Regulation of Cytoplasmic Division of *Xenopus* Embryo by *rho* p21 and Its Inhibitory GDP/GTP Exchange Protein (*rho* GDI)

Kiyohiko Kishi,* Takuya Sasaki,* Shinya Kuroda,* Takahito Itoh,* and Yoshimi Takai*‡

*Department of Biochemistry, Kobe University School of Medicine, Kobe 650, Japan; and ‡Department of Cell Physiology, National Institute for Physiological Sciences, Okazaki 444, Japan

Abstract. Evidence is accumulating that the *rho* family, a member of the *ras* p21-related small GTP-binding protein superfamily, regulates cell morphology, cell motility, and smooth muscle contraction through the actomyosin system. The actomyosin system is also known to be essential for cytoplasmic division of cells (cytokinesis). In this 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 *botulinum* ADP-ribosyltransferase C3, known to selectively ADP-ribosylate *rho* p21 and to impair its function, in the cytoplasmic division using *Xenopus* embryos. The sperm-induced cytoplasmic division of *Xenopus* embryos was not affected by microinjection into the embryos of either *smg* GDS or the guanosine-5'-(3-*O*-thio)triphosphate (GTPγS)-bound form of *rhoA* p21, one member of the *rho* family, but completely inhibited by microinjection of *rho* GDI or C3. Under these conditions, nuclear division occurred normally but the furrow formation, which was induced by the contractile ring consisting of actomyosin just beneath the plasma membrane, was impaired. Comicroinjection of *rho* GDI with the GTPγS-bound form of *rhoA* p21 prevented the *rho* GDI action. Moreover, the sperm-induced cytoplasmic division of *Xenopus* embryos was inhibited by microinjection into the embryos of the *rhoA* p21 pre-ADP-ribosylated by C3 which might serve as a dominant negative inhibitor of endogenous *rho* p21. These results indicate that *rho* p21 together with its regulatory proteins regulates the cytoplasmic division through the actomyosin system.

Two distinct cytoskeletal structures, a bipolar mitotic spindle and a contractile ring, appear transiently and play an active role in M phase in animal cells (for reviews see references 28, 46). A bipolar mitotic spindle is composed of microtubules and their associated proteins and divides the replicated chromosomes for each daughter cell. A contractile ring is composed of actin filaments and myosin just beneath the plasma membrane and divides the cell into two by pulling the membrane inward (cytoplasmic division) (44, 53). The spindle first appears and then the ring appears. These sequential events in cell division have been studied on many kinds of cell lines and eggs. It is, however, still unclear how cell division is regulated.

One of the most useful approaches to study the mechanism of cell division, especially of cytoplasmic division, is to use amphibian eggs. They have several merits such as firm and large-sized cell membrane which is resistant even to distilled water and makes it easy to be microinjected with any kinds of samples, and they form clearly visible cleavage furrows with contractile rings. The contraction and protein components of the contractile ring have been analyzed with isolated cleavage furrows of newt eggs (31), and actin-modulating proteins composing the contractile ring have been studied (for reviews see references 29, 45). It has been reported that inhibitors or binding agents for actin or myosin, such as phalloidin, cytochalasin B, or ML-9, block cytoplasmic division of several kinds of cells and eggs (13, 30, 53, 54). However, the regulatory mechanism of the actomyosin system for cytoplasmic division is still unclear.

The *rho* p21 family, consisting of three members, A, B, and C, belongs to the *ras* p21-related small GTP-binding protein superfamily (for reviews see references 12, 62). Among over 40 members of small GTP-binding proteins, only the *rho* p21 family is ADP-ribosylated by C3 and EDIN (5, 23, 38, 61). C3 and EDIN are the ADP-ribosyltransferases of *Clostridium botulinum* and *Staphylococcus aureus*, respectively (2, 60). Asn[41] of *rho* p21 is ADP-ribosylated by these enzymes (56, 61). This site is located in the putative effector domain of *rho* p21, and the ADP-ribosylation has been suggested to impair the *rho* p21 function (43, 56). Consistently, C3 and EDIN have been shown to affect various cell functions. C3 makes NIH/3T3 cells round and refractile (51), generates short neurites in PC-12 cells (40), and decreases the actin filaments in *Vero* cells and neutrophils (6, 51, 59). In *Xenopus* oocytes, C3 induces migration of germinal vesicles and enhances the progesterone-induced vesicle breakdown (51). The guanosine-5'-(3-*O*-thio)triphosphate (GTPγS)[1]-bound active form of *rhoA* p21 or *rhoA*[val14] p21, which is a

---

1. *Abbreviations used in this paper:* GDI, GDP dissociation inhibitor; GDS, GDP dissociation stimulator; GST, glutathione S-transferase; GTPγS, guanosine-5'-(3-*O*-thio)triphosphate.

point-mutated active form, into Swiss 3T3 cells, contracts the cells, and redistributes the pigments in *Xenopus* oocytes (35, 43). In these experiments, C3 is added or microinjected and *rho* p21 is microinjected into each cell. It has recently been shown that the assembly of focal adhesions and stress fibers induced by growth factors, such as PDGF and bombesin, and lysophosphatidic acid is inhibited in Swiss 3T3 cells when the function of endogenous *rho* p21 is blocked by microinjection of C3 or the *rho*A p21 pre-ADP–ribosylated by C3 which might serve as a dominant negative inhibitor of endogenous *rho* p21 (48). These results suggest that *rho* p21 regulates these cell functions through the actomyosin system. On the other hand, a GTP-binding protein has been suggested to decrease the Ca^{2+} concentrations necessary for the vasoconstrictor-induced smooth muscle contraction (10, 25, 41). We have found that *rho* p21 is a GTP-binding protein which is involved in this vasoconstrictor-induced Ca^{2+} sensitization mechanism (16). Moreover, we have recently shown that microinjection of *rho* GDI or C3 into Swiss 3T3 cells induces changes of morphology and stress fibers and moreover inhibits cell motility (32, 63). Thus, it has become evident that *rho* p21 regulates various cell functions in which the actomyosin system is involved.

There are two interconvertible forms for small GTP-binding proteins including *rho* p21: GDP-bound inactive and GTP-bound active forms. 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* GDP dissociation stimulator (GDS) and *rho* GDS, and the other is an inhibitory type, named *rho* GDP dissociation inhibitor (GDI) (11, 18, 20, 33, 42, 64, 66). These regulatory proteins are present in most cells, and 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 (24, 27). *rho* p21 is present in the GDP-bound inactive form which is complexed with *rho* GDI in the cytosol of resting cells (27, 47). The GDP-bound form of *rho*A p21 complexed with *rho* GDI is resistant to the ADP-ribosylation by EDIN (24).

rho p21 undergoes three kinds of posttranslational modifications in the COOH-terminal region: geranylgeranylation of the cysteine residue, removal of the three COOH-terminal amino acids, and carboxyl methylation of the exposed cysteine residue (22). Only the posttranslationally processed form of *rho* p21, but not the posttranslationally unprocessed form, are sensitive to the GDI and GDS actions, although both forms are sensitive to the action of the GTPase activating protein (17, 34, 62).

On the basis of these observations, we have proposed the following 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 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 *smg* GDS or *rho* 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.

Extending these earlier observations, we have investigated here the actions of *rho*A p21 and its regulatory proteins in the cytoplasmic division of *Xenopus* embryos.

Materials and Methods

Materials and Chemicals

Healthy *Xenopus laevis* females and males were obtained from Hamamatsu Biological Research Service Inc. (Shizuoka, Japan). The cDNA of *rho*A p21 was kindly provided by Dr. P. Madaule (Institut Pasteur, Paris, France). The baculovirus carrying the cDNA of *rac*1 p21 was kindly supplied by Drs. P. Polakis and F. McCormick (Onyx Pharmaceuticals, Emeryville, CA). C3 was kindly supplied from Dr. B. Syuto (Hokkaido University, Sapporo, Japan) (36). Mutagenesis of Asn to Ile at codon 41 of *rho*A p21 (*rho*A[Ile41] p21) was carried out by site-directed mutagenesis (52). *Spodoptera frugiperda* cells overexpressing *rho*A p21, *rho*A[Ile41] p21, or *rac* p21 were kindly provided by Dr. Y. Matsuura (National Institute of Health, Tokyo, Japan). *rho*A p21, *rho*A[Ile41] p21, and *rac*1 p21 were purified from the cytosol fraction of *Sp. frugiperda* cells overexpressing each small GTP-binding protein as described (33). *rho*A p21 lacking the three COOH-terminal amino acids (*rho*A p21[ΔLVL]) was purified from overexpressing *E. coli* (22). *rho* GDI was purified as glutathione S-transferase (GST) fusion proteins from *E. coli* overexpressing GST-*rho* GDI (9, 24, 58). It was confirmed that recombinant GST-*rho* GDI showed the same activity toward the GDP/GTP exchange reaction of *rho*A p21 as the *rho* GDI purified from bovine brain did in a cell-free system. *smg* GDS was purified from the cytosol fraction of overexpressing *E. coli*. The GTPγS-bound form of *rho*A p21, *rho*A[Ile41] p21, *rho*A p21[ΔLVL], and *rac*1 p21 were made by incubating each small GTP-binding protein with 60 μM GTPγS as described (3, 68). The GDP-bound form of *rho* p21 and *rac*1 p21 was made with 60 μM GDP by the same method. The ADP-ribosylated *rho*A p21 was made by incubation with C3 in a cell-free system as described (61). After the ribosylation, the ADP-ribosylated *rho*A p21 was purified by Mono Q HR5/5 column chromatography. This sample was not contaminated by C3. All the proteins used were concentrated in Centricon 10 (Amicon Corp., Danvers, MA) to the concentrations of 4–28 mg/ml. During the concentration, the buffers contained in the sample preparations were replaced by buffer A slightly modified as described (4). FITC-labeled phalloidin was purchased from Sigma Chemical Co. (St. Louis, MO).

Preparation of Embryos

Eggs were obtained from fully mature *X. laevis* females and fertilized by rubbing with a testis which was surgically obtained from *X. laevis* males. The fertilized eggs were dejellied and used for microinjection as previously described (39).

Microinjection

Each sample to be tested was microinjected into living *Xenopus* embryos in modified modified Ringer's (0.1 M NaCl, 2.0 mM KCl, 1.0 mM MgSO$_4$, 2.0 mM CaCl$_2$, 5.0 mM Hepes, 0.1 mM EDTA, pH 7.8), and 5% Ficoll (type 400; Sigma Chemical Co.) at 18°C with the microinjection system (IM-1, Narishige, Tokyo, Japan) as described (21, 39). Briefly, glass capillaries drawn, cut to a tip diameter of about 10 μm, and ground were used to microinject each sample. About 10 embryos were usually microinjected within 5 min. The embryos were incubated for indicated periods of time at 18°C.

About 5 $\times$ 10^{-8} liters of sample was microinjected by one injection. When the GTPγS-bound form of *rho*A p21 or the same form of *rac*1 p21 was used at 0.5 mg/ml each, the intracellular concentrations of the microinjected samples were calculated to be about 2.4 μM. Since the endogenous concentration of *rho*A p21 or *rac*1 p21 is not known, the levels of the exogenous and endogenous small GTP-binding proteins were not compared. The intracellular concentration of the microinjected *rho* GDI was calculated to be about 6.2 μM when GST-*rho* GDI was microinjected at 3.3 mg/ml. *smg* GDS was microinjected at the same concentration of *rho* GDI. However, the endogenous concentrations of *rho* GDI and *smg* GDS are not known. The intracellular concentration of the microinjected C3 was calculated to be about 1.5 nM when C3 was microinjected at 2.6 μg/ml.

Analyses of Furrow Formation and Actin Filament Reorganization

Furrow formation was analyzed by stereomicroscope (model SMZ-U; Nikon, Tokyo, Japan). Photomicrographs of the embryos were taken at the indicated time after the fertilization. For analysis of actin filament reorganization, the embryos at the indicated time after the fertilization were fixed in 20% DMSO/methanol overnight at −20°C, bleached for 48 h at room temperature in 20% DMSO/10% hydrogen peroxide/methanol, and washed twice in PBS for 20 min at room temperature as described with slight modifications (7). They were incubated in 10 μg/ml FITC-labeled phalloidin/PBS for 72 h at room temperature, washed in PBS, and examined by the laser scanning confocal imaging system (model MRC-600; Bio-Rad, Tokyo, Japan).

Analysis of Nuclear Division

To examine nuclear division, Xenopus embryos, which cleaved normally or were microinjected with rho GDI or C3 at 60 min after the fertilization, were picked up at 90 min and fixed in 4% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4) for 4–12 h at 4°C. They were dehydrated in a graded series of ethanol, transferred into 99% chloroform at room temperature, embedded in paraffin, sectioned at 7 μm, and stained with hematoxylin and eosin (57).

Determination

Protein concentrations were determined with BSA as a standard protein by densitometric tracing of protein bands stained with Coomassie brilliant blue on an SDS–polyacrylamide gel as described (65).

Results

Inhibition of the Furrow Formation by Microinjection of rho GDI or C3 into Xenopus Embryos

When Xenopus embryos were fertilized by Xenopus sperm, the furrow formation started at about 85 min after the fertilization and the cytoplasmic division completed within about 5 min. However, when rho GDI or C3 was microinjected in an appropriate amount into Xenopus embryos at 60 min after the fertilization, the furrow formation was completely blocked and a white belt appeared on the equatorial region in animal hemisphere of the embryos instead of the furrow (Fig. 1, B and C; and Tables I and II). This white belt remained persistently, and this inhibitory effect on the furrow formation was observed also at the next cleavage (Fig. 2, B and C). In the control embryos microinjected with buffer A, a well-developed cleavage furrow was observed (Fig. 1 A and 2 A; and Table I).

When rho GDI or C3 was microinjected into the embryos at the onset of the furrow formation, the following furrow formation was inhibited and only the white belt appeared as seen in Fig. 1, B and C (Tables I and II). The embryos microinjected with buffer A continued the normal furrow formation (Table I).

Reorganization of Actin Filaments by Microinjection of rho GDI or C3 into Xenopus Embryos

To show that rho GDI or C3 inhibits the furrow formation through at least the actomyosin system, an actin bundle was visualized by FITC-labeled phalloidin and analyzed by confocal microscopy. In the control embryos at 85 min, just at the onset of the furrow formation, an actin bundle was aligned along the cytoplasmic side of the cell membrane and observed along the equatorial region where the furrow formation had started (Fig. 3 A, 85 min). This actin bundle along the equatorial region was supposed to compose a con-
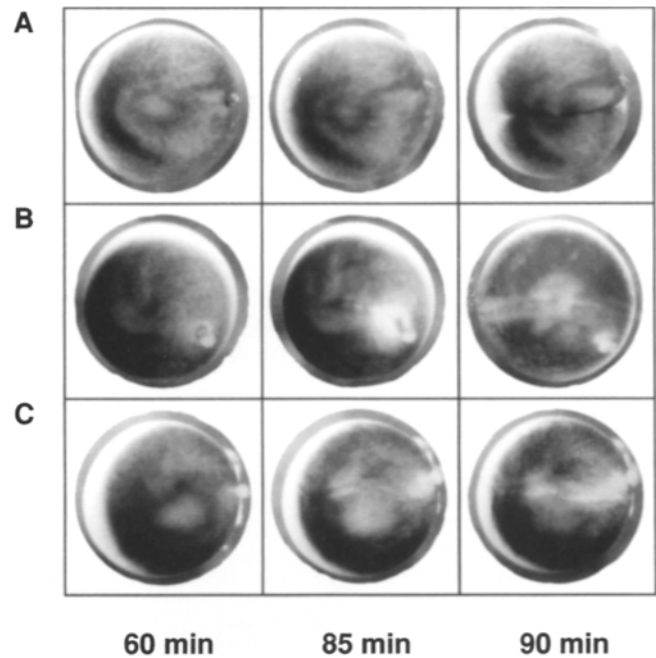
Figure 1. Effect of microinjection of rho GDI or C3 into Xenopus embryos on the furrow formation. rho GDI or C3 was microinjected into Xenopus embryos at 60 min after the fertilization. Photographs of the embryos were taken just after the microinjection and at 85 and 90 min after the fertilization. (A) Buffer A; (B) rho GDI at 3.3 mg/ml; (C) C3 at 2.6 μg/ml. The results shown are the representatives with typical phenotype of 40 embryos tested with buffer A as a control, 58 embryos tested with rho GDI, and 76 embryos tested with C3. The embryos were obtained from three different X. laevis females.

tractile ring. In the embryos microinjected with rho GDI or C3, the actin bundle along the equatorial region was not observed (Fig. 3, B and C, 85 min). In the control embryos which underwent the cytoplasmic division, at 90 min after the fertilization, the actin bundle was aligned along the circumference of the furrow in addition to the cytoplasmic side of the cell membrane (Fig. 3 A, 90 min). In the embryos microinjected with rho GDI or C3, an actin bundle was aligned along only the cytoplasmic side of the cell membrane, and a septum with an actin bundle was not observed (Fig. 3, B and C, 90 min).

Normal Nuclear Division by Microinjection of rho GDI or C3 into Xenopus Embryos

To make sure that the embryos arrested in cleavage by microinjection with either rho GDI or C3 underwent nuclear division as well as the normally cleaved embryos, the nuclear division was examined by a hematoxylin and eosin staining method. The nuclear division occurred at about 70 min after the fertilization in the control embryos and a pair of chromatin was observed to be aligned almost symmetrically (Fig. 4 A). In the embryos arrested in cleavage by microinjection with either rho GDI or C3, the normal nuclear division was observed (Fig. 4, B and C).

Specificity of rho p21 for the Cytoplasmic Division of Xenopus Embryos

rhoA p21, rhoB p21, racl p21, and rac2 p21 are substrate small GTP-binding proteins for rho GDI (15, 34, 64). The

Table I. Effect of rho GDI, rhoA p21, and Ribosylated rhoA p21 on the Furrow Formation

Substance microinjected			Inhibition of furrow formation	
rho GDI	rhoA p21	ADP-ribosylated rhoA p21	Inhibited embryos/ total embryos	
(mg/ml)	(mg/ml)	(mg/ml)		(%)
0*	0	0	0/40 (0/30)	0 (0)
2.5	0	0	6/40	15
3.3	0	0	37/58	64
3.3	0.5	0	0/46	0
5.0	0	0	43/66	65
10.0	0	0	37/44 (25/30)	84 (83)
0	0	1.25	29/41	71
0	0	2.5	32/44	73

rho GDI with or without the GTP-γS-bound form of rhoA p21, or ADP-ribosylated rhoA p21 was microinjected into Xenopus embryos at 60 min after the fertilization, and the furrow formation was examined at 90 min after the fertilization. The numbers in parentheses summarize the results of the experiment of microinjection of rho GDI just at the onset of the furrow formation.
* Microinjection of buffer A alone.

Table II. Effect of C3, rhoA p21, and rhoA^{Ile41} p21 on the Furrow Formation

Substance microinjected			Inhibition of furrow formation	
C3	rhoA p21	rhoA^{Ile41} p21	Inhibited embryos/ total embryos	
(µg/ml)	(mg/ml)	(mg/ml)		(%)
0*	0	0	0/40	0
1.0	0	0	0/33	0
2.0	0	0	0/36	0
2.6	0	0	51/76	67
2.6	1.25	0	19/47	40
2.6	0	1.25	8/58	15
6.5	0	0	24/36	67
32.5	0	0	36/46	78
65	0	0	35/35	100
130	0	0	38/38 (30/30)	100 (100)

C3 was microinjected with or without the GTP-γS-bound form of rhoA p21 or rhoA^{Ile41} p21 into Xenopus embryos at 60 min after the fertilization, and the furrow formation was examined at 90 min after the fertilization. The numbers in parentheses summarize the results of the experiment of microinjection of C3 just at the onset of the furrow formation.
* Microinjection of buffer A alone.

affinities of rho GDI for these small GTP-binding proteins are apparently similar (S. Ando, unpublished observation). The inhibitory action of rho GDI on the furrow formation was prevented by comicroinjection of rho GDI with the GTP-γS-bound form of rhoA p21 (Fig. 5 C; and Table I). The rho GDI action was not, however, prevented by comicroinjection of rho GDI with the same form of racl p21. It may be noted that the pigment of the embryos comicroinjected with rho GDI and racl p21 moved away from the point where microinjection capillary was inserted and a pigment circle with depigmented area inside remained around the point (Fig. 5 E). Moreover, when the rhoA p21 pre-ADP-ribosylated by C3 was microinjected into the embryos, the furrow formation was completely blocked and a white belt seen in Fig. 1, B and C appeared (Table I). This form of rhoA p21 has been reported to serve as a dominant negative inhibitor of endogenous rho p21 (48).

On the other hand, when rho GDI was comicroinjected with the GDP-bound form of rhoA p21 or the same form of racl p21, the rho GDI action was prevented (data not shown). In these experiments, the GDP-bound form of these small GTP-binding proteins were used in the same amounts as those used in Fig. 5. The actual reason why the GDP-bound form of these small GTP-binding proteins prevented the rho GDI action is not known, but they might interact with rho GDI to lower the concentration of rho GDI less than that necessary for its action.

Importance of the Posttranslational Modifications of the COOH-Terminal Region of rhoA p21 in Preventing the rho GDI Action
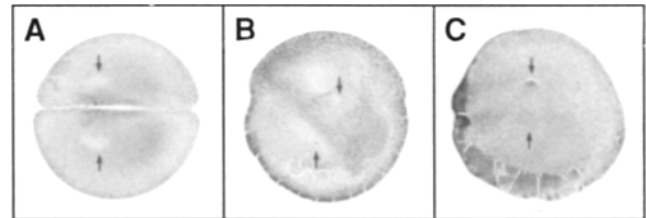
The rhoA p21 used in the above experiments was not posttranslationally modified with lipids. Nevertheless, the rho GDI action was prevented by comicroinjection of rho GDI with this form of rhoA p21 (Fig. 5 C; and Table I). This result suggests that the rhoA p21 microinjected into the embryos underwent the posttranslational modifications. Consistently, the rho GDI action was not prevented by comicroinjection of rho GDI with the rhoA p21 mutant which lacked the COOH-terminal three amino acids (rhoA p21^{ΔLVL}) (Fig. 5 D) This rhoA p21 mutant was previously confirmed not to be geranylgeranylated by a geranylgeranyltransferase in a cell-free system (67).
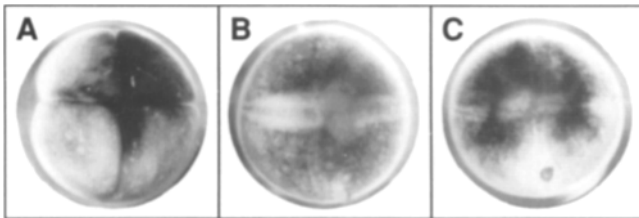
Figure 2. Effect of *rho* GDI and C3 on the second cleavage. Photographs were taken at 150 min after the fertilization. (*A*) Buffer A; (*B*) *rho* GDI at 3.3 mg/ml; (*C*) C3 at 2.6 µg/ml. The results shown are the representatives with typical phenotype of the embryos used in Fig. 1.

Figure 4. Effect of *rho* GDI or C3 on the nuclear division. *rho* GDI or C3 was microinjected into *Xenopus* embryos at 60 min after the fertilization. The embryos were picked up at 90 min, applied to paraffin sections, and nuclei was visualized by a hematoxylin and eosin staining method. (*A*) The normally cleaved embryo; (*B*) *rho* GDI at 3.3 mg/ml; (*C*) C3 at 2.6 µg/ml. Arrows indicate nuclei. The results shown are the representatives with typical phenotype of the embryos used in Fig. 1.

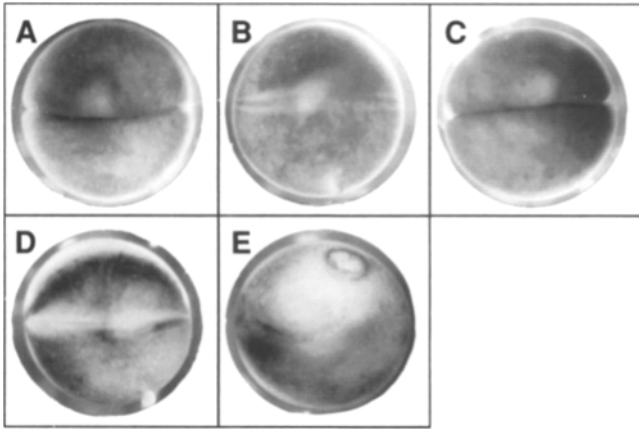## Prevention of the C3 Action by rhoA^{Ile41} p21

C3 ADP-ribosylates Asn^{41} in the putative effector domain of *rho* p21 and impairs its function (43, 56). We therefore examined the effect of *rho*A^{Ile41} p21, which was not ADP-ribosylated by C3, on the C3 action. Comicroinjection of *rho* GDI with the GTPγS-bound form of *rho*A p21 did not prevent the C3 action, because exogenous *rho*A p21 was also ADP-ribosylated and did not function (Table II). However, comicroinjection of C3 with the GTPγS-bound form of *rho*A^{Ile41} p21 prevented the C3 action (Table II).

Inability of rhoA p21 or smg GDS Alone to Affect the Cytoplasmic Division of Xenopus Embryos

Microinjection of *rho* GDI into *Xenopus* embryos inhibited the furrow formation 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 the comparable conditions, microinjection of the GTPγS-bound form of *rho*A p21 alone into the embryos did not affect the
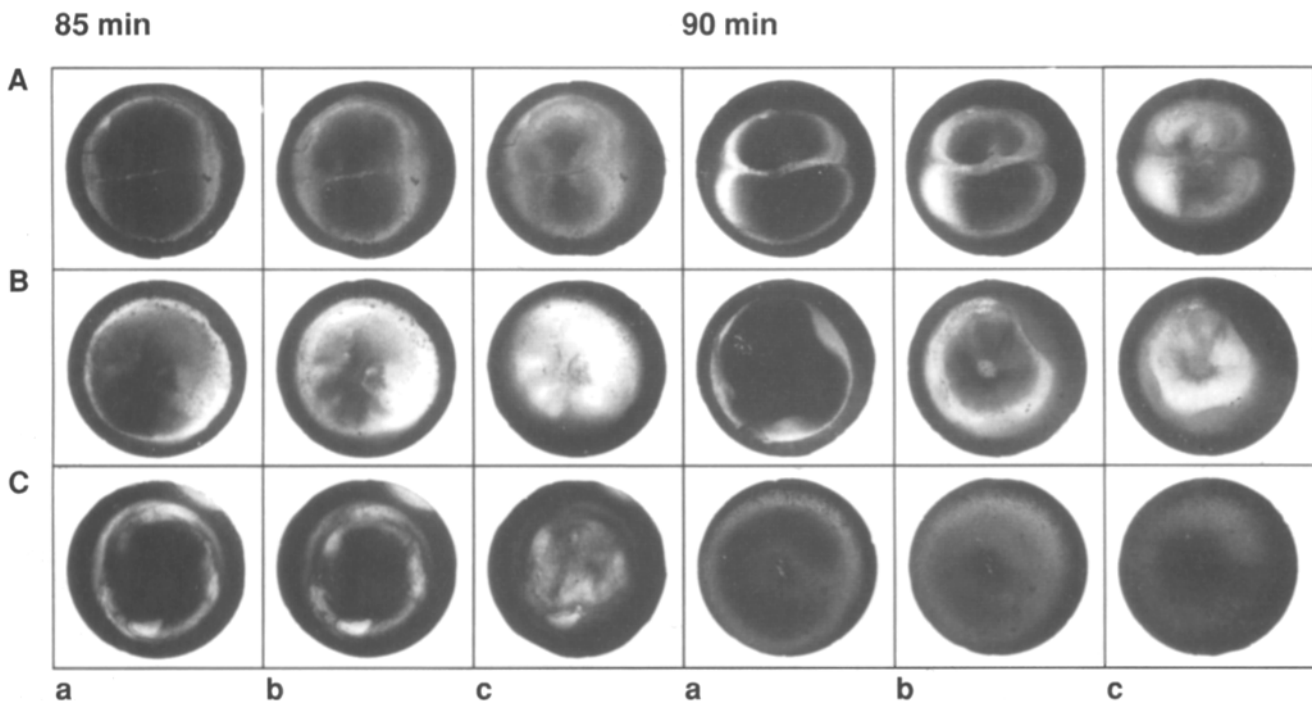
Figure 3. Effect of *rho* GDI and C3 on the reorganization of actin filaments. Actin filaments were stained with FITC-labeled phalloidin at 85 and 90 min after the fertilization. Photographs were taken at the three scanning levels of the animal hemisphere: (*a*) equatorial, (*b*) middle, (*c*) near the top of the hemisphere. (*A*) Buffer A; (*B*) *rho* GDI at 10 mg/ml; (*C*) C3 at 130 µg/ml. The results shown are the representatives with typical phenotype of 30 embryos tested with buffer A as a control, 30 embryos tested with *rho* GDI, and 30 embryos tested with C3. The embryos were obtained from three different *X. laevis* females. It may be noted that the embryos microinjected with buffer A were intact in shape, but that the embryos microinjected with *rho* GDI and C3 became flabby gradually, especially in the animal hemisphere. The top of the animal hemisphere was depressed in the embryos microinjected with *rho* GDI and C3. This may be due to the production of the cell surface membrane without the furrow formation. The actin bundle observed at the center of the embryos in *B* and *C* were thought to be the cortex of the depressed tops.

Figure 5. Prevention of the *rho* GDI action by *rho*A p21. The furrow formation was examined after comicroinjection of *rho* GDI with the GTPγS-bound form of *rho*A p21, *rho*A p21$^{\Delta LVL}$, or *rac*1 p21 at 60 min after the fertilization. Photographs of the embryos were taken at 90 min after the fertilization. (*A*) Buffer A; (*B*) *rho* GDI at 3.3 mg/ml; (*C*) *rho* GDI at 3.3 mg/ml plus *rho*A p21 at 0.5 mg/ml; (*D*) *rho* GDI at 3.3 mg/ml plus *rho*A p21$^{\Delta LVL}$ at 0.5 mg/ml; (*E*) *rho* GDI at 3.3 mg/ml plus *rac*1 p21 at 0.5 mg/ml. The results shown are the representatives of 40 embryos tested with buffer A as a control, 58 embryos tested with *rho* GDI alone, 46 embryos tested with *rho* GDI plus *rho*A p21, 45 embryos tested with *rho* GDI plus *rho*A p21$^{\Delta LVL}$, and 30 embryos tested with *rho* GDI plus *rac*1 p21. The embryos were obtained from three different *X. laevis* females.

furrow formation (data not shown). Moreover, microinjection of *smg* GDS alone into the embryos did not affect the furrow formation (data not shown). Comicroinjection of *rho* GDI with *smg* GDS did not prevent the *rho* GDI action (data not shown).

Discussion

In the cytoplasmic division, a contractile ring consisting of actomyosin is first produced. The contraction of this ring makes a furrow on the surface of the embryo, and finally a cell septum is produced (28, 44, 46, 55). Rubin et al. (51) have previously reported the phenomenon that addition of C3 to NIH/3T3 cells accumulates binucleate cells. Consistently, we have shown here that microinjection of *rho* GDI or C3 into *Xenopus* embryos inhibits the furrow formation induced by the contractile ring without affecting the nuclear division. This *rho* GDI action is prevented by comicroinjection of *rho* GDI with the GTPγS-bound active form of *rho*A p21 but not with the same form of another small GTP-binding protein, *rac*1 p21. The C3 action is also prevented by comicroinjection of C3 with the GTPγS-bound active form of a C3-insensitive mutant of *rho*A p21 (*rho*A^{Ile41} p21). We have moreover shown here that microinjection of the *rho*A p21 pre-ADP-ribosylated by C3, which has been reported to serve as a dominant negative inhibitor of endogenous *rho* p21 (48), also inhibits the furrow formation. Evidence is accumulating that *rho* p21 and *rho* GDI regulate various cell functions, including cell morphology, cell motility, and smooth muscle contraction, in which the actomyosin system is involved (16, 32, 63). Therefore, our present results together with these earlier observations indicate that *rho* p21 and *rho* GDI regulate the cytoplasmic division of *Xenopus*

embryos through the actomyosin system. It remains unclear, however, which type of *rho* p21 among the three is present in the embryos.

Cleavage furrow formation is thought of as occurring in two steps: the first would be the correct localization and building up of the contractile ring and the second the process of furrow formation itself. We have shown here that the inhibitory action of *rho* GDI or C3 is immediate, that the furrow formation on the way is immediately inhibited by *rho* GDI, and that the white belt appears at the site of normal furrowing seen in Fig. 1, *B* and *C*. These results suggest that *rho* GDI or C3 inhibits not only the building up the contractile ring but also the furrow formation itself.

We have shown here that microinjection of the GTPγS-bound form of *rho*A p21 does not affect the cytoplasmic division under the 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. We have recently described that microinjection of *rho* GDI into Swiss 3T3 cells induces changes of cell morphology and stress fibers and inhibits cell motility and that these *rho* GDI actions are prevented by comicroinjection of *rho* GDI with the GTPγS-bound form of *rho*A p21 (32, 63). Therefore, it is most likely that a part of endogenous *rho* p21 is in the GTP-bound active form, and that this active form regulates the actomyosin system.

We have previously shown in a cell-free experiment 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 (24, 27). Consistent with this earlier result, we have shown here that comicroinjection of *rho* GDI with *smg* GDS does not prevent the *rho* GDI action. Microinjection of *smg* GDS alone does not affect the cytoplasmic division. These results are consistent with our earlier observations for the actions of *smg* GDS in the regulation of cell morphology and motility of Swiss 3T3 cells (32, 63). This may be due to the action of endogenous *rho* GDI which is stronger than that of exogenous *smg* GDS or to an enough amount of the GTP-bound active form of endogenous *rho* p21 to induce the cytoplasmic division.

It is not known how microinjected *rho* GDI regulates the *rho* p21 activity and action in intact embryos. However, the following mechanisms may be the most likely. Namely, in *Xenopus* embryos under our experimental conditions, 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* GTPase activating protein, and *rho* p21 is present in the GTP-bound active form in an amount enough to induce the cytoplasmic division. When a large amount of *rho* GDI is artificially microinjected into the embryos, this steady state is changed: the GTP-bound form of *rho* p21 is first converted to the GDP-bound form by the action of *rho* GTPase activating protein. Then, this GDP-bound form of *rho* p21 is sequestered by *rho* GDI to make a complex. The GDP-bound form of *rho* p21 complexed with *rho* GDI is not converted to the GTP-bound form any more. In this way, the amount of the GTP-bound form is finally diminished. In the case of C3, microinjected C3 mainly ADP-ribosylates the GTP-bound active form of *rho* p21 which is free from *rho* GDI, because the GDP-bound inactive form of *rho* p21 complexed with *rho* GDI is resistant to this enzyme as far as examined in a cell-free system (24).

It still remains to be clarified how this steady state between the GDP-bound inactive form and the GTP-bound active form of *rho* p21 is regulated through its regulatory proteins and how the GTP-bound active form of *rho* p21 regulates the actomyosin system.

We have previously shown that *rho*A p21 undergoes the posttranslational modifications (22) and that the modifications are important for *rho* p21 to bind to membrane and to interact with *rho* GDI and *smg* GDS (17, 33, 62). The GDP-bound form of posttranslationally processed *rho*A p21, but not the GTPγS-bound form, interacts with *rho* GDI (17). We have also shown that the *rho*A p21 mutant (*rho*A p21^ΔLVL), which is not posttranslationally modified with lipids (22), does not prevent the *rho* GDI action in the regulation of cell morphology and motility of Swiss 3T3 cells (32, 63). Consistently, we have shown here that this mutant of *rho*A p21 does not prevent the *rho* GDI action in the cytoplasmic division of *Xenopus* embryos. These results indicate that the posttranslational modifications are also essential for the *rho* p21 action. Although the effector protein of *rho* p21 is not identified, it is possible that the posttranslational modifications of *rho* p21 are necessary for the interaction with its effector protein.

*rac*1 p21, *rac*2 p21, and G25K have about 50–60% amino acid homology to *rho*A p21 and the amino acid sequence of the putative effector domain of *rac*1 p21, *rac*2 p21, and G25K is the same as that of *rho*A p21 except one amino acid (8, 37). Moreover, these small GTP-binding proteins are substrates for *rho* GDI and *smg* GDS. However, the GTPγS-bound form of *rac*1 p21 does not prevent the *rho* GDI- or C3-inhibited cytoplasmic division. This result indicates that this small GTP-binding protein is either not directly involved in the regulation of the cytoplasmic division, or its activity is not affected by microinjection of *rho* GDI in the embryos due to an unknown reason. However, comicroinjection of *rho* GDI with *rac*1 p21 induces an appearance of the embryo surface slightly different from that induced by comicroinjection of *rho* GDI with *rho*A p21 (see Fig. 5 *E*). It remains unclear what this phenotype means. It may be noted that 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, 15, 26, 34). Furthermore, it has recently been reported that *rac* p21 is involved in the membrane ruffling induced by microinjection of the point-mutated active form of *ras* p21 or by growth factors, such as PDGF and bombesin, in Swiss 3T3 cells (49). The function of G25K is still unknown, but its yeast counterpart has been shown to regulate the budding and cell polarity (19). It has been reported that overexpression of *dbl*, a stimulatory GDP/GTP exchange protein for G25K, results in multinucleate phenotype in NIH/3T3 cells, and suggested from this result that G25K is involved in the control of cytoplasmic division (50). The *dbl* protein has been shown to be active on G25K and partly on *rac*1 p21, but it has not been clarified whether this exchange protein is also active on *rho* p21 (14). Moreover, our preliminary results have revealed that the inhibitory action of *rho* GDI on the furrow formation in *Xenopus* embryos is not prevented by the GTPγS-bound form of GST-G25K. We have shown here that the furrow formation is inhibited by C3, known to ADP-ribosylate *rho* p21 but not G25K, and also inhibited by the pre-ADP-ribosylated *rho*A p21. Therefore, it is less likely that G25K is also in-

volved in the control of the cytoplasmic division. Further investigation is necessary for understanding the function of *rac* p21 and G25K in *Xenopus* embryos.

References

1. Abo, A., E. Pick, A. Hall, N. Totty, C. G. Teahan, and A. W. Segal. 1991. Activation of the NADPH oxidase involves the small GTP-binding protein p21^rac1. *Nature (Lond.).* 353:668–670.
2. Aktories, K., S. Rösener, U. Blaschke, and G. S. Chhatwal. 1988. Botulinum ADP-ribosyltransferase C3: purification of the enzyme and characterization of the ADP-ribosylation reaction in platelet membranes. *Eur. J. Biochem.* 172:445–450.
3. Araki, S., A. Kikuchi, Y. Hata, M. Isomura, and Y. Takai. 1990. Regulation of reversible binding of *smg* p25A, a *ras* p21-like GTP-binding protein, to synaptic plasma membranes and vesicles by its specific regulatory protein, GDP dissociation inhibitor. *J. Biol. Chem.* 265:13007–13015.
4. Birchmeier, C., D. Broek, and M. Wigler. 1985. *RAS* proteins can induce meiosis in Xenopus oocytes. *Cell.* 43:615–621.
5. Braun, U., B. Habermann, I. Just, K. Aktories, and J. Vandekerckhove. 1989. Purification of the 22kDa protein substrate of botulinum ADP-ribosyltransferase C3 from porcine brain cytosol and its characterization as a GTP-binding protein highly homologous to the *rho* gene product. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 243:70–76.
6. Chardin, P., P. Boquet, P. Madaule, M. R. Popoff, E. J. Rubin, and D. M. Gill. 1989. The mammalian G protein *rho*C is ADP-ribosylated by *Clostridium botulinum* exoenzyme C3 and affects actin microfilaments in Vero cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:1087–1092.
7. Dent, J. A., and M. W. Klymkowsky. 1989. Whole-mount analyses of cytoskeletal reorganization and function during oogenesis and early embryogenesis in *Xenopus. In* The Cell Biology of Fertilization. H. Schatten and G. Schatten, editors. Academic Press, Inc., Orlando, FL. 63–103.
8. Didsbury, J., R. F. Weber, G. M. Bokoch, T. Evans, and R. Snyderman. 1989. *rac*, a novel *ras*-related family of proteins that are botulinum toxin substrates. *J. Biol. Chem.* 264:16378–16382.
9. Eaton, D., H. Rodriguez, and G. A. Vehar. 1986. Proteolytic processing of human factor VIII. Correlation of specific cleavages by thrombin, factor Xa, and activated protein C with activation and inactivation of factor VIII coagulant activity. *Biochemistry.* 25:505–512.
10. Fujiwara, T., T. Itoh, Y. Kubota, and H. Kuriyama. 1989. Effects of guanosine nucleotides on skinned smooth muscle tissue of the rabbit mesenteric artery. *J. Physiol.* 408:535–547.
11. Fukumoto, Y., K. Kaibuchi, Y. Hori, H. Fujioka, S. Araki, T. Ueda, A. Kikuchi, and Y. Takai. 1990. Molecular cloning and characterization of a novel type of regulatory protein (GDI) for the *rho* proteins, *ras* p21-like small GTP-binding proteins. *Oncogene.* 5:1321–1328.
12. Hall, A. 1990. The cellular functions of small GTP-binding proteins. *Science (Wash. DC).* 249:635–640.
13. Hamaguchi, Y., and I. Mabuchi. 1982. Effects of phalloidin microinjection and localization of fluorescein-labeled phalloidin in living sand dollar eggs. *Cell Motility.* 2:103–113.
14. Hart, M. J., A. Eva, T. Evans, S. A. Aaronson, and R. A. Cerione. 1991. Catalysis of guanine nucleotide exchange on the CDC42Hs protein by the *dbl* oncogene product. *Nature (Lond.).* 354:311–314.
15. Hiraoka, K., K. Kaibuchi, S. Ando, T. Musha, K. Takaishi, T. Mizuno, M. Asada, L. Ménard, E. Tomhave, J. Didsbury, R. Snyderman, and Y. Takai. 1992. Both stimulatory and inhibitory GDP/GTP exchange proteins, *smg* GDS and *rho* GDI, are active on multiple small GTP-binding proteins. *Biochem. Biophys. Res. Commun.* 182:921–930.
16. Hirata, K., A. Kikuchi, T. Sasaki, S. Kuroda, K. Kaibuchi, Y. Matsuura, H. Seki, K. Saida, and Y. Takai. 1992. Involvement of *rho* p21 in the

GTP-enhanced calcium ion sensitivity of smooth muscle contraction. *J. Biol. Chem.* 267:8719–8722.

17. Hori, Y., A. Kikuchi, M. Isomura, M. Katayama, Y. Miura, H. Fujioka, K. Kaibuchi, and Y. Takai. 1991. Post-translational modifications of the C-terminal region of the *rho* protein are important for its interaction with membranes and the stimulatory and inhibitory GDP/GTP exchange proteins. *Oncogene.* 6:515–522.

18. Isomura, M., K. Kaibuchi, T. Yamamoto, S. Kawamura, M. Katayama, and Y. Takai. 1990. Partial purification and characterization of GDP dissociation stimulator (GDS) for the *rho* proteins from bovine brain cytosol. *Biochem. Biophys. Res. Commun.* 169:652–659.

19. Johnson, D. I., and J. R. Pringle. 1990. Molecular characterization of *CDC42*, a *Saccharomyces cerevisiae* gene involved in the development of cell polarity. *J. Cell Biol.* 111:143–152.

20. Kaibuchi, K., T. Mizuno, H. Fujioka, T. Yamamoto, K. Kishi, Y. Fukumoto, Y. Hori, and Y. Takai. 1991. Molecular cloning of the cDNA for stimulatory GDP/GTP exchange protein for *smg* p21s (*ras* p21-like small GTP-binding proteins) and characterization of stimulatory GDP/GTP exchange protein. *Mol. Cell. Biol.* 11:2873–2880.

21. Karsenti, E., J. Newport, R. Hubble, and M. Kirschner. 1984. Interconversion of metaphase and interphase microtubule arrays, as studied by the injection of centrosomes and nuclei into *Xenopus* eggs. *J. Cell Biol.* 98:1730–1745.

22. Katayama, M., M. Kawata, Y. Yoshida, H. Horiuchi, T. Yamamoto, Y. Matsuura, and Y. Takai. 1991. The posttranslationally modified C-terminal structure of bovine aortic smooth muscle *rho*A p21. *J. Biol. Chem.* 266:12639–12645.

23. Kikuchi, A., K. Yamamoto, T. Fujita, and Y. Takai. 1988. ADP-ribosylation of the bovine brain *rho* protein by botulinum toxin type C1. *J. Biol. Chem.* 263:16303–16308.

24. Kikuchi, A., S. Kuroda, T. Sasaki, K. Kotani, K. Hirata, M. Katayama, and Y. Takai. 1992. Functional interactions of stimulatory and inhibitory GDP/GTP exchange proteins and their common substrate small GTP-binding protein. *J. Biol. Chem.* 267:14611–14615.

25. Kitazawa, T., S. Kobayashi, K. Horiuti, A. V. Somlyo, and A. P. Somlyo. 1989. Receptor-coupled, permeabilized smooth muscle: role of the phosphatidylinositol cascade, G-proteins, and modulation of the contractile response to Ca²⁺. *J. Biol. Chem.* 264:5339–5342.

26. Knaus, U. G., P. G. Heyworth, T. Evans, J. T. Curnutte, and G. M. Bokoch. 1991. Regulation of phagocyte oxygen radical production by the GTP-binding protein Rac 2. *Science (Wash. DC.).* 254:1512–1515.

27. Kuroda, S., A. Kikuchi, K. Hirata, T. Masuda, K. Kishi, T. Sasaki, and Y. Takai. 1992. Cooperative function of *rho* GDS and *rho* GDI to regulate *rho* p21 activation in smooth muscle. *Biochem. Biophys. Res. Commun.* 185:473–480.

28. Mabuchi, I. 1986. Biochemical aspects of cytokinesis. *Int. Rev. Cytol.* 101:175–213.

29. Mabuchi, I. 1990. Cleavage furrow formation and actin-modulating proteins. *Ann. N. Y. Acad. Sci.* 582:131–146.

30. Mabuchi, I., and H. Takano-Ohmuro. 1990. Effects of inhibitors of myosin light chain kinase and other protein kinases on the first cell division of sea urchin eggs. *Dev. Growth Differ.* 32:549–556.

31. Mabuchi, I., S. Tsukita, S. Tsukita, and T. Sawai. 1988. Cleavage furrow isolated from newt eggs: contraction, organization of the actin filaments, and protein components of the furrow. *Proc. Natl. Acad. Sci. USA.* 85:5966–5970.

32. Miura, Y., A. Kikuchi, T. Musha, S. Kuroda, H. Yaku, T. Sasaki, and Y. Takai. 1992. Regulation of morphology by *rho* p21 and its inhibitory GDP/GTP exchange protein (*rho* GDI) in Swiss 3T3 cells. *J. Biol. Chem.* In press.

33. Mizuno, T., K. Kaibuchi, T. Yamamoto, M. Kawamura, T. Sakoda, H. Fujioka, Y. Matsuura, and Y. Takai. 1991. A stimulatory GDP/GTP exchange protein for smg p21 is active on the post-translationally processed form of c-Ki-ras p21 and rhoA p21. *Proc. Natl. Acad. Sci. USA.* 88:6442–6446.

34. Mizuno, T., K. Kaibuchi, S. Ando, T. Musha, K. Hiraoka, K. Takaishi, M. Asada, H. Nunoi, I. Matsuda, and Y. Takai. 1992. Regulation of the superoxide-generating NADPH oxidase by a small GTP-binding protein and its stimulatory and inhibitory GDP/GTP exchange proteins. *J. Biol. Chem.* 267:10215–10218.

35. Mohr, C., I. Just, A. Hall, and K. Aktories. 1990. Morphological alterations of *Xenopus* oocytes induced by valine-14 p21ʳʰᵒ depend on isoprenylation and are inhibited by *Clostridium botulinum* C3 ADP-ribosyltransferase. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 275:168–172.

36. Moriishi, K., B. Syuto, N. Yokosawa, K. Oguma, and M. Saito. 1991. Purification and characterization of ADP-ribosyltransferases (exoenzyme C3) of *Clostridium botulinum* type C and D strains. *J. Bacteriol.* 173:6025–6029.

37. Munemitsu, S., M. A. Innis, R. Clark, F. McCormick, A. Ullrich, and P. Polakis. 1990. Molecular cloning and expression of a G25K cDNA, the human homolog of the yeast cell cycle gene *CDC42*. *Mol. Cell. Biol.* 10:5977–5982.

38. Narumiya, S., A. Sekine, and M. Fujiwara. 1988. Substrate for botulinum ADP-ribosyltransferase, Gb, has an amino acid sequence homologous to a putative *rho* gene product. *J. Biol. Chem.* 263:17255–17257.

39. Newport, J., and M. Kirschner. 1982. A major developmental transition in early Xenopus embryos. I. Characterization and timing of cellular changes at the midblastula stage. *Cell.* 30:675–686.

40. Nishiki, T., S. Narumiya, N. Morii, M. Yamamoto, M. Fujiwara, Y. Kamata, G. Sakaguchi, and S. Kozaki. 1990. ADP-ribosylation of the *rho/rac* proteins induces growth inhibition, neurite outgrowth and acetylcholine esterase in cultured PC-12 cells. *Biochem. Biophys. Res. Commun.* 167:265–272.

41. Nishimura, J., M. Kolber, and C. van Breemen. 1988. Norepinephrine and GTP-γ-S increase myofilament Ca²⁺ sensitivity in α-toxin permeabilized arterial smooth muscle. *Biochem. Biophys. Res. Commun.* 157:677–683.

42. Ohga, N., A. Kikuchi, T. Ueda, J. Yamamoto, and Y. Takai. 1989. Rabbit intestine contains a protein that inhibits the dissociation of GDP from and the subsequent binding of GTP to *rho*B p20, a *ras* p21-like GTP-binding protein. *Biochem. Biophys. Res. Commun.* 163:1523–1533.

43. Paterson, H. F., A. J. Self, M. D. Garrett, I. Just, K. Aktories, and A. Hall. 1990. Microinjection of recombinant p21ʳʰᵒ induces rapid changes in cell morphology. *J. Cell Biol.* 111:1001–1007.

44. Perry, M. M., H. A. John, and N. S. T. Thomas. 1971. Actin-like filaments in the cleavage furrow of newt egg. *Exp. Cell Res.* 65:249–253.

45. Pollard, T. D., and J. A. Cooper. 1986. Actin and actin-binding proteins. A critical evaluation of mechanisms and functions. *Annu. Rev. Biochem.* 55:987–1035.

46. Rappaport, R. 1986. Establishment of the mechanism of cytokinesis in animal cells. *Int. Rev. Cytol.* 105:245–281.

47. Regazzi, R., A. Kikuchi, Y. Takai, and C. B. Wollheim. 1992. The small GTP-binding proteins in the cytosol of insulin-secreting cells are complexed to GDP-dissociation inhibitor proteins. *J. Biol. Chem.* 267:17512–17519.

48. Ridley, A. J., and A. Hall. 1992. The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell.* 70:389–399.

49. Ridley, A. J., H. F. Paterson, C. L. Johnston, D. Diekmann, and A. Hall. 1992. The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. *Cell.* 70:401–410.

50. Ron, D., M. Zannini, M. Lewis, R. B. Wickner, L. T. Hunt, G. Graziani, S. R. Tronick, S. A. Aaronson, and A. Eva. 1991. A region of proto-*dbl* essential for its transforming activity shows sequence similarity to a yeast cell cycle gene, *CDC24*, and the human breakpoint cluster gene, *bcr*. *New Biologist.* 3:372–379.

51. Rubin, E. J., D. M. Gill, P. Boquet, and M. R. Popoff. 1988. Functional modification of a 21-kilodalton G protein when ADP-ribosylated by exoenzyme C3 of *Clostridium botulinum*. *Mol. Cell. Biol.* 8:418–426.

52. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 545 pp.

53. Schroeder, T. E. 1968. Cytokinesis: filaments in the cleavage furrow. *Exp. Cell Res.* 53:272–276.

54. Schroeder, T. E. 1970. The contractile ring. I. Fine structure of dividing mammalian (HeLa) cells and the effects of cytochalasin B. *Z. Zellforsch. Mikrosk. Anat.* 109:431–449.

55. Schroeder, T. E. 1973. Actin in dividing cells: contractile ring filaments bind heavy meromyosin. *Proc. Natl. Acad. Sci. USA.* 70:1688–1692.

56. Sekine, A., M. Fujiwara, and S. Narumiya. 1989. Asparagine residue in the *rho* gene product is the modification site for botulinum ADP-ribosyltransferase. *J. Biol. Chem.* 264:8602–8605.

57. Shiokawa, K., M. Yoshida, H. Fukamachi, Y. Fu, K. Tashiro, and M. Sameshima. 1992. Cytological studies of large nucleus-like structures formed by exogenously-injected linear and circular DNAs in fertilized eggs of *Xenopus laevis*. *Dev. Growth Differ.* 34:79–90.

58. Smith, D. B., and K. S. Johnson. 1988. Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. *Gene.* 67:31–40.

59. Stasia, M.-J., A. Jouan, N. Bourmeyster, P. Boquet, and P. V. Vignais. 1991. ADP-ribosylation of a small size GTP-binding protein in bovine neutrophils by the C3 exoenzyme of *Clostridium botulinum* and effect on the cell motility. *Biochem. Biophys. Res. Commun.* 180:615–622.

60. Sugai, M., T. Enomoto, K. Hashimoto, K. Matsumoto, Y. Matsuo, H. Ohgai, Y.-M. Hong, S. Inoue, K. Yoshikawa, and H. Suginaka. 1990. A novel epidermal cell differentiation inhibitor (EDIN): purification and characterization from *Staphylococcus aureus*. *Biochem. Biophys. Res. Commun.* 173:92–98.

61. Sugai, M., K. Hashimoto, A. Kikuchi, S. Inoue, H. Okumura, K. Matsumoto, Y. Goto, H. Ohgai, K. Moriishi, B. Syuto, K. Yoshikawa, H. Suginaka, and Y. Takai. 1992. Epidermal cell differentiation inhibitor ADP-ribosylates small GTP-binding proteins and induces hyperplasia of epidermis. *J. Biol. Chem.* 267:2600–2604.

62. Takai, Y., K. Kaibuchi, A. Kikuchi, and M. Kawata. 1992. Small GTP-binding proteins. *Int. Rev. Cytol.* 133:187–230.

63. Takaishi, K., A. Kikuchi, S. Kuroda, K. Kotani, T. Sasaki, and Y. Takai. 1993. Involvement of *rho* p21 and its inhibitory GDP/GTP exchange protein (*rho* GDI) in cell motility. *Mol. Cell. Biol.* 13:72–79.

64. Ueda, T., A. Kikuchi, N. Ohga, J. Yamamoto, and Y. Takai. 1990.

Purification and characterization from bovine brain cytosol of a novel regulatory protein inhibiting the dissociation of GDP from and the subsequent binding of GTP to *rho*B p20, a *ras* p21-like GTP-binding protein. *J. Biol. Chem.* 265:9373-9380.

65. Weber, K., J. R. Pringle, and M. Osborn. 1972. Measurement of molecular weights by electrophoresis on SDS-acrylamide gel. *Methods Enzymol.* 26:3-27.

66. Yamamoto, T., K. Kaibuchi, T. Mizuno, M. Hiroyoshi, H. Shirataki, and Y. Takai. 1990. Purification and characterization from bovine brain cytosol of proteins that regulate the GDP/GTP exchange reaction of *smg*

p21s, *ras* p21-like GTP-binding proteins. *J. Biol. Chem.* 265:16626-16634.

67. Yoshida, Y., M. Kawata, M. Katayama, H. Horiuchi, Y. Kita, and Y. Takai. 1991. A geranylgeranyltransferase for *rho*A p21 distinct from the farnesyltransferase for *ras* p21s. *Biochem. Biophys. Res. Commun.* 175:720-728.

68. Yoshida, Y., M. Kawata, Y. Miura, T. Musha, T. Sasaki, A. Kikuchi, and Y. Takai. 1992. Microinjection of *smg/rap*1/*Krev*-1 p21 into Swiss 3T3 cells induces DNA synthesis and morphological changes. *Mol. Cell. Biol.* 12:3407-3414.