Regulation of Morphology by rho p21 and Its Inhibitory GDP/GTP Exchange Protein (rho GDI) in Swiss 3T3 Cells*

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rho GDP dissociation inhibitor (GDI) is an inhibitory GDP/GTP exchange protein for a group of small GTP-binding proteins including at least rhoA p21, rhoB p21, rac1 p21, rac2 p21, and G25K. Microinjection of rho GDI into Swiss 3T3 cells made the cells round and refractile. This morphological change was accompanied by the disappearance of stress fibers. The rho GDI action was prevented by coinjection of rho GDI with the guanosine 5'- (3-O-thio) triphosphate (GTPγS)-bound form of rhoA p21, but not with the GTPγS-bound form of rhoA p21 lacking the C-terminal three amino acids, which was not post-translationally modified with lipids. The GTPγS-bound form of rac1 p21, the same form of G25K, the same form of smg p21B, or Ki-ras-212 p21 was ineffective. Microinjection of the bacterial ADP-ribosyltransferase C3 specific for rho p21 into Swiss 3T3 cells induced the similar changes of morphology and stress fibers. This C3 action was not prevented by coinjection of C3 with the GTPγS-bound form of rhoA p21, but was prevented by coinjection with the same form of a rhoA p21 mutant which was not ADP-ribosylated by C3. These results indicate that the rho GDI-rho p21 system regulates cell morphology presumably through the actomyosin system in Swiss 3T3 cells.

The rho p21 family, consisting of three members A, B, and C, belongs to the ras p21-related small G protein superfami

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1The abbreviations used are: G protein, GTP-binding protein; EDIN, epidermal differentiation inhibitor; GTPγS, guanosine 5'- (3-O-thio) triphosphate; GEP, GDP/GTP exchange protein; GAP, GTPase activating protein; GDS, GDP dissociation stimulator; GDI, GDP dissociation inhibitor; GST, glutathione S-transferase; FITC, fluorescein isothiocyanate; FCS, fetal calf serum; PBS, phosphate-buffered saline.

this result that the ADP-ribosylation impairs the rho p21 function (9). Consistently, C3 and EDIN have been shown to affect various cell functions. Addition of C3 to NIH/3T3 cells made the cells round and refractile (10). Addition of C3 to PC-12 cells generates short neurites, and addition of C3 to Vero cells and neutrophils decreases the actin filaments (10-13). Microinjection of C3 into Xenopus oocytes induces the migration of germinal vesicles and enhances the progesterone-induced vesicle breakdown (10). Microinjection of the GTPγS-bound active form of rhoA p21 or rhoA™24 p21, which is a point-mutated active form, into Swiss 3T3 cells contracts the cells, and microinjection of rhoA™24 p21 into Xenopus oocytes redistributes the pigments (14, 15). These results suggest that rho p21 regulates these cell functions through the actomyosin system. On the other hand, GTPγS has been shown to decrease the Ca²⁺ concentrations necessary for the vasoconstrictr-induced smooth muscle contraction, and a G protein has been suggested to be involved in this Ca²⁺ sensitization mechanism (16-18). We have found that C3 and EDIN inhibit this GTPγS-induced Ca²⁺ sensitization in smooth muscle contraction and that this inhibitory action of EDIN is overcome by the GTPγS-bound active form of rho p21 (19). This result indicates that rho p21 is a G protein which is involved in the vasoconstrictr-induced Ca²⁺ sensitization mechanism, and moreover provides the definitive evidence for the involvement of rho p21 in the regulation of the actomyosin system.

The rho p21 activity is regulated by both GEP and GAP (1, 2). The conversion from the GDP-bound inactive form to the GTP-bound active form is regulated by GEP and the reverse conversion is regulated by GAP. There are two types of GEP for rho p21: one is a stimulatory type, named smg GDS and rho GDS, and the other is an inhibitory type, named rho GDI (20-26). These regulatory proteins are present in most cells, and the intracellular amount of rho GDI is larger than that of smg GDS (27). 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 in their simultaneous presence (26, 27). rho p21 is present in the GDP-bound inactive form complexed with rho GDI in the cytosol of resting cells such as insulinoma cells and smooth muscle cells (26, 28). The GDP-bound form of rhoA p21 complexed with rho GDI is resistant to the ADP-ribosylation by EDIN (27). Meanwhile rho p21 undergoes three kinds of post-translation modifications in the C-terminal region: geranylgeranylation of the cysteine residue, removal of the three C-terminal amino acids, and carboxyl methylation of the exposed cysteine residue (29). Only the post-translationally processed form of rho p21, but not the post-translationally unprocessed form, is sensitive to these GDI and GDS actions, although both forms are sensitive to the GAP action (2, 25, 30). On the basis of these observations, we have tentatively proposed the
follow ing modes of action and activation 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 masked by rho GDI. Upon stimulation of cells with some agonists, the inhibitory action of rho GDI is released in rho p21 with unknown properties, the GDP-bound inactive form of rho p21 becomes sensitive to the smg GDS action, and the GTP-bound active form is produced. By this activation, rho p21 opens the effector region, interacts with its effector protein, and exerts its biological function through this effector protein.

In the present study, to prove these mechanisms of activation and action of rho p21 and to clarify the physiological function of rho GDI, we have investigated the actions of rho p21 and rho GDI in intact cells and examined their effects on the morphology of Swiss 3T3 cells.

**EXPERIMENTAL PROCEDURES**

**Materials and Chemicals—**Swiss 3T3 cells were kindly supplied by Dr. E. Rongeur (Imperial Cancer Research Fund, London, United Kingdom). The cDNAs of rhoA p21 and Ki-ras \(^{\text{ALVL}}\) p21 were kindly provided by Dr. P. Madaule (Centre National de la Recherche Scientifique, Labastide, France) and Dr. R. A. Weinberg (Massachusetts Institute of Technology), respectively. The cDNA of G25K and the baculovirus carrying the cdna of rac1 p21 were kindly provided by Dr. P. Poleakis and F. McCormick (Chiron Corp.). The baculoviruses carrying the cDNA of rhoA p21, rhoA \(^{\text{ALVL}}\) p21, smg p21B, or Ki-ras \(^{\text{ALVL}}\) p21 were kindly provided by Dr. Y. Matsuura (National Institute of Health, Tokyo, Japan). C, was kindly supplied by Dr. B. Syuto (Hokkaido University, Sapporo, Japan) (31). Mutagenesis of Aan to Icde at codon 41 of rhoA p21 (rhoA \(^{\text{ALVL}}\) p21) was carried out by site-directed mutagenesis (14, 32). rhoA p21, rhoA \(^{\text{ALVL}}\) p21, rac1 p21, smg p21B, and Ki-ras \(^{\text{ALVL}}\) p21 were expressed in Sf9 cells and purified from the cytosol fraction of Sf9 cells overexpressing each small G protein (25). rhoA p21 lacking the three C-terminal amino acids (rhoA \(^{\text{ALVL}}\) p21) was purified from rhoA \(^{\text{ALVL}}\) overexpressing Escherichia coli (29). rho GDI and G25K were purified as GST fusion proteins from E. coli overexpressing GST-rho GDI and GST-G25K, respectively (27, 33, 34). It was confirmed that the recombinant GST-rho GDI showed the same activity toward the GDP/GTP exchange reaction of rhoA p21 as the 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 the GST-rho GDI produced in the similar method shows the same activity as rhoA p21 purified from rhoA overexpressing Sf9 cells (33). smg GDS was purified from smg GDS-overexpressing E. coli. The GTPyS-bound form of rhoA p21, rhoA \(^{\text{ALVL}}\) p21, rhoA \(^{\text{ALVL}}\) p21, rac1 p21, GST-G25K, or smg p21B was made by incubating each small G protein with 60 \(\mu\)M GTPyS (35, 36). All the proteins used were concentrated in Centricon-10 (Amicon) to the concentrations of 2–20 mg/ml. During the concentration, the buffer contained in the sample preparations were replaced by buffer A (20 \(\mu\)M Tris/ HCl at pH 7.4 containing 20 \(\mu\)M NaCl, 2 \(\mu\)M MgCl\(_2\), 100 \(\mu\)M ATP, 0.1 mM EDTA, and 1 mM 2-mercaptoethanol). FITC-labeled phalloidin was purchased from Sigma.

**Cell Culture—**Stock cultures of Swiss 3T3 cells were maintained at 37 °C in a humidified atmosphere of 3% CO\(_2\) and 97% air in Dulbecco’s modified Eagle’s medium containing 10% FCS, penicillin (100 units/ml), and streptomycin (100 \(\mu\)g/ml). To analyze cell morphology and stress fibers, the cells were seeded into 35-mm glass tissue culture dishes (Nunc Inc.) at a density of 1.4 \(\times\) 10^5 cells/dish in 2.5 ml of Dulbecco’s modified Eagle’s medium containing 10% FCS, and the dishes were incubated at 37 °C for 5 days. These cells were subconfluent.

**Microinjection—**Each sample to be tested was microinjected into living Swiss 3T3 cells (36, 37). Briefly, glass capillaries drawn to a tip diameter of less than 1 \(\mu\)m were used to microinject each sample. About 50 cells in an area surrounded by four grids were usually microinjected with 5 min, and all cells were confirmed to be microinjected by lucifer yellow. Trypan blue exclusion test showed that more than 90% of the cells survived the microinjection procedure. The cells were returned to the incubator and incubated for various periods of time as indicated at 37 °C.

About 5 \(\times\) 10^4 liter of sample was microinjected by one injection (37). When the GTPyS-bound form of rhoA p21, the same form of rhoA \(^{\text{ALVL}}\) p21, the same form of rhoA p21 \(^{\text{ALVL}}\), the same form of rac1 p21, the same form of smg p21B, or Ki-ras \(^{\text{ALVL}}\) p21 was microinjected at 0.5 mg/ml each and the GTPyS-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 \(\mu\)M. Since the endogenous concentration of rhoA p21, rac1 p21, or G25K was unknown, the levels of the exogenous and endogenous small G proteins were not compared. On the other hand, the concentrations of the microinjected smg p21B and Ki-ras \(^{\text{ALVL}}\) p21 were about 15-fold more than their endogenous levels. When GST-rho GDI and smg GDS were microinjected at 5 and 8 mg/ml, respectively, the intracellular concentrations of the microinjected rho GDI and smg GDS were calculated to be about 13 and 19 \(\mu\)M, respectively. The intracellular concentrations of the microinjected rho GDI and smg GDS were about 80- and 150-fold more than their endogenous levels, respectively. C, was microinjected at 100 \(\mu\)g/ml, and the intracellular concentration of the microinjected C, was calculated to be about 0.57 \(\mu\)g/ml.

**Analysis of Cell Morphology and Stress Fibers—**Cell morphology was analyzed by phase-contrast microscopy (model IMT-2, Olympus, Tokyo, Japan) at various periods of time after microinjection. For analysis of stress fibers, cells grown on glass coverslips were washed with PBS and fixed in 3.7% formaldehyde/PBS for 50 min at room temperature. About 200 \(\mu\)g/ml FITC-phalloidin was applied to each coverslip. The coverslips were incubated in a humidified chamber for 60 min at room temperature, washed in PBS, and mounted in 20% glycerol/PBS containing 1 mg/ml p-phenylenediamine, and the edges were sealed with nail polish. The samples were examined by a fluorescence microscope (model D-7082, Carl Zeiss, Oberkochen, Germany).

**Results**

**Changes of Morphology and Stress Fibers by Microinjection of rho GDI into Swiss 3T3 Cells—**When rho GDI was microinjected into Swiss 3T3 cells, the cells became round and refractile within 1 h after the microinjection as analyzed by phase-contrast microscopy (Fig. 1). These cells left beaded dendritic processes attached to the dish. The morphological change continued for 6 h after the microinjection, but thereafter the cells regained their normal morphology. In the normal control cells, well-developed stress fibers were observed, but in the rounded cells, stress fibers disappeared as analyzed by staining the fibers by FITC-labeled phalloidin (Fig. 2). These results suggest that rho GDI induces the morphological change through at least the actomyosin system and exerts this action through its substrate small G protein(s).

**Prevention of the rho GDI Action by rhoA p21—**rhoA p21, rac1 p21, rac2 p21, and G25K \(^{2}\) are known to be substrates for rho GDI (20, 39). The rho GDI-induced morphological change was prevented by coinoculation of rho GDI with the GTPyS-bound form of rhoA p21 (Fig. 3). However, the rho GDI action was not prevented by coinoculation of rho GDI with the same form of rac1 p21 or the same form of G25K. The rho GDI action was not prevented by coinoculation of rho GDI with the same form of smg p21B or Ki-ras \(^{\text{ALVL}}\) p21 either. Neither smg p21B nor Ki-ras p21 is a substrate for rho GDI (20).

**Inability of rhoA p21 and smg GDS to Induce Morphological Change—**Microinjection of rho GDI into Swiss 3T3 cells induced the morphological change, and this rho GDI action was prevented by coinoculation of rho GDI with the GTPyS-bound form of rhoA p21 as described above. However, under the comparable conditions, microinjection of the GTPyS-bound form of rhoA p21 alone into Swiss 3T3 cells did not affect the morphology (Fig. 4). Microinjection of smg \(^{2}\) R. A. Cerione, personal communication.
Function of the rho GDI-rho p21 System

FIG. 1. Morphological change by microinjection of rho GDI into Swiss 3T3 cells. Swiss 3T3 cells were microinjected with rho GDI and incubated for various periods of time as indicated. After the cells were fixed, the specimen was analyzed by a phase-contrast microscopy. A, before microinjection; B, 1 h after microinjection; C, 2 h after microinjection; D, 6 h after microinjection; E, 10 h after microinjection; F, 16 h after microinjection; G, 24 h after microinjection. The results shown are the representative of three independent experiments. Bar indicates 10 μm. All photographs were taken with the same magnification.

FIG. 2. Disappearance of stress fibers by microinjection of rho GDI into Swiss 3T3 cells. Swiss 3T3 cells were microinjected with buffer A or rho GDI and incubated for 2 h. After the cells were fixed and stained with FITC-labeled phalloidin, the specimen was analyzed by a fluorescence microscopy. A, with buffer A; B, with rho GDI. The results shown are the representative of three independent experiments. Bar indicates 10 μm. Both photographs were taken with the same magnification.

FIG. 3. Prevention of the rho GDI action by rhoA p21. Morphological change of Swiss 3T3 cells was examined at 2 h after comicroinjection of rho GDI with the GTPγS-bound form of rhoA p21, the same form of rac1 p21, the same form of G25K, the same form of smg p21B, or Ki-rasK p21. A, with buffer A alone; B, with rho GDI alone; C, with rho GDI plus rhoA p21; D, with rho GDI plus rac1 p21; E, with rho GDI plus G25K; F, with rho GDI plus smg p21B; G, with rho GDI plus Ki-rasK p21. The results shown are the representative of three independent experiments. Bar indicates 10 μm. All photographs were taken with the same magnification.

GDS alone into the cells did not affect the morphology either (Fig. 4). Moreover, comicroinjection of rho GDI with smg GDS did not prevent the rho GDI action.

Requirement of the Post-translational Modifications of the C-terminal Region of rhoA p21 for the Prevention of the rho GDI Action—The rhoA p21 used in the above experiments was not post-translationally modified with lipids. Nevertheless, the rho GDI action was prevented by comicroinjection of rho GDI with this form of rhoA p21. This result suggests that the rhoA p21 microinjected into the cells underwent the post-translational modifications. Consistently, the rho GDI action was not prevented by comicroinjection of rho GDI with the rhoA p21 mutant which lacked the C-terminal three amino acids (rhoA p21ΔV1) (Fig. 5). This rhoA p21 mutant was previously confirmed not to be geranylgeranylated by geranylgeranyltransferase in a cell-free system (29, 40). The morphological change induced by comicroinjection of rho GDI with the GTPγS-bound form of rhoA p21ΔV1 was, however, slightly different from that induced by microinjection of rho GDI alone. The dendritic processes of the cells microinjected with both rho GDI and the GTPγS-bound form of rhoA p21ΔV1 was far less than those of the cells microinjected with rho GDI alone. The reason for this difference is not known.

Changes of Morphology and Stress Fibers by Microinjection of C3 into Swiss 3T3 Cells—Microinjection of C3 into Swiss 3T3 cells induced morphological change similar to that in...
duced by microinjection of rho GDI (Fig. 6). The C3-induced morphological change was observed within 1 h and continued at least for 24 h, although the rho GDI-induced morphological change continued only for 6 h and thereafter the cells regained their normal morphology (Fig. 1). This morphological change was also accompanied with disappearance of stress fibers (Fig. 7). The C3 action in morphological change was not prevented by the GTPγS-bound form of rhoA p21 (Fig. 8). This might be due to the ADP-ribosylation by C3 of the microinjected as well as endogenous rhoA p21s. Consistently, the C3 action was prevented by comicroinjection of C3 with the rhoA p21 mutant, in which Asn51 at the ADP-ribosylation site was changed to Ile (rhoA51Ie p21) (Fig. 8). This rhoA p21 mutant was not ADP-ribosylated by C3 in the cell-free experiments (data not shown).

**DISCUSSION**

We have first shown here that microinjection of rho GDI into Swiss 3T3 cells leads to morphological change and the disappearance of stress fibers. This rho GDI action is prevented by comicroinjection of rho GDI with the GTPγS-bound active form of rhoA p21 but not with other small G proteins including rac1 p21, G25K, smg p21B, and Ki-ras120 p21. Moreover, we have shown here that the changes of morphology and stress fibers induced by rho GDI are similar to those induced by C3 that were previously reported (11, 14). These results together with the previous fact that only rho p21 is ADP-ribosylated by C3 indicate that the rho GDI-induced morphological change is mediated at least through the actomyosin system and that the rho GDI action is mediated through rho p21. It is 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 form of rho p21 and not with the GTP-bound form, and inhibits its GDP/GTP exchange reaction (20). We have moreover suggested that rho GDI prevents interaction of rho p21 with the effector protein by masking directly or indirectly the effector domain (27). Therefore, our present results together with these previous observations obtained in the cell-free experiments indicate that rho GDI indeed functions and negatively regulates the rho p21 activity and/or the rho p21 action in intact cells.

It has previously been reported that microinjection of the GTPγS-bound form of rhoA p21 or rhoa111 p21 induces morphological change in Swiss 3T3 cells (14). However, in our experiments, microinjection of the GTPγS-bound form of rhoA p21 does not induce morphological change in Swiss 3T3 cells under the conditions where rho GDI induces the morphological change, and this rho GDI action is prevented by comicroinjection of rho GDI with the GTPγS-bound form of rhoA p21. These results, together with other present findings that rho GDI and C3 induce the morphological change and that this action is prevented by rho p21 or its appropriate mutant, suggest that endogenous rho p21 is present in the GTP-bound active form in an amount that maintains normal cell morphology in Swiss 3T3 cells under our experimental conditions.

We have previously shown in the cell-free experiments that the inhibitory action of rho GDI is stronger than the stimulatory action of smg GDS in their simultaneous presence in the GDP/GTP exchange reaction of rho p21 (27). Consistently with this earlier result, we have shown here that comicroinjection of rho GDI with smg GDS does not prevent the rho GDI action. Moreover, microinjection of smg GDS alone does not induce morphological change. This may be due to the presence of endogenous rho GDI which suppresses the action of exogenous smg GDS or to the presence of the endogenous GTP-bound active form of rho p21 in an amount that maintains normal cell morphology.

It is not known how the microinjected rho GDI regulates the rho p21 activity and action in intact Swiss 3T3 cells. However, the following mechanisms may be most likely. Namely, in Swiss 3T3 cells under our experimental conditions where the cells are subconfluent and are cultured in the medium containing 10% FCS which include various growth factors, nutritional factors, and factors necessary for cell-substratum adhesion, the GDP-bound inactive form and the GTP-bound active form are present in an appropriate steady state by the actions of rho GDI, rho GDS, smg GDS, and rho

**Fig. 6. Morphological change by microinjection of C3 into Swiss 3T3 cells.** Swiss 3T3 cells were microinjected with C3 and incubated for various periods of time as indicated. After the cells were fixed, the specimen was analyzed by a phase-contrast microscopy. A, before microinjection; B, 1 h after microinjection; C, 2 h after microinjection; D, 6 h after microinjection; E, 10 h after microinjection; F, 16 h after microinjection; G, 24 h after microinjection. The results shown are the representative of three independent experiments. Bar indicates 10 μm. All photographs were taken with the same magnification.

**Fig. 5. Requirement of the post-translational modifications of the C-terminal region of rhoA p21 for the prevention of the rho GDI action.** Morphological change of Swiss 3T3 cells was examined at 2 h after microinjection with buffer A, rho GDI, rho GDI plus the GTPγS-bound form of rhoA p21, rho GDI plus the GTPγS-bound form of rhoA p21 ASV, A, with buffer A; B, with rho GDI; C, with rho GDI plus rhoA p21; D, with rho GDI plus rhoA p21 ASV. The results shown are the representative of three independent experiments. Bar indicates 10 μm. All photographs were taken with the same magnification.
post-translational modifications with lipids (29) and that the modifications are important for rho p21 to bind to membrane and to interact with rho GDI and smg GDS (2, 25, 30). The GDP-bound form of post-translationally modified rho p21, but not the GTPγS-bound form, interacts with rho GDI (30). We have made here the rhoA p21 mutant (rhoA p21ΔLVL) which is not post-translationally modified with lipids (29) and have shown that the GTPγS-bound form of this rhoA p21 mutant does not prevent the rho GDI action. This result indicates that the post-translationally modifications of rhoA p21 are essential for the rho p21 action. This result is also consistent with previous report that a hydroxymethylglutaryl coenzyme A reductase inhibitor makes NIH/3T3 cells round and refractile (43). Although the effector protein of rho p21 has not been identified, it is possible that the post-translational modifications of rho p21 are also necessary for interaction with its effector protein.

We have previously reported that both Ki-ras4112 p21 and smg p21B induce membrane ruffling in Swiss 3T3 cells and that Ki-ras4112 p21 leads to transformation and disappearance of stress fibers in the same cells (36). The morphological change induced by Ki-ras4112 p21 is different from that induced by rho GDI or C3. Moreover, it takes about 20 h for Ki-ras4112 and smg p21B to induce these changes, and neither Ki-ras4112 nor smg p21B shows these actions at least by 1 h. While rho p21 directly or indirectly regulates the cytoskeletal system, it is likely that the effect of Ki-ras4112 p21 and smg p21B on the cytoskeletal system is due to their secondary effect.

rac1 p21, rac2 p21, and G25K have about 50–60% amino acid homology to rhoA p21, and the amino acid sequence of the putative effector domain of rac1 p21, rac2 p21, and G25K is the same as that of rhoA p21 except for one amino acid (44, 45). Moreover, these small G proteins are substrates for rho GDI and smg GDS (39, 45). However, neither the GTPγS-bound form of rac1 p21 nor the same form of G25K overcomes the rho GDI- or C3-induced cell morphological change. These results indicate that these small G proteins are not at least directly involved in the regulation of cell morphology. Consistently, several groups including our own have shown that rac p21, rho GDI, and smg GDS regulate NADPH oxidase-catalyzed superoxide generation in phagocytes (46–48). Furthermore, after the submission of this paper, it was reported that rac1 p21, but not rho p21, is involved in the membrane ruffling induced by microinjection of the point-mutated active form of Ha-ras p21 or by growth factors such as platelet-derived growth factor and bombesin in Swiss 3T3 cells (49). It has also been shown that rac1 p21 induces through rhoA p21 the assembly of focal adhesions and stress fibers which may cause morphological change (49). In our experiments, when rho GDI was comicroinjected with the GTPγS-bound form of rac1 p21, the endogenous rho p21 activity was inhibited by the rho GDI action and therefore rac p21 might not affect cell morphology. The function of G25K is still unknown, but its yeast counterpart has been shown to regulate budding and cell polarity (50).

REFERENCES

Function of the rho GDI-rho p21 System