf serum for 24 hr and cell lysates were prepared as in Fig. 3. For the exchange factor activity assay, [^{3}H]GDP–Ras complexes (0.4 μg) were incubated with extracts (25 μg) or the homogenization buffer in the presence of 0.4 mM GTP, and the bound nucleotides were measured by immunoprecipitation. Activity [mean ± SD (n = 3)] is shown as described in Fig. 1. [^{3}H]GDP (33,000 cpm) was bound to Ras. For the GAP activity assay, [α-^{32}P]GTP–Ras (0.4 μg) was incubated with extracts (30 μg) and analyzed as described in Fig. 2. For Src kinase assay, cell extracts (500 μg) were immunoprecipitated with 4 μg of rabbit anti-Src antibody. Immunoprecipitates were subjected to the *in vitro* kinase reaction using [γ-^{32}P]ATP, and the amounts of ^{32}P incorporated into $pp60^{c\text{-}Src}$ were analyzed by SDS/PAGE, as described (26). GAP activity is (GTP × 100)/(GTP + GDP). Specific activity: ±, very weak; +, moderate; +++, very strong.

ating protein(s) might be tyrosine-phosphorylated. To test this hypothesis, we fractionated activated *src*-transformed cell extracts by affinity chromatography using immobilized anti-phosphotyrosine antibody. More than 80% of the exchange factor activity was immunodepleted by the immunoaffinity resins. The fractions that eluted with 10 mM phenyl phosphate enhanced the exchange activity of Ras and did not have any effect on other types of small molecular mass G proteins, Rap1A and Rab1B, suggesting that the exchange factor bound was Ras-specific (Fig. 4). Immunoblot analysis with anti-phosphotyrosine antibody after gel electrophoresis showed that the eluted fraction contained phosphotyrosine-containing proteins with molecular masses between 30 and 180 kDa (B.-Q.L., M.S., H.-f.K., and T.K., unpublished data). Similar results were obtained with EGF- or PDGF-treated cells. The results indicate that the exchange factor could be either tyrosine-phosphorylated or associated with a phosphotyrosine-containing protein(s).

The antibody against a 1.4-kb carboxyl-terminal region of $CDC25^{mM}$ containing its catalytic domain (provided by Dan Broek, University of Southern California) was used to detect the protein of the exchange factor family in the eluates. No protein in either whole-cell extracts or eluted fractions specifically reacted with the antibody on an immunoblot, raising the possibility that $CDC25^{mM}$ is not expressed in NIH 3T3 cells (B.-Q.L., M.S., H.-f.K., and T.K., unpublished data).

DISCUSSION

Present biochemical studies demonstrate that the Ras guanine nucleotide exchange factor activity, but not GAP activity, was enhanced in EGF- or PDGF-stimulated cells and in oncogenic TPK-transformed cells. Since the increase in the amount of Ras–GTP complex correlated with the enhancement of the exchange factor activity, the activated factor might stimulate Ras activities by promoting the conversion of Ras–GDP to Ras–GTP in these biological systems, Ras–GAP or neurofibromatosis type 1 protein might simply play a role as a negative regulator and/or effector for Ras. This is consistent with the observation based on molecular genetic studies that stimulation of nucleotide exchange on Ras would be the predominant mechanism in PDGF-, EGF-, and insulin-treated cells (25, 26). In our previous study (24), nerve growth factor was found to stimulate both the exchange factor and GAP activities in PC12 cells during the early stage of cell differentiation. In activated T lymphocytes, the activation of Ras appeared to involve a protein kinase C-induced decrease in GAP activity without alteration of the exchange factor activity (21). The present results seem to differ from those described in the two systems above. Therefore, the mode of action of these Ras regulatory proteins could be different depending on the biological systems used.

Another major finding is that activation of the exchange factor is triggered by phosphorylation events induced by ligand stimulation of receptor TPKs and oncogenic TPKs.

Table 2. Suppression of Ras guanine nucleotide exchange factor activity by genistein

Cell type	[^{3}H]GDP released, %	
	− genistein	+ genistein
NIH 3T3	10.0 ± 0.9	2.9 ± 0.6
NIH 3T3 (Tyr-527 → Phe, activated Src)	41.0 ± 0.2	7.0 ± 0.9
EGF-treated NIH 3T3	34.0 ± 2.0	2.2 ± 0.2
PDGF-treated NIH 3T3	37.0 ± 3.7	2.0 ± 0.2

Confluent NIH 3T3 cells or NIH (pcSrc 527/foc/Ep) cells were serum-starved for 24 hr and treated with genistein (30 μg/ml) for 15 min. Then NIH 3T3 cells were treated with EGF (150 ng/ml) or PDGF (50 ng/ml) for 10 min in the presence of the drug. Cell extracts were prepared and 20 μl of extract (25 μg) was tested for the dissociation of [^{3}H]GDP from Ras (0.4 μg), as described in Fig. 3. Activity [mean ± SD (n = 3)] is shown as described in Fig. 1. [^{3}H]GDP (32,000 cpm) was bound to Ras without addition. The GDP release by buffer alone (2.0%) was subtracted.

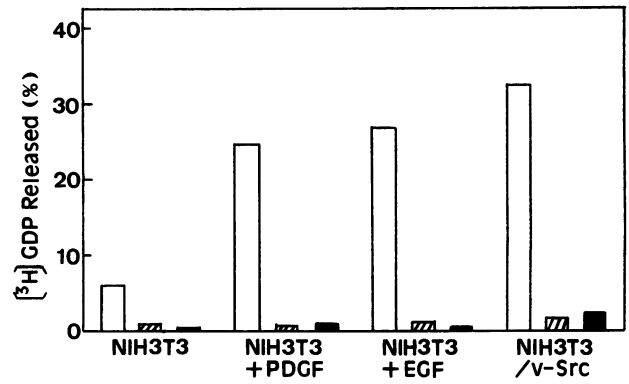

FIG. 4. Immunoaffinity purification of the exchange factor activity by anti-phosphotyrosine antibody. Effects of eluted proteins on the exchange activity of Ras. Cell lysates (5×10^7 cells)from NIH 3T3 cells treated with EGF (100 ng/ml), NIH 3T3 cells treated with PDGF (50 ng/ml), or v-*src*-transformed NIH 3T3 cells were prepared. Extracts were mixed with anti-phosphotyrosine antibody-agarose and eluted proteins (5 μg) were tested for the activity to stimulate the dissociation of GDP from 0.1 μg of Ras (open bars), Rap1A (hatched bars), and Rab1B (solid bars) as described in Fig. 3. The activity is given as in Fig. 1 and data are the mean of duplicate experiments. Similar results were obtained in different sets of experiments.

This can be drawn by the following facts. (*i*) Constitutive activation of the exchange factor was detected in Src or ErbB2 oncogenic TPK-transformed cells, whereas the response of the factor to EGF or PDGF was transient. (*ii*) Exchange factor activity was stimulated in the cells carrying a TPK-activated Src mutant and was suppressed in the cells carrying its TPK-defective mutant. (*iii*) Enhancement of the activity by EGF, PDGF, and activated Src was inhibited by treatment of cells with genistein, a TPK inhibitor, at a dose that inhibits TPK activity. The mechanism by which TPKs activate the exchange factor remains to be determined. Since the exchange factor activity was retained on an anti-phosphotyrosine antibody-coupled resin, the simplest model would be the direct tyrosine phosphorylation of the factor by EGF/PDGF receptor TPKs and oncogenic Src/ErbB2 TPKs or by an as yet unidentified TPK activated by the above mentioned TPKs. Alternatively, tyrosine phosphorylation of a protein associating with the exchange factor could modulate the exchange factor activity. Recently, two Src homology 2 (SH2)-containing proteins, SHC and GRB2, have been found to bind to tyrosine-phosphorylated EGF/PDGF receptors via its SH2 domain (36, 37). SHC protein was phosphorylated by activated EGF receptor or v-Src, whereas GRB2 was not phosphorylated but formed a specific complex with phosphorylated SHC (38). These observations suggest that SHC and GRB2 couple growth factor receptors to a signaling pathway involved in cell proliferation. If the association of a phosphotyrosine-containing protein(s) with the exchange factor is required for its activation, a protein such as SHC or GRB2 might be a good candidate for regulator of the factor.

Which member of the exchange factor family catalyzes the exchange reaction of Ras in NIH 3T3 cells remains to be determined. In our data, the growth responsive exchange factor in NIH 3T3 cells specifically interacted with Ras but not Rap1A and Rab1B. The exchange factor affinity-purified by anti-phosphotyrosine antibody-conjugated resins from total cell lysates did not crossreact with the anti-CDC25^{mM} antibody, although Cen *et al.* (10) reported the crossreactivity of a 95-kDa protein in NIH 3T3 cells with the anti-CDC25^{mM} antibody. Several investigators also found that neither the transcript nor the protein of the murine homologue of yeast *S. cerevisiae* CDC25 gene was detected in NIH 3T3 cells and that the gene was specifically expressed in brains (8, 11, 39). Since mSOS is widely expressed in various types of cells including BALB/c 3T3 cells, mSOS may mediate the postulated regulatory roles of Ras in growth signaling.

Note. After the submission of our work, several investigators (40–46) published work describing the association of GRB2 with mSOS-1 and the complex formation of GRB2 with phosphorylated SHC in signal transmission from the EGF receptor or Src TPKs to Ras.

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