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Involvement of *rho* p21 and Its Inhibitory GDP/GTP Exchange Protein (*rho* GDI) in Cell Motility

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Evidence is accumulating that *rho* p21, a *ras* p21-related small GTP-binding protein (G protein), regulates the actomyosin system. The actomyosin system is known to be essential for cell motility. In the present study, we examined the action of *rho* p21, its inhibitory GDP/GTP exchange protein (named *rho* GDI), its stimulatory GDP/GTP exchange protein (named *smg* GDS), and *Clostridium botulinum* ADP-ribosyltransferase C_3, known to selectively ADP-ribosylate *rho* p21 and to impair its function, in cell motility (chemokinesis) of Swiss 3T3 cells. We quantitated the capacity of cell motility by measuring cell tracks by phagokinesis. Microinjection of the GTPγS-bound active form of *rho*A p21 or *smg* GDS into Swiss 3T3 cells did not affect cell motility, but microinjection of *rho* GDI into the cells did inhibit cell motility. This *rho* GDI action was prevented by comicroinjection of *rho* GDI with the GTPγS-bound form of *rho*A p21 but not with the same form of *rho*A p21 lacking the C-terminal three amino acids which was not posttranslationally modified with lipids. The *rho* GDI action was not prevented by Ki-*ras*$^{Val-12}$ p21 or any of the GTPγS-bound form of other small GTP-binding proteins including *rac*1 p21, G25K, and *smg* p21B. Among these small G proteins, *rho*A p21, *rac*1 p21, and G25K are known to be substrates for *rho* GDI. The *rho* GDI action was not prevented by comicroinjection of *rho* GDI with *smg* GDS. Microinjection of C_3 into Swiss 3T3 cells also inhibited cell motility. These results indicate that the *rho* GDI-*rho* p21 system regulates cell motility, presumably through the actomyosin system.

Cell motility is essential for inflammatory reactions, tissue repair, and immune-system interactions in normal cells (for a review, see reference 48). Moreover, cell motility is essential for malignant cancer cells to infiltrate surrounding tissues and for smooth muscle cells in the media to migrate to the intima at the atherosclerotic lesion of vascular vessels (17; for a review, see reference 42). Evidence is accumulating that various kinds of cytoskeletal proteins and interacting proteins between cells and their substrates or neighboring cells regulate cell motility (48). Among these proteins, actomyosin is suggested to be one of the most important molecules in cell motility, on the basis of the observations that cell motility is sensitive to the actin-binding compounds cytochalasin D and phalloidin, which prevent polymerization and depolymerization of actin filaments, respectively (36, 46; for reviews, see references 13 and 28), and that an antibody against myosin affects cell motility (21). However, it is not clear how this actomyosin system is regulated to cause cell motility.

Evidence is accumulating that small G proteins regulate various cell functions including cell growth, cell differentiation, gene expression, vesicle transport, cell morphological change, smooth muscle contraction, and superoxide generation in phagocytes (1, 20, 27; for reviews, see references 5, 6, 8, 18, and 51). Of the many small G proteins, the *rho* p21 family, consisting of three members (A, B, and C), has been suggested to regulate the actomyosin system on the basis of the observations that C_3, which selectively ADP-ribosylates the *rho* p21 family (9, 34, 44; for reviews, see references 18 and 51), changes both stress fibers and cell morphology (12, 35, 38, 43) and that *rho*$^{Val-14}$ p21, a point-mutated active form of *rho* p21, also affects cell morphology (38). C_3 is an ADP-ribosyltransferase of *Clostridium botulinum* (2). Furthermore, this role of *rho* p21 has been substantiated by our recent observations that C_3 and epidermal cell differentiation inhibitor (EDIN) inhibit the action of guanosine 5′-(3-*O*-thio)triphosphate (GTPγS), which lowers the Ca^{2+} concentration necessary for the vasoconstrictor-induced smooth muscle contraction, and that this inhibitory action of EDIN is overcome by *rho* p21 (20). EDIN is also a *Staphylococcus aureus* ADP-ribosyltransferase which selectively ADP-ribosylates the *rho* p21 family (49, 50).

rho p21 has GDP-bound inactive and GTP-bound active forms which are interconvertible by GDP/GTP exchange and GTPase reactions (18, 51). The conversion from the GDP-bound inactive form to the GTP-bound active form is regulated by GDP/GTP exchange proteins and the reverse conversion is regulated by GTPase-activating proteins. There are two types of GDP/GTP exchange proteins for *rho* p21: one is a stimulatory type, named *smg* GDS and *rho* GDS, and the other is an inhibitory type, named *rho* GDI (16, 24, 31, 52). These regulatory proteins are present in most cells, and *rho* GDI is more abundant than *smg* GDS in HL-60 cells, human platelets, and bovine brain (26). Moreover, the inhibitory action of *rho* GDI in the GDP/GTP exchange reaction is stronger than the stimulatory action of *smg* GDS or *rho* GDS if both are present (26, 29). *rho* p21 is present in the GDP-bound inactive form complexed with *rho* GDI in the cytosol of resting smooth muscle and insulinoma cells (29, 39). The GDP-bound form of *rho*A p21 complexed with *rho* GDI is resistant to ADP-ribosylation by EDIN (26). *rho* p21 undergoes three kinds of posttranslational modifications in the C-terminal region: geranylgeranylation of the cysteine residue, removal of the three C-terminal amino acids, and carboxylmethylation of the exposed cysteine residue (25). Only the posttranslationally processed form of *rho*A p21 (not the posttranslationally unprocessed form) is sensitive to these GDI and GDS actions (22, 31). On the basis of these observations, we have tentatively proposed the following

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modes of activation and action of *rho* p21. In resting cells, the posttranslationally processed form of *rho* p21 is present in the cytosol in the GDP-bound inactive form complexed with *rho* GDI and its effector region is directly or indirectly masked by *rho* GDI. Upon stimulation of cells with some agonists, the inhibitory action of *rho* GDI is released in an unknown manner, the GDP-bound inactive form of *rho* p21 becomes sensitive to the action of *smg* GDS or *rho* GDS, and the GTP-bound active form is produced. By this activation, *rho* p21 opens its effector domain, interacts with its effector protein, and exerts its biological function through this effector protein.

In the light of these observations, we examined whether the *rho* GDI-*rho* p21 system regulates cell motility. In this paper, we demonstrate that microinjection of *rho* GDI or C_3 into Swiss 3T3 cells inhibits the cell motility and that the inhibitory action of *rho* GDI is prevented by comicroinjection of *rho* GDI with the GTPγS-bound active form of *rho*A p21.

MATERIALS AND METHODS

Materials and chemicals. Swiss 3T3 cells were kindly supplied by E. Rozengurt, Imperial Cancer Research Fund, London, England. The cDNAs of *rho*A p21 and Ki-*ras*$^{Val\text{-}12}$ p21 were kindly provided by P. Madaule, Centre National de la Recherche Scientifique Laboratoire, Gif sur Yvette, France, and R. A. Weinberg, Massachusetts Institute of Technology, Boston, Mass., respectively. The cDNA of G25K and the baculovirus carrying the cDNA of *rac*1 p21 were kindly supplied by P. Polakis and F. McCormick, Chiron Corp. The baculoviruses carrying the cDNA of *rho*A p21, Ki-*ras*$^{Val\text{-}12}$ p21, or *smg* p21B were kindly provided by Y. Matsuura, National Institute of Health, Tokyo, Japan. *rho*A p21, *rac*1 p21, Ki-*ras*$^{Val\text{-}12}$ p21, and *smg* p21B were expressed in *Spodoptera frugiperda* cells (Sf9 cells) and purified from the cytosol fraction of Sf9 cells overexpressing each small G protein as described previously (31). *rho*A p21 lacking the three C-terminal amino acids (*rho*A p21$^{\Delta LVL}$) was purified from *rho*A p21$^{\Delta LVL}$-overexpressing *Escherichia coli* (25). *rho* GDI and G25K were purified as glutathione *S*-transferase (GST) fusion proteins from *E. coli* overexpressing GST-*rho* GDI and GST-G25K, respectively (15, 26, 45). It was confirmed that recombinant GST-*rho* GDI showed the same activity in the GDP/GTP exchange reaction of *rho*A p21 as did *rho* GDI purified from bovine brain in the cell-free experiments. Although it is not known whether GST-G25K shows the same activity as G25K, it was confirmed that GST-*rho*A p21 produced by a similar method showed the same activity as did *rho*A p21 purified from *rho*A p21-overexpressing Sf9 cells. *smg* GDS was purified from *smg* GDS-overexpressing *E. coli* (31). Active protein concentrations of these small G proteins were determined by their [^{35}S]GTPγS-binding activity, which was assayed by the filtration method described previously (52). The GTPγS-bound form of *rho*A p21, *rho*A p21$^{\Delta LVL}$, *rac*1 p21, GST-G25K, and *smg* p21B was made by incubating each small G protein with 60 μM GTPγS as described previously (55). The GDP-bound form of all of these small G proteins was made by the same method with 60 μM GDP. All the proteins used were concentrated in Centricon-10 (Amicon) to 2 to 20 mg/ml. During the concentration, the buffers contained in the sample preparations were replaced by buffer A (20 mM Tris HCl [pH 7.4] containing 20 mM NaCl, 2 mM $MgCl_2$, 100 μM ATP, 0.1 mM EDTA, and 1 mM 2-mercaptoethanol). C_3 was kindly supplied by B. Syuto, Hokkaido University, Sapporo, Japan (32).

Cell culture. Stock cultures of Swiss 3T3 cells were maintained at 37°C in a humidified atmosphere of 10% CO_2 and 90% air in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml).

Cell motility assay. Tissue culture dishes (Nunc Inc.) were coated with colloidal gold as described previously (3, 4). Briefly, 9 ml of 14.5 mM $AuCl_4H$ and 30 ml of 36.5 mM Na_2CO_3 were added to 55 ml of H_2O, and the solution was heated in a glass beaker. Immediately after reaching the boiling point, the solution was removed from the heat and 9 ml of a 0.1% formaldehyde solution was quickly added. Colloidal gold was formed within 1 min. The solution was cooled to about 50°C and poured into bovine serum albumin-coated 35-mm grid tissue culture dishes. After a 45-min incubation, the solution was removed and the dishes were washed once with phosphate-buffered saline. For cell motility assay, the cells were seeded on colloidal gold-coated dishes at a density of 2×10^3 cells per dish in 2 ml of Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Photographs were taken, and cell motility was evaluated by measuring areas free of the gold particles.

Microinjection. Each sample to be tested was microinjected into living Swiss 3T3 cells as described previously (7, 55). Briefly, glass capillaries drawn to a tip diameter of less than 1 μm were used to microinject each sample. About 30 cells were microinjected within 20 min at 4 h after the cells were seeded on colloidal gold-coated dishes. About 30 to 40% of the microinjected cells could still attach to the dish and move after a 24-h incubation.

About 5×10^{-14} liter of sample was microinjected during one injection (7). When the GTPγS-bound form of *rho*A p21, the same form of *rac*1 p21, the same form of *smg* p21B, or Ki-*ras*$^{Val\text{-}12}$ p21 was microinjected at 0.5 mg/ml and the GTPγS-bound form of GST-G25K was microinjected at 1 mg/ml, the intracellular concentrations of the microinjected samples were calculated to be about 3.5 μM. Since the endogenous concentration of *rho*A p21, *rac*1 p21, or G25K is not known, the levels of the exogenous and endogenous small G proteins were not compared. On the other hand, the concentrations of microinjected *smg* p21B and Ki-*ras*$^{Val\text{-}12}$ p21 were about 15-fold higher than the endogenous levels. When GST-*rho* GDI and *smg* GDS were microinjected at 5 and 8 mg/ml, respectively, the intracellular concentrations of microinjected *rho* GDI and *smg* GDS were calculated to be about 13 and 19 μM, respectively. The intracellular concentrations of microinjected *rho* GDI and *smg* GDS were about 80- and 150-fold higher than the endogenous levels, respectively. The endogenous levels of *smg* p21B, Ki-*ras*$^{Val\text{-}12}$, *rho* GDI, and *smg* GDS were quantitated by using immunoblots with their respective antibodies. C_3 was microinjected at 40 μg/ml, and the intracellular concentration of the microinjected C_3 was calculated to be about 0.21 μM.

Determination of protein concentrations. Protein concentrations were determined, with bovine serum albumin as a standard, by densitometric tracing of protein bands stained with Coomassie brilliant blue on a sodium dodecyl sulfate-polyacrylamide gel as described previously (53).

RESULTS

Inhibition of cell motility by microinjection of *rho* GDI or C_3 into Swiss 3T3 cells. The gold particles were observed as a homogeneous layer of fine black particles (Fig. 1). When

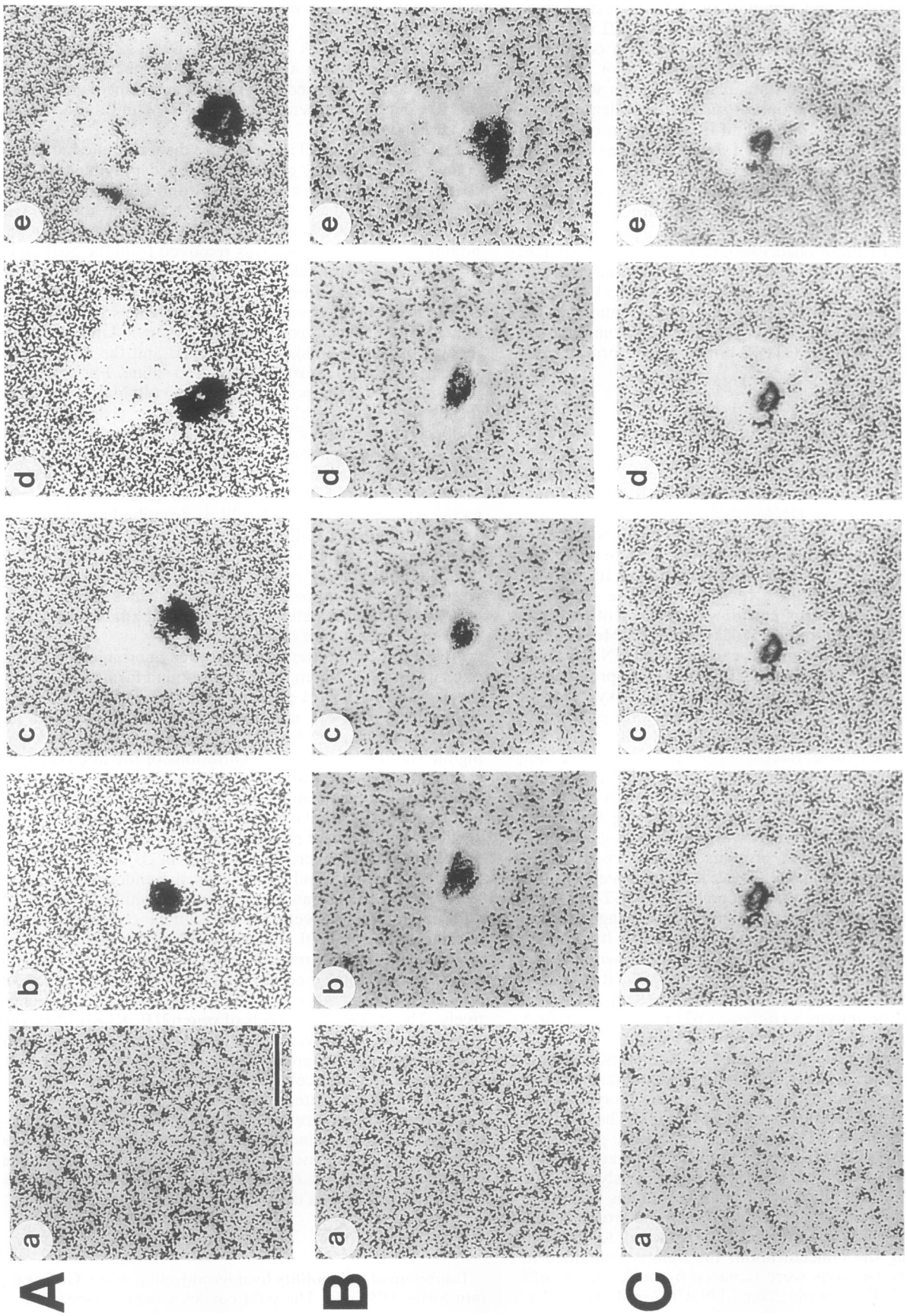

FIG. 1. Inhibition of cell motility by microinjection of *rho* GDI or C_3 into Swiss 3T3 cells. After microinjection of buffer A (A) *rho* GDI (B), or C_3 (C) into Swiss 3T3 cells and incubation for various periods as indicated, the cell motility was analyzed by phase-contrast microscopy. (a) Gold particles only; (b) before microinjection; (c) 2 h after microinjection; (d) 6 h after microinjection; (e) 12 h after microinjection. The results shown are representative of three independent experiments. Bar, 30 μm. All photographs were taken with the same magnification.

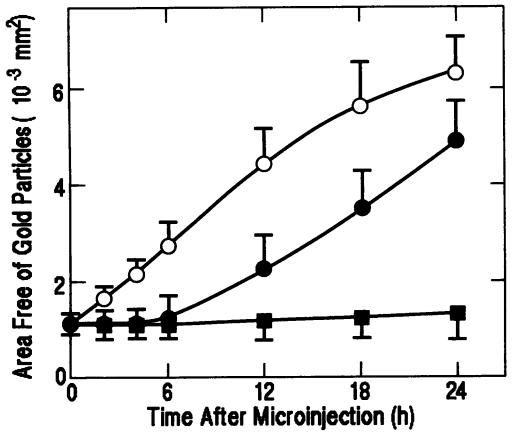

FIG. 2. Time courses of the motility of Swiss 3T3 cells. After microinjection of buffer A, *rho* GDI, or C_3 into Swiss 3T3 cells and incubation for various periods as indicated, the cell motility was examined. Symbols: ○, with buffer A; ●, with *rho* GDI; ■, with C_3. The results shown are the mean ± standard error of three independent experiments.

Swiss 3T3 cells migrated on this substrate, they phagocytized and removed the gold particles to produce a white track free of the particles (Fig. 1A). One migrating cell was visible inside the white track as a black body. The area of the track increased linearly until 24 h after microinjection of buffer A into Swiss 3T3 cells (Fig. 2). Microinjection of *rho* GDI into Swiss 3T3 cells inhibited cell motility for 6 h after microinjection (Fig. 1B). After 6 h, the cell began to move again, and the migration area reached about 80% of that of the control cell at 24 h (Fig. 2). Thus, *rho* GDI action was reversible, and microinjection of *rho* GDI did not kill the cells. The minimum concentration of *rho* GDI necessary to inhibit cell motility was about 2.5 mg/ml. Microinjection of C_3 into Swiss 3T3 cells also inhibited cell motility for 6 h after microinjection (Fig. 1C), but in this case the cell did not begin to move again even at 24 h (Fig. 2). Under these conditions, about 70 to 80% of the cells into which C_3 was microinjected were detached from the dish, but 20 to 30% of the cells still attached to the dish and did not appear to die under these conditions, as estimated by a trypan blue exclusion test (data not shown). It was previously reported that intracellular vesicle traffic of endocytosis and exocytosis might also be involved in cell motility (10). We therefore examined the effect of *rho* GDI on pinocytosis by measuring the accumulation of fluorescein isothiocyanate-dextran by the method of Oliver et al. (37). Microinjection of *rho* GDI did not affect pinocytosis (data not shown).

Prevention of *rho* GDI action by *rho*A p21. We have previously shown that *rho* GDI is active on *rho*A p21, *rho*B p21, *rac*1 p21, and *rac*2 p21 and that *rho* GDI interacts with the GDP-bound form of *rho*A p21 and *rho*B p21 much more preferentially than with the GTP-bound form (19, 52). It has also been found that *rho* GDI is active on G25K (11). The time-dependent cell motility was inhibited by microinjection of *rho* GDI as described above, and this *rho* GDI action was prevented by comicroinjection of *rho* GDI with the GTPγS-bound form of *rho*A p21 (Fig. 3 and 4). The minimum ratio of the GTPγS-bound form of *rho*A p21 to *rho* GDI for preventing *rho* GDI action was about 1/10. However, *rho* GDI action was not prevented by comicroinjection of *rho* GDI with the same form of *rac*1 p21 or G25K. In contrast, *rho* GDI action was also prevented by comicroinjection of *rho* GDI with the GDP-bound form of *rho*A p21, *rac*1 p21, or G25K (data not shown). In these experiments, the GDP-bound forms of these small G proteins were used in the same amounts as those used in the experiments shown in Fig. 3 and 4. The reason why the GDP-bound forms of all of these small G proteins prevented *rho* GDI action is not known, but they might interact with *rho* GDI and effectively lower the concentration of *rho* GDI to a level below that necessary for its action. *rho* GDI action was not prevented by comicroinjection of *rho* GDI with the GTPγS-bound form of *smg* p21B, the GDP-bound form of *smg* p21B, or Ki-*ras*$^{Val\text{-}12}$ p21 (Fig. 3). Neither *smg* p21B nor Ki-*ras* p21 is a substrate for *rho* GDI (52).

Inability of *rho*A p21 and *smg* GDS to affect cell motility. Microinjection of *rho* GDI into Swiss 3T3 cells inhibited cell motility, and this *rho* GDI action was prevented by comicroinjection of *rho* GDI with the GTPγS-bound form of *rho*A p21 as described above. However, under comparable conditions, microinjection of the GTPγS-bound form of *rho*A p21 alone into Swiss 3T3 cells did not affect cell motility (Fig. 5). Microinjection of *smg* GDS alone into the cells did not affect cell motility. Moreover, comicroinjection of *rho* GDI with *smg* GDS did not prevent *rho* GDI action. The level of endogenous *rho* GDI was about 1.5-fold higher than that of endogenous *smg* GDS in Swiss 3T3 cells as estimated by the results of immunoblots with their respective antibodies (data not shown).

Requirement of the post-translational modifications of the C-terminal region of *rho*A p21 for the prevention of the *rho* GDI action. *rho*A p21 used in the above experiments was not posttranslationally modified with lipids. Nevertheless, *rho* GDI action was prevented by comicroinjection of *rho* GDI with this form of *rho*A p21 as described above. This result suggests that *rho*A p21 microinjected into the cells underwent the posttranslational modifications. Also, *rho* GDI action was not prevented by comicroinjection of *rho* GDI with the *rho*A p21 mutant lacking the C-terminal three amino acids (*rho*A p21$^{\Delta LVL}$) (Fig. 6). This *rho*A p21 mutant was previously confirmed not to be geranylgeranylated by its geranylgeranyltransferase in the cell-free experiments (25, 54).

DISCUSSION

We have first shown here that microinjection of *rho* GDI into Swiss 3T3 cells inhibits cell motility, generally called chemokinesis. This *rho* GDI action is prevented by comicroinjection of *rho* GDI with the GTPγS-bound active form of *rho*A p21 but not by comicroinjection with other small G proteins including *rac*1 p21, G25K, *smg* p21B, and Ki-*ras*$^{Val\text{-}12}$ p21. Moreover, we have shown here that C_3, which is known to selectively ADP-ribosylate *rho* p21, also inhibits cell motility. This result is consistent with a recent observation that C_3 inhibits spontaneous and chemoattractant-induced motility of neutrophils (47).

Evidence is accumulating that *rho* p21 affects the actomyosin system (38), and recently we have consistently obtained evidence that *rho* GDI induces morphological change and disappearance of stress fibers through *rho* p21 in Swiss 3T3 cells (30). Furthermore, the actomyosin system is known to be essential for cell motility (13, 28, 36, 46, 48). We have confirmed that cytochalasin D inhibits the motility of Swiss 3T3 cells under our assay conditions, whereas colchicine, a microtubule-disrupting agent, has no effect on cell motility (data not shown). Therefore, our present results, together with these earlier observations, indicate that *rho* GDI inhibits *rho* p21 activation and/or action and thereby blocks cell motility, presumably through the actomyosin system. It is

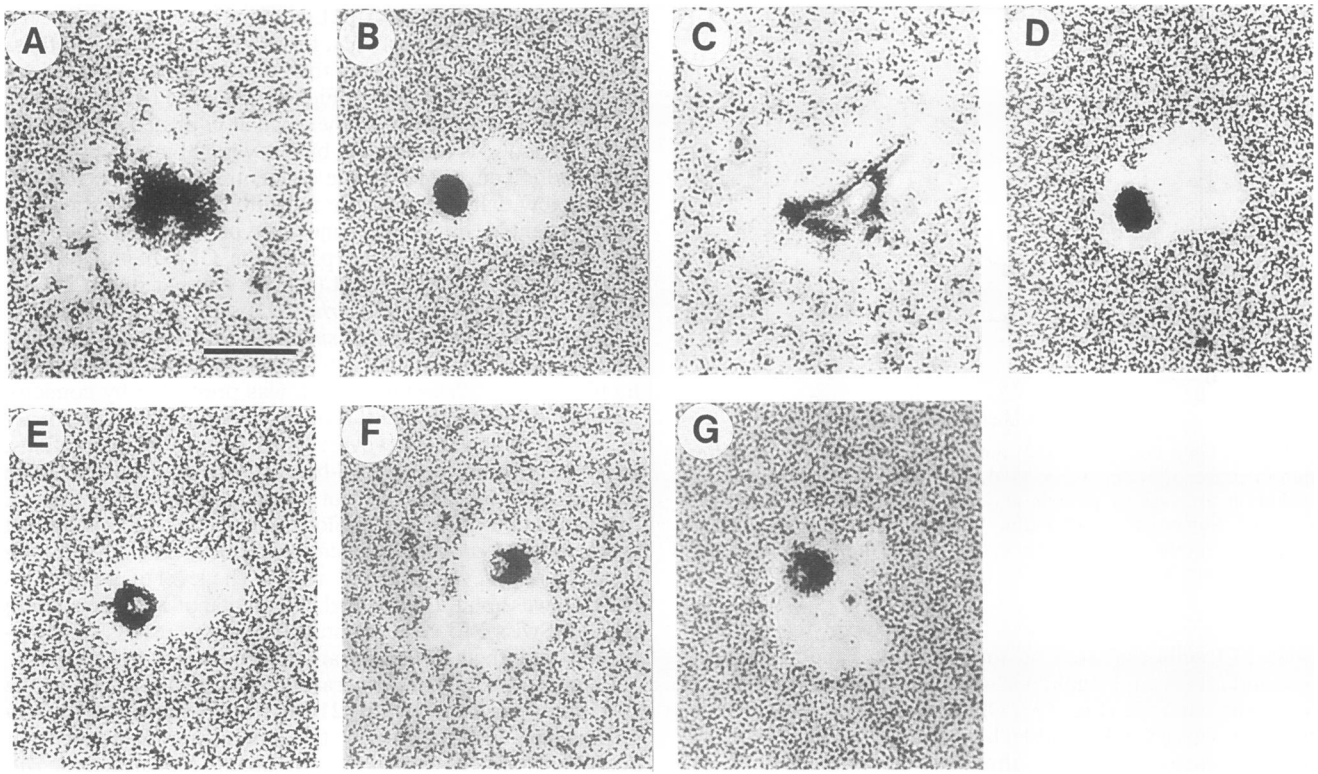

FIG. 3. Prevention of *rho* GDI action by *rho*A p21. The motility of Swiss 3T3 cells was examined at 6 h after microinjection of buffer A (A) or *rho* GDI (B) or comicroinjection of *rho* GDI with the GTPγS-bound form of *rho*A p21 (C), the same form of *rac*1 p21 (D), the same form of G25K (E), the same form of *smg* p21B (F), or Ki-*ras*$^{Val-12}$ p21 (G). The results are representative of three independent experiments. Bar, 30 μm. All photographs were taken with the same magnification.

unclear which type of *rho* p21 among the three members of the *rho* p21 family is present in Swiss 3T3 cells.

We have previously reported that *rho* GDI forms a complex with the GDP-bound inactive form of *rho* p21 much more preferentially than with the GTP-bound active form (52). Moreover, we have suggested that *rho* GDI masks directly or indirectly the effector domain of *rho* p21 and prevents interaction of *rho* p21 with the effector protein (26). Therefore, our present results, together with these previous observations obtained in the cell-free experiments, indicate that *rho* GDI indeed negatively regulates *rho* p21 activity and/or *rho* p21 action in intact cells.

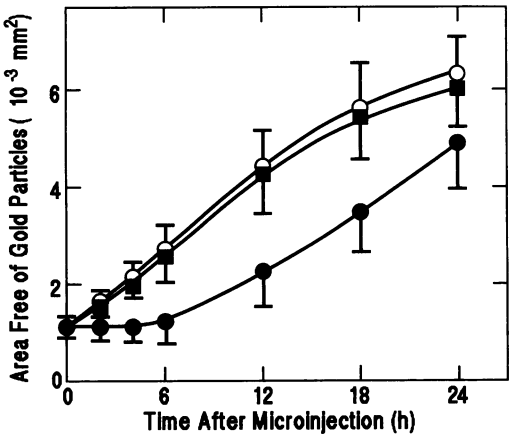

FIG. 4. Time courses of the effect of *rho*A p21 on *rho* GDI action. After comicroinjection of *rho* GDI with buffer A or the GTPγS-bound form of *rho*A p21 into Swiss 3T3 cells and incubation for various periods as indicated, the cell motility was examined. Symbols: ○, with buffer A; ●, with *rho* GDI; ■, with *rho* GDI plus the GTPγS-bound form of *rho*A p21. The results shown are the mean ± standard error of three independent experiments.

We have shown here that microinjection of the GTPγS-bound form of *rho*A p21 does not affect cell motility under conditions where *rho* GDI inhibits it and that this *rho* GDI action is prevented by comicroinjection of *rho* GDI with the GTPγS-bound form of *rho*A p21. Our results suggest that enough endogenous *rho* p21 is present in the GTP-bound active form to maintain normal cell motility of Swiss 3T3 cells under our experimental conditions.

We have previously shown in the cell-free experiments that the inhibitory action of *rho* GDI in the GDP/GTP exchange reaction of *rho* p21 is stronger than the stimulatory action of *smg* GDS when they are both present (26). Consistent with this earlier result, we have shown here that comicroinjection of *rho* GDI with *smg* GDS does not prevent *rho* GDI action. We have shown here that microinjection of *smg* GDS alone into Swiss 3T3 cells does not affect cell motility. This might be due to the presence of enough endogenous GTP-bound active form of *rho* p21 and/or enough endogenous *rho* GDI to prevent the action of exogenous *smg* GDS.

It is not known how microinjected *rho* GDI regulates *rho* p21 activity and action in intact Swiss 3T3 cells. However, the following mechanisms may be the most likely. In Swiss

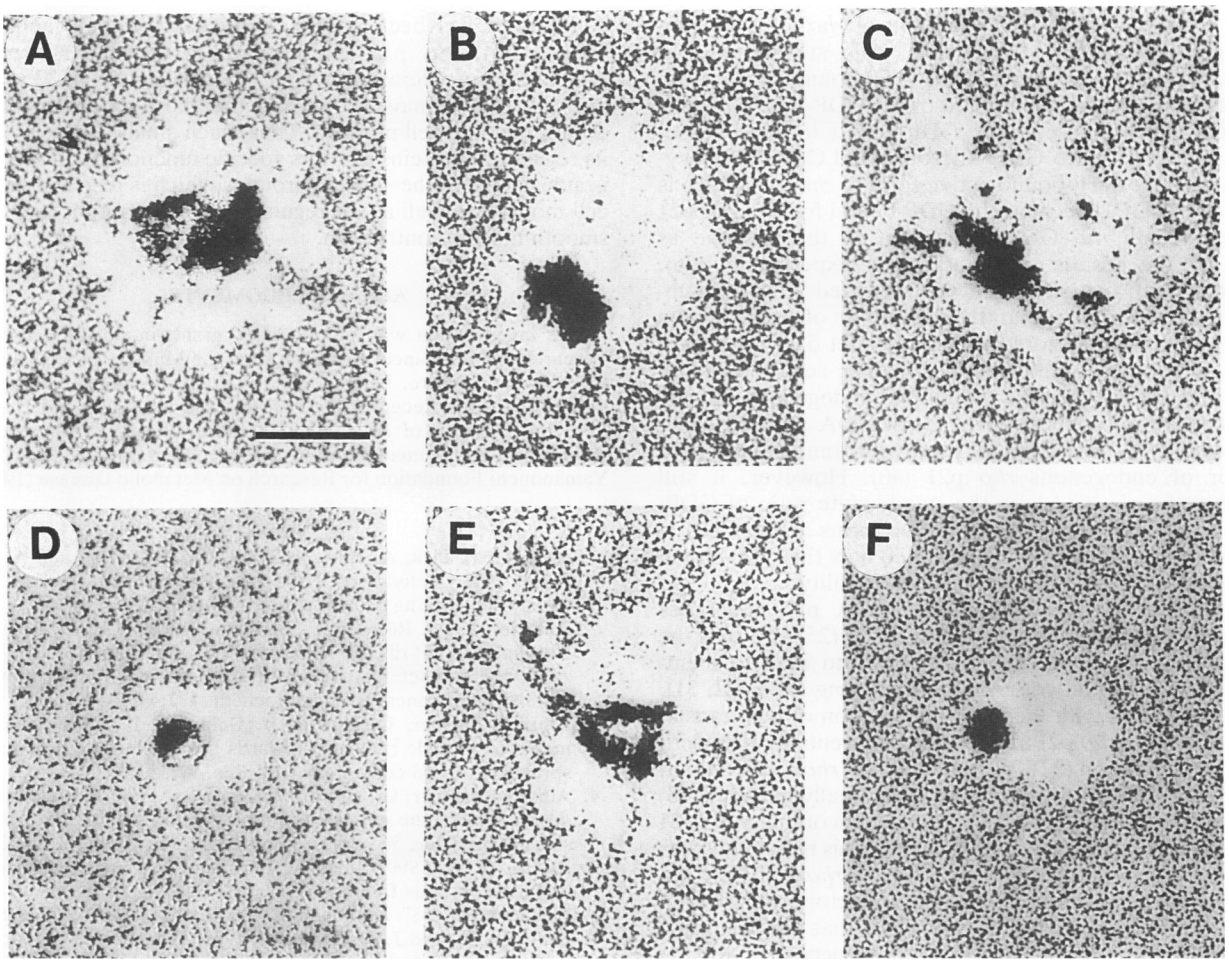

FIG. 5. Inability of *rho*A p21 and *smg* GDS to affect cell motility. The motility of Swiss 3T3 cells was examined at 6 h after microinjection with buffer A (A), the GTPγS-bound form of *rho*A p21 (B), *smg* GDS (C), *rho* GDI (D), *rho* GDI plus the GTPγS-bound form of *rho*A p21 (E), or *rho* GDI plus *smg* GDS (F). The results shown are representative of three independent experiments. Bar, 30 μm. All photographs were taken with the same magnification.

3T3 cells under our experimental conditions, the GDP-bound inactive and GTP-bound active forms are present in an appropriate steady state as a result of the actions of *rho* GDI, *rho* GDS, *smg* GDS, and *rho* GAP, and enough *rho* p21 is present in the GTP-bound active form to maintain the normal cell motility, because the experiments are complete 28 h after the cells are seeded and the cells are still in the medium containing 10% fetal calf serum, which includes various

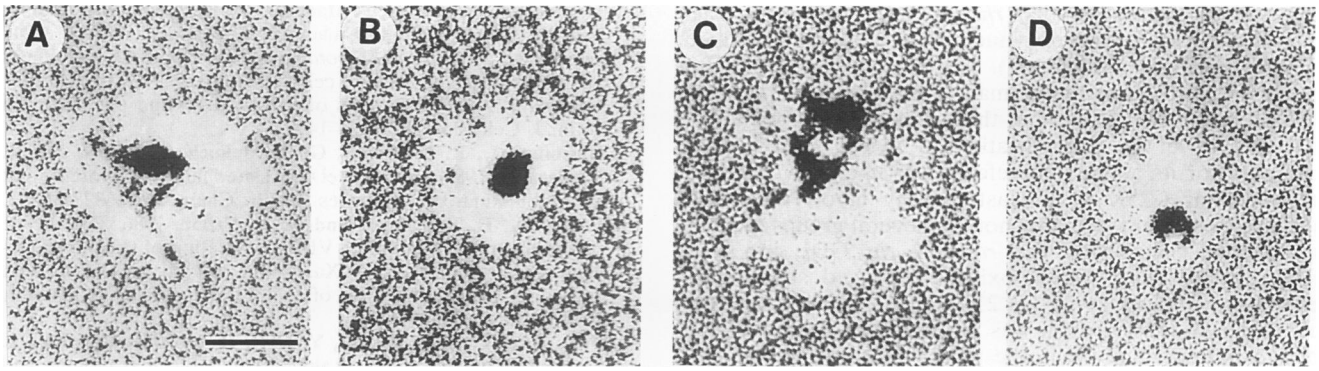

FIG. 6. Requirement of posttranslational modifications of the C-terminal region of *rho*A p21 for the prevention of the *rho* GDI action. The motility of Swiss 3T3 cells was examined at 6 h after microinjection with buffer A (A), *rho* GDI (B), *rho* GDI plus the GTPγS-bound form of *rho*A p21 (C), or *rho* GDI plus the GTPγS-bound form of *rho*A $p21^{\Delta LVL}$ (D). The results shown are representative of three independent experiments. Bar, 30 μm. All photographs were taken with the same magnification.

growth factors. When a large amount of *rho* GDI is artificially microinjected into the cells, this steady state is changed so that the amount of the GTP-bound form of *rho* p21 is diminished and the amount of the GDP-bound form is concomitantly increased; this GDP-bound form is subsequently trapped by *rho* GDI. Microinjected C_3 ADP-ribosylates mainly the GTP-bound active form of *rho* p21 which is free from *rho* GDI, because the GDP-bound form of *rho* p21 complexed with *rho* GDI is resistant to this enzyme as shown by the results of the cell-free experiments (26). Consistent with our results, it was reported after the submission of this paper that the assembly of stress fibers induced by growth factors, such as platelet-derived growth factor and bombesin, and lysophosphatidic acid is inhibited in Swiss 3T3 cells when the function of endogenous *rho* p21 is blocked by microinjection of C_3 or *rho*A p21 pre-ADP-ribosylated by C_3, which might serve as a dominant negative inhibitor of endogenous *rho* p21 (40). However, it still remains to be clarified how the steady-state ratio of GDP-bound inactive and GTP-bound active forms of *rhoA* p21 is regulated by its regulatory proteins and how the GTP-bound active form of *rho*A p21 regulates cell motility.

We have previously shown that *rho*A p21 undergoes posttranslational modifications with lipids (25) and that the modifications are important for *rho* p21 to bind to membranes and to interact with *rho* GDI and *smg* GDS (22, 31). *rho* GDI interacts with the GDP-bound form of posttranslationally modified *rho* p21 much more preferentially than with the GTP-bound form (22). We have made a *rho*A p21 mutant (*rho*A $p21^{\Delta LVL}$) which is not posttranslationally modified (25) and have shown that the GTPγS-bound form of this *rho*A p21 mutant does not prevent *rho* GDI action. This result indicates that the posttranslational modifications of *rho*A p21 are essential for *rho* p21 action. Although the effector protein of *rho* p21 has not been identified, it is possible that the posttranslational modifications of *rho* p21 are also necessary for the interaction with its effector protein.

rac p21 and G25K have about 50 to 60% amino acid homology to *rho*A p21, and the amino acid sequences of the putative effector domains of *rac* p21 and G25K are the same as that of *rho*A p21 except for one amino acid (14, 33). Moreover, these small G proteins are substrates for *rho* GDI and *smg* GDS (11, 19, 31). We have shown here that neither the GTPγS-bound form of *rac*1 p21 nor the same form of G25K prevents the action of *rho* GDI in inhibiting cell motility under conditions where the same form of *rho*A p21 prevents *rho* GDI action, but that the GDP-bound forms of *rho*A p21, *rac*1 p21, and G25K prevent *rho* GDI action. We have previously shown that *rho* GDI interacts with the GDP-bound form of *rho* p21 much more preferentially than with the GTP-bound form (52). The reason why the GDP-bound forms of all of these small G proteins prevents *rho* GDI action is not known, but they might interact with *rho* GDI and lower its concentration to a level below that necessary for its action. Therefore, it is likely that neither *rac*1 p21 nor G25K is at least directly involved in cell motility. Consistent with this notion, several groups including our own have shown that *rac* p21, *rho* GDI, and *smg* GDS regulate the NADPH oxidase-catalyzed superoxide generation in phagocytes (1, 27). Furthermore, after the submission of this paper, it was reported that *rac*1 p21, but not *rho* p21, is involved in the pinocytosis and membrane ruffling induced by microinjection of the point-mutated active form of *rac*1 p21 or Ha-*ras* p21 or by growth factors such as platelet-derived growth factor and bombesin in Swiss 3T3 cells (41). The function of G25K is still unknown, but its yeast counterpart has been shown to regulate budding and cell polarity (24). *ras* p21 and *smg* p21 have been shown to regulate cell proliferation in NIH 3T3 cells and Swiss 3T3 cells (6, 7, 51, 55). We have shown here that these small G proteins do not regulate cell motility. Thus, each small G protein and its regulatory proteins exert its specific functions, and *rho* p21 is at least one of the small G proteins which is responsible for cell motility as well as for regulation of cell morphology and smooth muscle contraction.

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