Roles of Rho-associated Kinase in Cytokinesis; Mutations in Rho-associated Kinase Phosphorylation Sites Impair Cytokinetic Segregation of Glial Filaments

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Abstract. Rho-associated kinase (Rho-kinase), which is activated by the small GTPase Rho, regulates formation of stress fibers and focal adhesions, myosin fiber organization, and neurite retraction through the phosphorylation of cytoskeletal proteins, including myosin light chain, the ERM family proteins (ezrin, radixin, and moesin) and adducin. Rho-kinase was found to phosphorylate a type III intermediate filament (IF) protein, glial fibrillary acidic protein (GFAP), exclusively at the cleavage furrow during cytokinesis. In the present study, we examined the roles of Rho-kinase in cytokinesis, in particular organization of glial filaments during cytokinesis. Expression of the dominant-negative form of Rho-kinase inhibited the cytokinesis of *Xenopus* embryo and mammalian cells, the result being production of multinuclei. We then constructed a series of mutant GFAPs, where Rho-kinase phosphorylation sites were variously mutated, and expressed them in type III IF-negative cells. The mutations induced impaired segregation of glial filament (GFAP filament) into postmitotic daughter cells. As a result, an unusually long bridge-like cytoplasmic structure formed between the unseparated daughter cells. Alteration of other sites, including the cdc2 kinase phosphorylation site, led to no remarkable defect in glial filament separation. These results suggest that Rho-kinase is essential not only for actomyosin regulation but also for segregation of glial filaments into daughter cells which in turn ensures correct cytokinetic processes.

Key words: intermediate filament • glial fibrillary acidic protein (GFAP) • Rho • Rho-associated kinase • cytokinesis

THE Rho family of small GTPases appear to be key players in various cellular processes; Rho, Rac, and Cdc42, currently the best understood molecules, control the adhesion, morphology, and motility of mammalian cells, and also regulate signal transduction pathways that affect gene transcription in the nucleus (for review see Machesky and Hall, 1996; Hall, 1998). Rho may also be involved in cytokinesis since inhibition of endogenous Rho by *botulinum* ADP-ribosyltransferase C3 blocked cytokinesis in *Xenopus* embryo (Kishi et al., 1993) and sand dollar (Mabuchi et al., 1993), perhaps due to inhibition of actin reorganization and contractile ring formation. In addition, in *Xenopus* embryos, Rho is apparently important for assembly of actin filaments and proper constriction of the contractile ring, and Cdc42 has a role in furrow ingression (Drechsel et al., 1996).

Rho cycles between GDP-bound inactive and GTP-bound active forms, which binds to specific targets and then exerts biological functions. Several Rho targets have been identified: protein kinase N (PKN)[1] (Amano et al., 1996*a*; Watanabe et al., 1996), Rho-kinase/ROK$_\alpha$ (Leung et al., 1995; Matsui et al., 1996), and the myosin-binding subunit (MBS) of myosin phosphatase (Kimura et al., 1996). p160^{ROCK} is an isoform of Rho-kinase (Ishizaki et al., 1997). Rho-kinase regulates the phosphorylation of myo-

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1. *Abbreviations used in this paper:* aa, amino acid(s); CF, cleavage furrow; EL cells, L cells that express E-cadherin; ERM, ezrin, radixin, and moesin; IF, intermediate filament; GFAP, glial fibrillary acidic protein; MBS, myosin-binding subunit of myosin phosphatase; MLC, myosin light chain; PH, pleckstrin homology; PI, propidium iodide; PKN, protein kinase N.

sin light chain (MLC) of myosin II by direct phosphorylation of MLC and by inactivation of myosin phosphatase through phosphorylation of MBS (Amano et al., 1996*b*; Kimura et al., 1996; Chihara et al., 1997). In addition to MLC and MBS, Rho-kinase phosphorylates the ERM family proteins (ezrin, radixin, and moesin) and adducin both in vitro and in vivo (Matsui et al., 1998; Fukata et al., 1998; Kimura et al., 1998). Rho-kinase has been shown to regulate the formation of actin stress fibers and focal adhesions (Amano et al., 1997; Leung et al., 1996; Ishizaki et al., 1997), smooth muscle contraction (Kureishi et al., 1997), myosin fiber organization and *c-fos* expression (Chihara et al., 1997), and neurite retraction (Amano et al., 1998).

Intermediate filaments (IFs) constitute major components of the cytoskeleton and the nuclear envelope in most cell types (for reviews see Eriksson et al., 1992; Fuchs and Weber, 1994). Although IFs were considered to be relatively stable compared with other cytoskeletons such as actin filaments and microtubules, intensive in vitro investigations revealed that site-specific phosphorylation of the head domains of IF proteins by several kinases, such as protein kinase A (PKA), protein kinase C (PKC), Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and cdc2 kinase, dynamically alters the filament structure (for review see Inagaki et al., 1996). However, it has remained to be determined if the results obtained in vitro reflect the physiological significance of IF phosphorylation in vivo.

During cytokinesis of cell division, the cleavage furrow forms between the daughter nuclei, after which the essential cell components are segregated into postmitotic daughter cells. Protein phosphorylation/dephosphorylation is thought to play pivotal roles in mitotic processes (Norbury and Hunt, 1991; Nurse, 1992; Nigg, 1993), and it has been suggested that it may also regulate cellular separation in cytokinesis (Satterwhite et al., 1992; Murray and Hunt, 1993; Yamakita et al., 1994). We detected protein kinase activity in the cytokinetic cells and this activity phosphorylates a type III IF protein, glial fibrillary acidic protein (GFAP) (Nishizawa et al., 1991; Matsuoka et al., 1992; Sekimata et al., 1996; Kosako et al., 1997) at metaphase–anaphase transition at the cleavage furrow. We named the putative kinase cleavage furrow (CF) kinase. We then found that in vivo phosphorylation sites of GFAP (Thr-7, Ser-13, and Ser-38) by CF kinase is located in the head domain and completely overlapped with in vitro GFAP phosphorylation sites by Rho-kinase, and that the phosphorylation of GFAP head domain by Rho-kinase led to disassembly of the filament structure in vitro (Kosako et al., 1997). Moreover, Rho-kinase and its regulator Rho were immunocytochemically shown to be concentrated at the cleavage furrow (Takaishi et al., 1995; Kosako et al., 1998). Taken together, we consider that Rho-kinase functions as a CF kinase for GFAP.

In the present study, we examined roles of Rho-kinase in cytokinesis, and found that the dominant-negative form of Rho-kinase inhibited cleavage furrow formation in *Xenopus* embryos and cytokinesis of mammalian EL cells. We also analyzed functions of the specific phosphorylation of GFAP by Rho-kinase during cytokinesis. For this purpose, we constructed a series of mutant human GFAPs, where Rho-kinase phosphorylation sites Thr-7, Ser-13, and/or Ser-38 are substituted to Ala, and expressed them in type III IF-negative cells, T24. Mutations in the Rho-kinase phosphorylation sites specifically impaired segregation of glial filament into postmitotic daughter cells. Consequently, an unusually long bridge-like cytoplasmic structure formed between unseparated daughter cells, and Ser-38 was found to be prerequisite for this phenotype. On the basis of these observations, we propose that Rho-kinase is involved in not only the actomyosin system but also in IF reorganization during cytokinesis.

Materials and Methods

Gene Construction

For expression of wild-type GFAP, cDNA for human GFAP (Reeves et al., 1989) was introduced in the expression vector pDR2 (Clontech, Palo Alto, CA). For site-directed mutagenesis, we used polymerase chain reaction (PCR) with oligonucleotide mutation primers and the template GFAP cDNA. The cDNA fragment of RB/PH (TT), encoding Rho-binding domain with point mutations (Asn1036 and Lys1037 to Thr) and pleckstrin homology (PH) domain, was subcloned into the pMAL-c2 vector and expressed in *Escherichia coli* as maltose-binding protein (MBP) fusion protein and purified on amylose resin (New England Biolabs, Beverly, MA). RB/PH (TT) was reported to function as a dominant-negative version of Rho-kinase (Amano et al., 1998). The mutation sites were confirmed by sequencing using the dideoxy termination method and a DNA sequencer (Applied Biosystems, Foster City, CA).

Microinjection into Xenopus Eggs

Proteins used were concentrated and replaced by microinjection buffer (88 mM NaCl, 20 mM Tris-Cl, pH 7.5) with Centricon 10 (Amicon, Beverly, MA). Eggs from *Xenopus laevis* were fertilized in vitro and cultured in 0.1× MBS (8.8 mM NaCl, 0.1 mM KCl, 0.041 mM $CaCl_2$, 0.033 mM $Ca(NO_3)_2$, 0.082 mM $MgSO_4$, 0.24 mM $NaHCO_3$, 1 mM Hepes, pH 7.4) containing 3% Ficoll. The embryos were selected at the beginning of the first furrow and 10 nl of protein sample was injected ~100 min after fertilization into one blastomere. Cleavage arrest of the injected blastomere was observed 2.5 h after fertilization.

Transfection

Mouse fibroblastic L cells which express E-cadherin (EL cells) were maintained in DME supplemented with 10% FCS. Transfection of plasmids into EL cells was carried out using lipofectamine (GIBCO BRL, Gaithersburg, MD). Human bladder cell carcinoma T24 cells maintained in DME supplemented with 10% FCS in a 37°C, 5% CO_2 incubator were cotransfected with pCMVEBNA (Clontech) and pSV2neo (Clontech) plasmids using lipofectamine. Cells expressing pCMVEBNA were cloned and maintained in the presence of 500 μg/ml G418 sulfate (GIBCO BRL). The EBNA-expressing T24 cells were transiently transfected with wild-type or mutant GFAP cDNA in pDR2, using lipofectamine, then were examined immunocytochemically or by immunoblotting 48 h after the transfection. In some experiments, mitotic cells were prepared as follows. 48 h after transfection, cells were treated with 15 ng/ml 3-(1-anillinoethylidene)-5-benzylpyrrolidine-2,4-dione (TN-16) (Wako Chemical, Neuss, Germany) for 4 h and mitotic cells were collected. After washing with DME to remove TN-16, cells were plated on glass coverslips, and then incubated for 3 h at 37°C in DME containing 10% FCS to allow cell cycle progression. Then, the GFAP bridge formation was analyzed by immunocytochemistry.

Immunocytochemistry and Immunoblotting

EL cells were seeded at a density of 1.7×10^4 cells onto 13-mm glass coverslips coated with polylysine (Sigma Chemical Co., St. Louis, MO). pEF-Bos-myc vectors encoding the coil region of Rho-kinase (amino acids [aa] 421–701) or RB/PH (TT) were transfected using lipofectamine reagent (GIBCO BRL). The nuclei and expressed proteins were stained with bisbenzimide (Molecular Probes, Eugene, OR) and polyclonal anti-myc antibody (Santa Cruz Biotechnology, Santa Cruz, CA), respectively (Amano et al., 1998). For the control experiment, pEF-Bos-myc vector was mixed

in a 4:1 ratio with pME18S-lacZ. To visualize cells expressing β-galactosidase, the cells were placed in fixation solution (2% formaldehyde, 0.2% glutaraldehyde in PBS) for 10 min at room temperature and washed three times with PBS. Cells were stained with anti–β-galactosidase antibody (Chemicon, Temecula, CA).

Immunocytochemical procedures using the antibodies MO389, YC10, KT13, KT34, pG1-T and the following propidium iodide (PI) staining have been described elsewhere in detail (Matsuoka et al., 1992; Sekimata et al., 1996). For double immunostaining with anti-lamin A/C and anti-GFAP antibodies, cells were fixed with −20°C methanol for 10 min and then were incubated with monoclonal anti-lamin A/C antibody (provided by Y. Yoneda, Osaka University, Osaka, Japan) diluted 1:1,000 in PBS and polyclonal anti-GFAP antibody (Dako, Carpinteria, CA) diluted 1:200 in PBS for 2 h. The lamin A/C immunoreactivity was visualized by incubation with biotinylated anti-mouse IgM antibody (Vector Laboratories, Burlingame, CA), followed by incubation with streptavidin-Texas red (Amersham, Arlington Heights, IL), whereas the GFAP immunoreactivity was visualized by FITC-conjugated anti-rabbit antibodies (BioSource, Camarillo, CA). For double immunostaining with anti-tubulin and anti-GFAP antibodies, cells fixed as above were incubated with monoclonal anti–α-tubulin antibody (Sigma Chemical Co.) diluted 1:500 in PBS and the polyclonal anti-GFAP antibody for 2 h, and the former immunoreactivity was visualized by use of Texas red–conjugated anti-mouse IgG antibody (Amersham). For double staining of actin and GFAP, cells fixed with 3.7% formaldehyde in PBS for 15 min, followed by treatment with 0.1% Triton X-100 in PBS, and then stained by incubation with rhodamine phalloidin (Molecular Probes) diluted 1:1,000 in PBS and the polyclonal anti-GFAP antibody for 2 h.

For immunoblotting, lysates of 2×10^4 cells were loaded in the lanes, resolved by SDS-PAGE, and then transferred onto a polyvinylide difluoride membrane (Immobilon-P; Millipore, Waters Chromatography, Milford, MA). The blots were then incubated overnight with polyclonal anti-GFAP antibody (Dako) diluted 1:500 in TBS-T (20 mM Tris, pH 7.6, 137 mM NaCl, 0.1% Tween 20). Immunoreactive bands were visualized by use of horseradish peroxidase-conjugated anti-rabbit antibody (Amersham) and the enhanced chemiluminescence Western blotting detection system (Amersham).

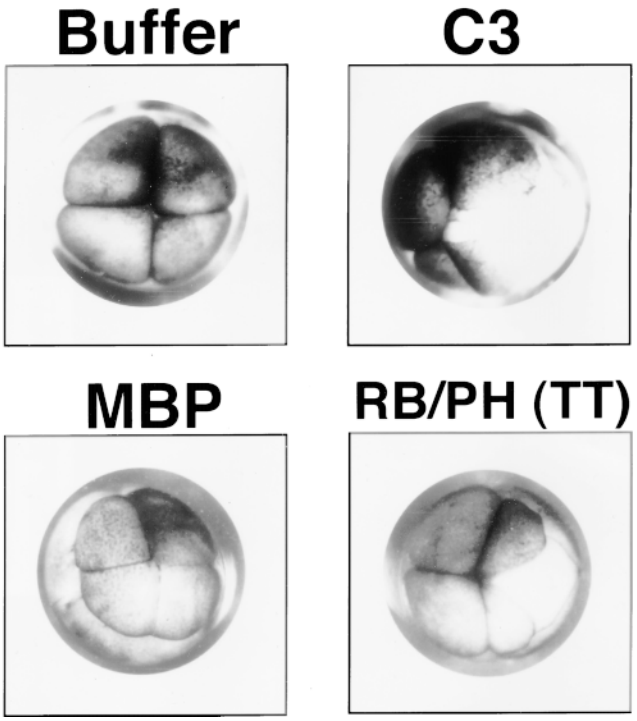

Figure 1. Effects of C3 or the dominant-negative form of Rho-kinase on furrow formation in *Xenopus* embryos. Microinjection buffer, C3 (2.5 μg/ml), MBP (10 mg/ml), or MBP-RB/PH (TT) (10 mg/ml) was injected into one blastomere at the two-cell stage. Photographs of the embryos were taken 2.5 h after fertilization. The results shown are representatives with typical phenotype.

Results

Dominant-negative Rho-Kinase Blocks Cytokinesis in Xenopus Embryos and Mammalian EL Cells

To analyze the function of Rho-kinase on cytokinesis, we used a polypeptide, RB/PH (TT), which contains a mutated Rho-binding domain and PH-domain of Rho-kinase (aa 941–1,388) and acts as a dominant-negative form of Rho-kinase (Amano et al., 1998). We have recently found that RB/PH (TT) specifically inhibits the kinase activity of Rho-kinase, but not the activity of PKN or myotonic dystrophy kinase-related Cdc42-binding kinase (MRCK) (Leung et al., 1998), which has a kinase domain similar to that of Rho-kinase (our unpublished observation). When *Xenopus* embryos, which are known to have a counterpart of Rho-kinase (Farah et al., 1998), were fertilized by *Xenopus* sperm, the furrow formation started ~90 min after fertilization and the cytoplasmic division was completed within ~5 min. When MBP-fused RB/PH (TT) (10 mg/ml) or recombinant C3 (2.5 μg/ml) was microinjected into one blastomere of *Xenopus* embryo at 100 min after the fertilization (two-cell stage), the cleavage furrow formation was blocked, and a white belt appeared on the equatorial region in the animal hemisphere of the embryos, instead of the furrow (Fig. 1 and Table I). Under these conditions, a well-developed cleavage furrow was observed in the control embryos microinjected with buffer or MBP (Fig. 1). Interference with cleavage furrow formation was also observed when the PH-domain of Rho-kinase (aa 1,125–1,388), which is known to be a weak dominant-negative form (Amano et al. 1998), was microinjected; the furrow formation was somewhat delayed compared with findings with the buffer control (data not shown). When we analyzed the effect of dominant-negative Rho-kinase using mammalian cells, expression of the RB/PH (TT) in EL cells also interfered with cleavage furrow formation (Fig. 2). By contrast, chromosome separation and movement towards the poles of mitotic apparatus, as well as daughter nuclei formation, occurred normally. Consequently, the transfected cells became multinuclear (Fig. 2). Approximately 20% of the cells expressing RB-PH (TT) had multinuclei 120 h after transfection (Fig. 2 *B*). We conclude that Rho-kinase plays essential roles in cleavage furrow formation.

Table I. Effects of C3 or the Dominant-negative Form of Rho-kinase on Furrow Formation

		Number of embryos		
		Total	Failed cleavage with white belt	Failed cleavage
				%
Buffer	—	11	0	0
C3	2.5 μg/ml	29	27	93
MBP	10 mg/ml	22	0	0
MBP-RB/PH (TT)	10 mg/ml	45	28	62

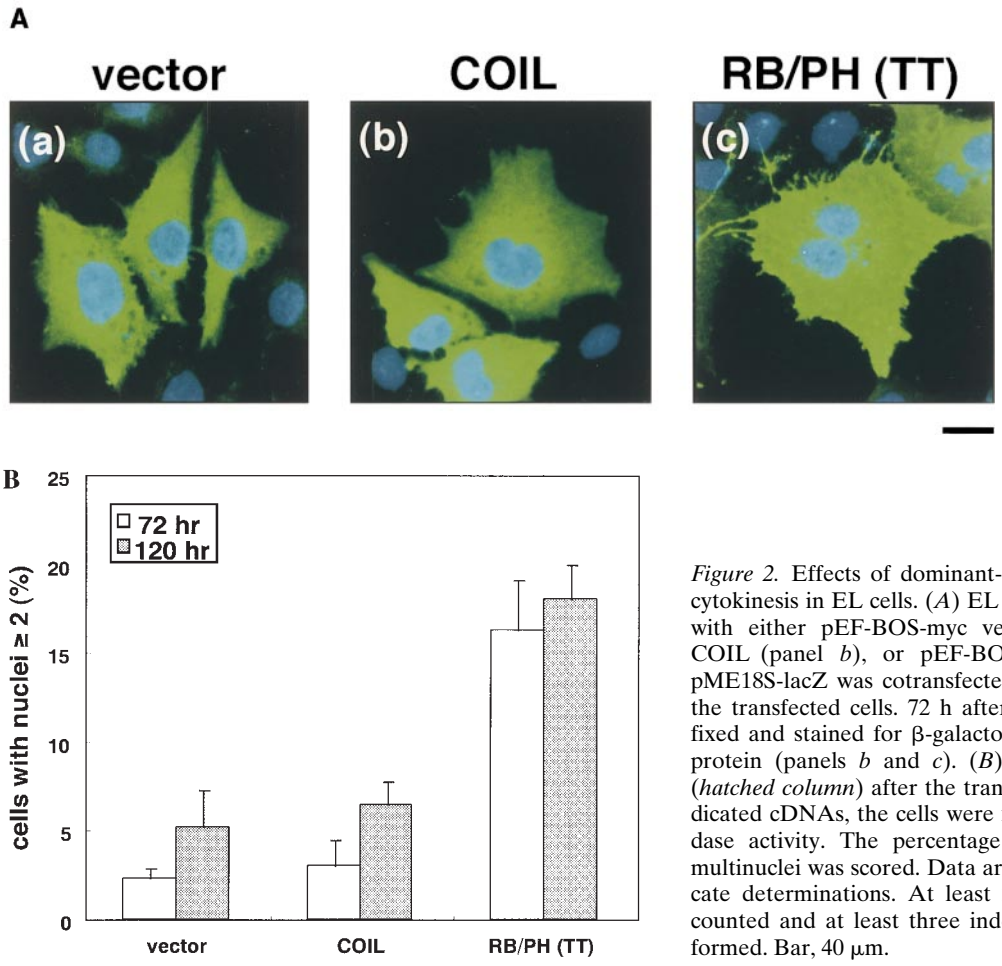

Figure 2. Effects of dominant-negative form of Rho-kinase on cytokinesis in EL cells. (*A*) EL cells were transiently transfected with either pEF-BOS-myc vector (panel *a*), pEF-BOS-myc-COIL (panel *b*), or pEF-BOS-myc-RB/PH (TT) (panel *c*). pME18S-lacZ was cotransfected with control vector to identify the transfected cells. 72 h after the transfection, the cells were fixed and stained for β-galactosidase (panel *a*) and myc-tagged protein (panels *b* and *c*). (*B*) 72 h (*open column*) or 120 h (*hatched column*) after the transfection of plasmids encoding indicated cDNAs, the cells were fixed and stained for β-galactosidase activity. The percentage of LacZ-positive cells bearing multinuclei was scored. Data are means ± SEM of at least triplicate determinations. At least 200 cells per each sample were counted and at least three independent experiments were performed. Bar, 40 μm.

Expression of GFAP in T24 Cells Lacking Type III IFs

CF kinase/Rho-kinase activity was reported to phosphorylate Thr-7, Ser-13, and Ser-38 of human GFAP, an astrocyte specific IF protein, at the cleavage furrow of cytokinetic cells (Nishizawa et al., 1991; Matsuoka et al., 1992; Sekimata et al., 1996; Kosako et al., 1997) (Fig. 3 *A*). It has been considered that Rho-kinase acts as a CF kinase toward GFAP and vimentin (Kosako et al., 1997; Goto et al., 1998; Kosako et al., 1998). We asked whether ectopically expressed GFAP can be phosphorylated even in cells lacking type III IFs. As shown in Fig. 3 *B*, wild-type GFAP transiently expressed in T24 cells, which do not express any type III IFs, was diffusely distributed in the cytoplasm. We stained the cells with monoclonal antibodies that recognize site-specific phosphorylation of GFAP (Matsuoka et al., 1992; Sekimata et al., 1996) and rabbit polyclonal antibody for GFAP (Dako). The obtained results confirmed that the ectopically expressed wild-type GFAP was also phosphorylated at Thr-7, Ser-13, and Ser-38 at the cleavage furrow of cytokinetic T24 cells (Fig. 3 *B*). Another mitotic kinase, cdc2 kinase, also phosphorylated GFAP at Ser-8 in metaphase cells (Fig. 3 *B*), as noted earlier (Matsuoka et al., 1992; Inagaki et al., 1996). The obtained data coincide with the finding that Rho-kinase and cdc2 kinase are expressed ubiquitously. T24 cells expressing wild-type GFAP underwent normal mitosis and cell growth (Fig. 3 *B* and Fig. 4 *B*).

Effects on Cytokinesis of Mutation of GFAP in Rho-Kinase Phosphorylation Sites

To determine the significance of GFAP phosphorylation by Rho-kinase, we constructed a mutant GFAP, m(7,13,38), with mutations in Rho-kinase phosphorylation sites (Fig. 3 *A*) and transiently expressed this mutant in T24 cells, using the pDR2 vector system. Fig. 4 *A* indicates that expression patterns of wild-type GFAP and m(7,13,38) mutant in interphase cells are indistinguishable; in some cells, expressed wild-type GFAP or the m(7,13,38) mutant appeared to be enriched at the perinuclear area and, in other cells, expressed GFAP formed an extended network from the perinuclear region to the cell periphery. Cells expressing the mutant had a normal morphology at prometaphase, metaphase, anaphase, and telophase (data not shown). At T24 cells expressing m(7,13,38), the percentage of mitotic cell population in the total transfected cells was 3.4%, a value comparable to that in the case of cells expressing wild-type and other mutated GFAP (Fig. 5 *A*). However, cells expressing m(7,13,38) showed a striking phenotype after passing through telophase. The mutant GFAP filaments failed to segregate into daughter cells and

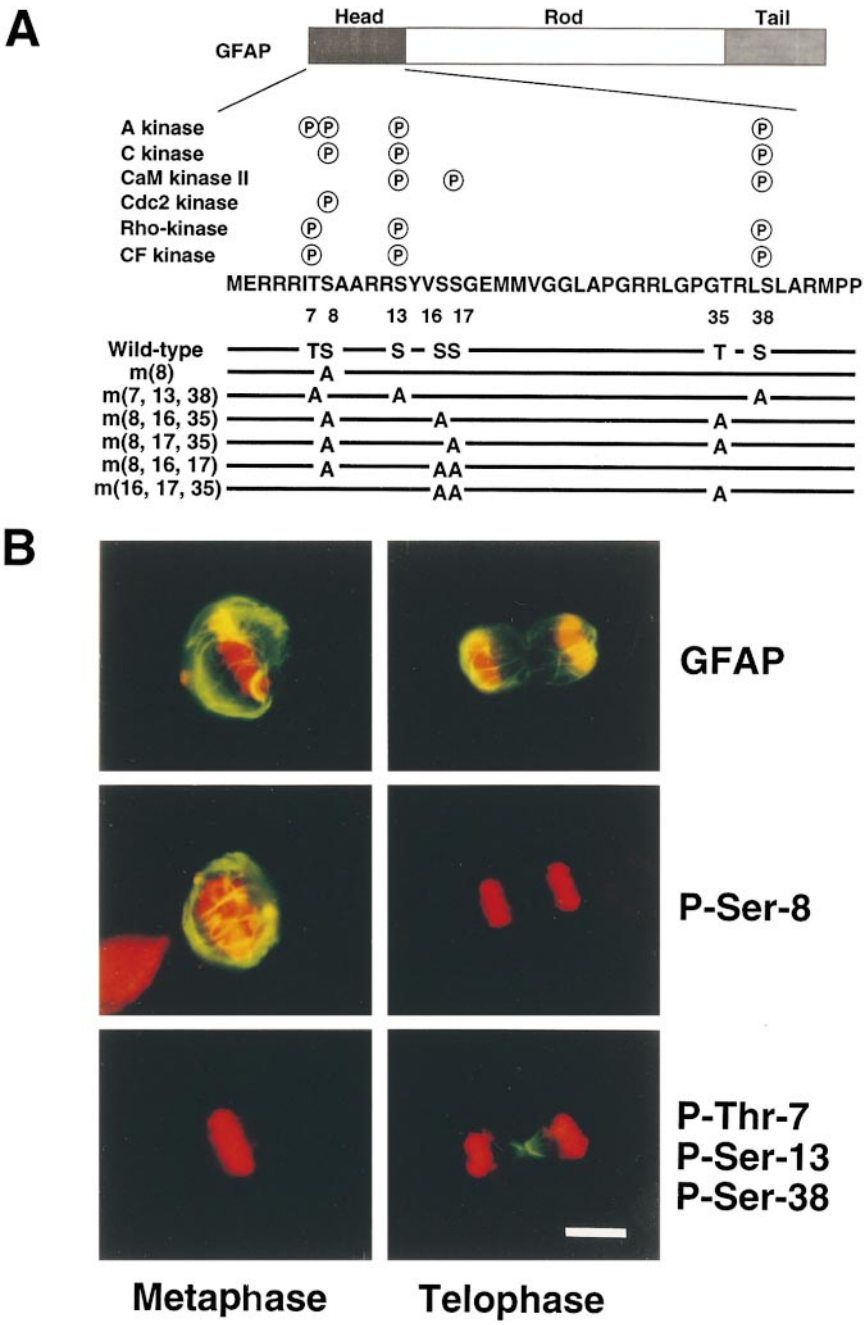

Figure 3. (*A*) Schemes showing GFAP phosphorylation sites and GFAP mutants produced in this study. Phosphorylation sites for PKA, PKC, CaMKII, cdc2 kinase, and Rho-kinase were identified in in vitro studies, whereas those for CF kinase were identified in in vivo sites. The sites are indicated by *P* within a circle. Note that Ser-38 of human GFAP (Reeves et al., 1989) corresponds to Ser-34 of bovine GFAP (Inagaki et al., 1996). (*B*) Cell cycle-dependent site-specific phosphorylation of wild-type GFAP expressed in T24 cells. The cells were immunostained by anti-GFAP antibody MO389 (*top*), YC10 that recognizes GFAP phosphorylation at Ser-8 (*middle*), or by KT13 that recognizes GFAP phosphorylation at Ser-13 (*bottom*). Ser-8 of GFAP was phosphorylated by cdc2 kinase at metaphase, whereas Ser-13 was phosphorylated by CF kinase in the cleavage furrow of cytokinetic cells. The antibodies pG1-T and KT34 that recognize GFAP phosphorylation at Thr-7 and Ser-38, respectively, showed that these sites are also phosphorylated by CF kinase at the cleavage furrow (data not shown). The green fluorescence represents the immunoreactions. The chromosomes were stained by PI (*red fluorescence*). Bar, 10 μm.

formed an unseparated bridge-like structure between them (Fig. 4 *B*, Fig. 5 *A*, and Fig. 6). As a result, the daughter cells were left unseparated. Cells forming such an unusual bridge accounted for 7.3% of the total m(7,13,38)-expressing cells 48 h after the transfection (Fig. 5 *A*). As shown in Fig. 5 *B*, Western blot analysis confirmed that the GFAP mutant was expressed comparably to the wild type. To further confirm the effects of m(7,13,38) parallel to wild type on cytokinesis, we synchronized the cells to mitotic phase with TN-16 as described in Materials and Methods and analyzed the GFAP bridge formation. As shown in Table II, the percentage of the cells with GFAP bridge dramatically increased (68%) by m(7,13,38), whereas the wild-type showed no bridge formation (0%), supporting the hypothesis that phosphorylation of GFAP by Rho-kinase is essential for proper cytokinesis. Fig. 6 shows examples of unseparated cells expressing m(7,13,38). Cells often formed very long GFAP bridges, some of which reached more than five times the cell diameter (Fig. 6 *g*). In addition, bridges were often torn off between daughter cells (Fig. 6, *e* and *f*, *arrowheads*).

Intracellular Bridge Formation Is a Specific Phenomenon in m(7,13,38)-expressing Cells

To rule out the possibility that the phenotype can be induced by nonsite-specific mutation of three possible GFAP phosphorylation sites, we prepared the mutants

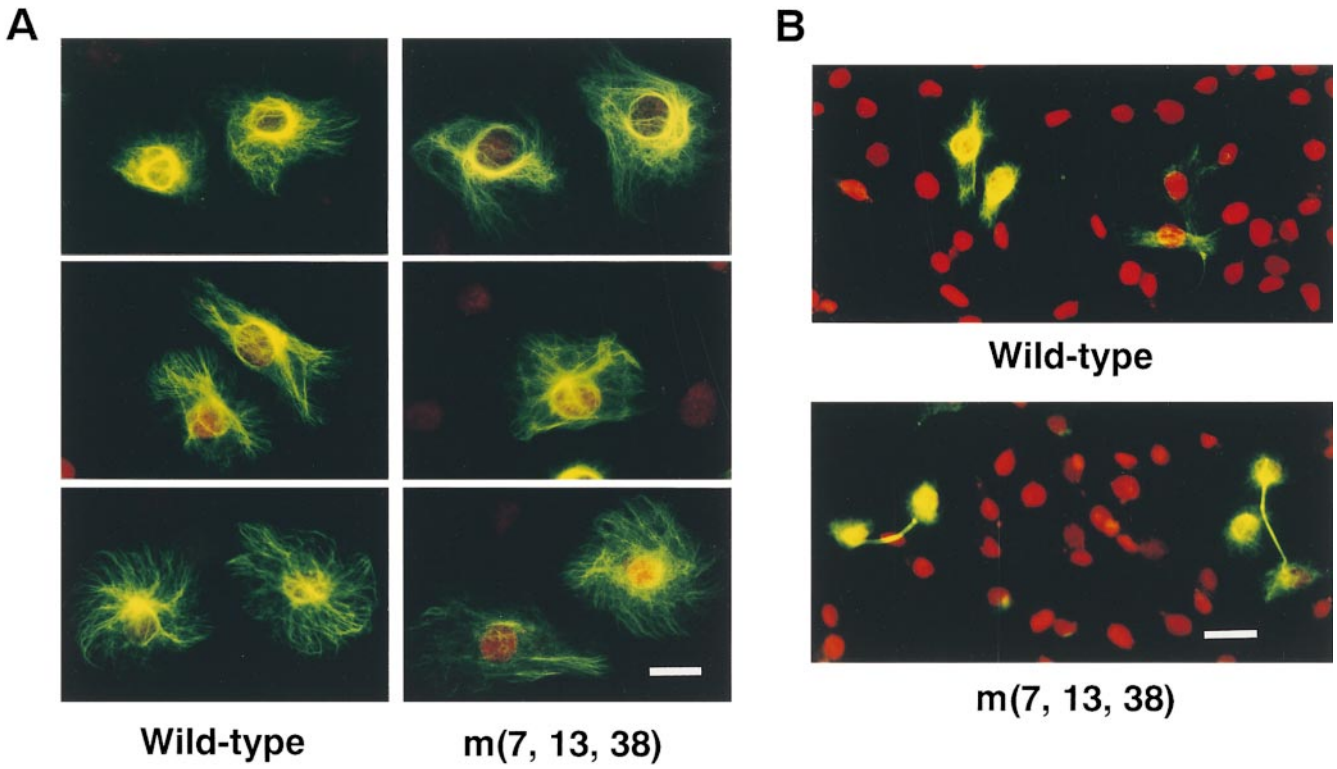

Figure 4. Mutations in the CF kinase/Rho-associated kinase phosphorylation sites impair GFAP segregation into postmitotic daughter cells. (*A*) Localization of wild-type GFAP and m(7,13,38) in interphase T24 cells. (*B*) Postmitotic T24 cells expressing wild-type GFAP and the mutant m(7,13,38). The green fluorescence represents GFAP immunoreactivity stained with MO389, whereas the chromosomes were stained by PI (*red fluorescence*). Bars: (*A*) 10 μm; (*B*) 40 μm.

m(8,16,35), m(8,17,35), m(8,16,17), and m(16,17,35) in which three Ser/Thr different from the Rho-kinase sites were altered (Fig. 3 *A*). None of these mutants showed any remarkable phenotype (Fig. 5 *A*). Previous studies demonstrated that mutations in cdc2 kinase phosphorylation sites of other IF proteins, lamin and vimentin, led to inhibition of nuclear lamina and vimentin filament disassembly, respectively, in early mitotic phases but not in cytokinesis (Heald and McKeon, 1990; Chou et al., 1996). We produced a mutant m(8) in which the cdc2 kinase phosphorylation site Ser-8 of GFAP (Inagaki et al., 1996) was altered (Fig. 3 *A*). In contrast to m(7,13,38), T24 cells expressing m(8) underwent cytokinesis normally and did not show GFAP bridge formation (Fig. 5 *A*).

As a next set of experiments, we tried to establish the stable cell lines expressing m(7,13,38) by use of various expression plasmids such as pDR2, pCMVneo, and pEF-Bos. We realized that it was difficult to establish m(7,13,38)-expressing cells under the conditions in which many hygromycin- or G418-resistant cell lines were obtained, since

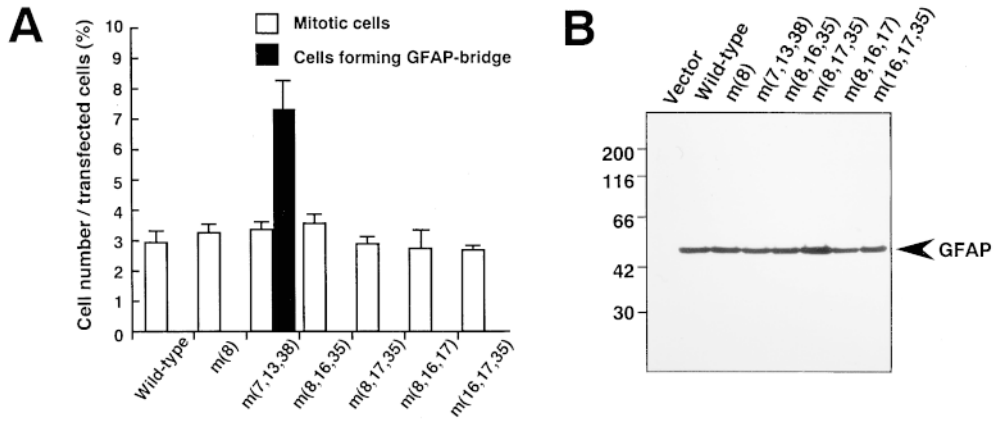

Figure 5. Effects by various mutants on the formation of GFAP bridge-like structure. (*A*) Population of mitotic cells (*open column*) and cells forming the bridge-like structure (*closed column*) expressing GFAP of wild-type, m(8), m(7,13,38), m(8,16,35), m(8,17,35), m(8,16,17), or m(16,17,35). The two daughter cells linked by one bridge-like structure was counted as one cell. (*B*) Western blot analysis of wild-type and mutant GFAPs expressed in T24 cells.

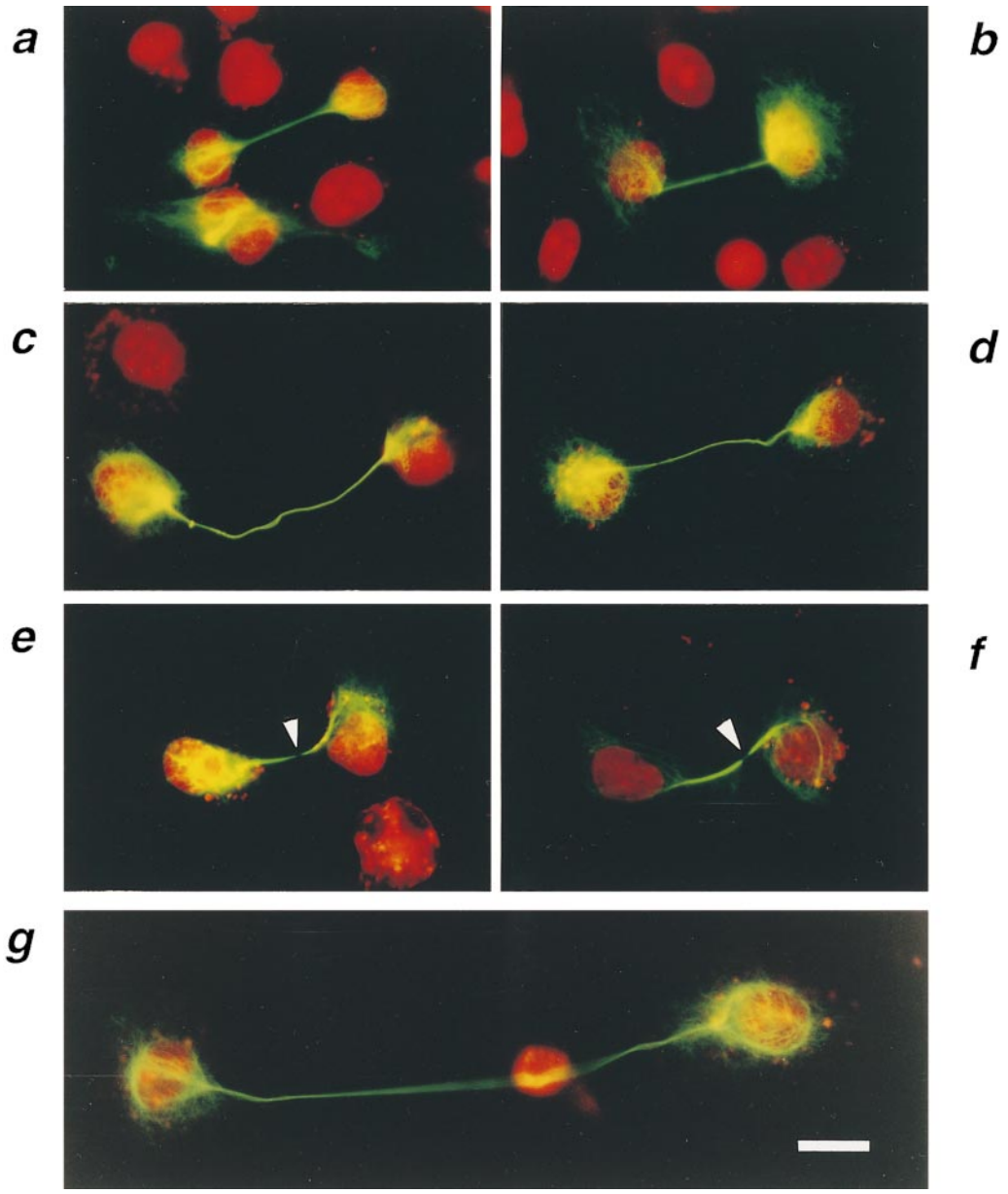

Figure 6. GFAP bridge-like structures in T24 cells expressing m(7,13,38). The green color represents GFAP immunoreactivity stained with MO389, whereas the red color shows chromosomes stained by PI. Bar, 10 μm.

the expression level of GFAP is so low that the protein did not form filamentous structure throughout cytoplasm (data not shown).

To minimize the artifact due to the lipofectamine transient transfection, we coexpressed m(7,13,38) with wild-type GFAP, and found that wild-type GFAP reversed the phenotype of m(7,13,38)-transfected cells (data not shown). This observation indicates that wild-type GFAP titrated out the mutant GFAP in cells and consequently the intercellular GFAP bridge formation was not observed. Thus, we conclude that the bridge formation caused by the m(7,13,38) is not an artifact due to the lipofectamine transient transfection.

Taken together, the obtained results indicate that the GFAP bridge was induced by site-specific mutations at Rho-kinase phosphorylation sites. Thus, this kinase plays an essential role in proper cytokinetic segregation of glial filaments.

Effects of Single and Double Mutations at Rho-Kinase Phosphorylation Sites on Formation of the GFAP Bridge-like Structure

In the next set of experiments, we wanted to determine the phosphorylation site which induces the m(7,13,38) phenotype. We introduced single or double mutations at Thr-7, Ser-13, and Ser-38. As depicted in Table III, Ser-38 was found to be the most important phosphorylation site for the proper segregation of glial filaments, since the mutation in this position resulted in the most prominent rela-

Table II. Effect of Synchronization of the Cells on Formation of GFAP Bridge-like Structures

	Number of cells		
	Total	Formation of GFAP bridge	Percent
Wild type	200	0 ± 0	0 ± 0
m(7,13,38)	200	135 ± 15	68 ± 8

The results are expressed as the mean ± SD of three independent experiments. At least 200 cells per each sample were counted.

Table III. Effect of Single and Double Mutations at Thr 7, Ser 13, and Ser 38 on Formation of GFAP Bridge-like Structures

Mutants	Relative scores of bridge-like structure
	%
m(7,13,38)	100
Wild type	0 ± 0
m(7)	1.3 ± 0.49
m(13)	4.8 ± 3.4
m(38)	26 ± 1.6
m(7,13)	8.7 ± 1.8
m(7,38)	43 ± 12
m(13,38)	81 ± 2.5

Population of cells with GFAP bridge-like structures relative to that of m(7, 13, 38) was scored. The results are expressed as the mean ± SD of three independent experiments. In each experiment, at least 2,000 cells were counted.

tive score of bridge-like structure. A single mutation at Thr-7 or Ser-13 was much less effective compared with Ser-38. Interestingly, an additional single mutation to Ser-38 had a synergistic effect on formation of the bridge-like structure.

Effects of Mutation in Rho-Kinase Phosphorylation Sites on the Reorganization of Other Cytoskeletal Components

To examine effects of the m(7,13,38) mutant on functions of other cytoskeletal components during cytokinesis, we analyzed the unseparated cells expressing the mutant m(7,13,38) (Fig. 7). Immunostaining with anti-lamin A/C antibody revealed the existence of a reformed nuclear lamina in each daughter cell (Fig. 7, *a* and *b*). The contractile ring of actin filaments and the midbody of microtubules had disappeared in the bridge between the daughter cells (Fig. 7, *c–f*), which means that bridge-forming cells had completed cytokinetic processes but that segregation of the GFAP filaments was not finished. Under this condition, keratin 7 and 18, the endogenous IFs of T24 cells, was segregated properly (data not shown).

Discussion

Rho-kinase is a downstream target for Rho (Leung et al., 1995; Matsui et al., 1996; Ishizaki et al., 1997). Rho has been considered to participate in cytokinesis (Mabuchi et al., 1993; Kishi et al., 1993; Drechsel et al., 1996). Molecular mechanisms of Rho functions in this cellular process are not clear. Here, by using dominant-negative Rho-kinase, RB/PH (TT), we first provide direct evidence that this kinase plays an essential role during cytokinesis, since it blocks cleavage furrow formation in the *Xenopus* embryo system. Inhibition of cytokinesis was also observed in RB/

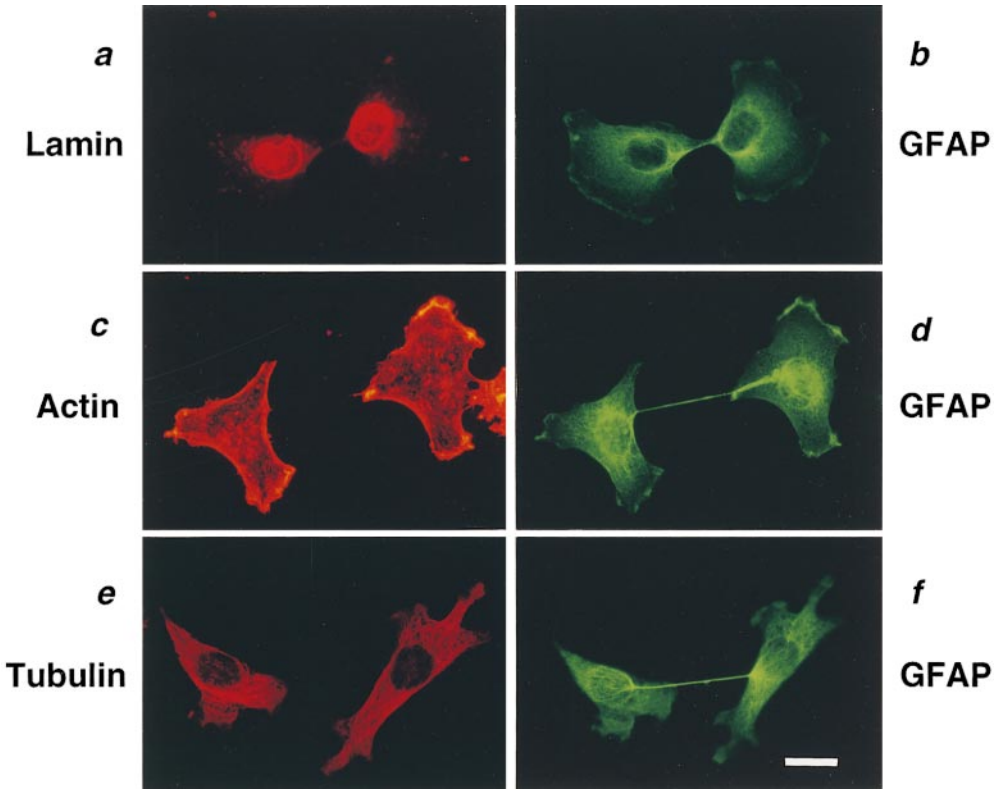

Figure 7. Analysis of the nuclear membrane, actin filaments, and microtubles in T24 cells forming GFAP bridge-like structures. T24 cells expressing m(7,13,38) were double-stained by anti-lamin A/C antibody (*a*) and polyclonal anti-GFAP antibody (Dako) (*b*), rhodamine phalloidin (*c*), and polyclonal anti-GFAP antibody (Dako) (*d*), or anti-tubulin antibody (*e*) and polyclonal anti-GFAP antibody (Dako) (*f*). Bar, 10 μm.

PH (TT)-expressing mammalian EL cells, where the cells became multinuclear. Together with the finding that RB/PH (TT) inhibits only Rho-kinase activity but not PKN (Mukai and Ono, 1994) or MRCK (Leung et al., 1998) activity, these results strongly suggest that Rho-kinase regulates cytokinesis. One possible explanation for the results obtained with *Xenopus* embryos and EL cells is that Rho-kinase phosphorylates substrate(s) such as MBS (Kimura et al., 1996) and myosin light chain (Amano et al., 1996*b*), which are involved in contractile ring formation and its maintenance, and therefore RB/PH (TT) would be expected to interfere with the cytokinesis process at the step of cleavage furrow formation.

To elucidate the Rho-kinase function in spatiotemporal organization of glial filaments during cytokinetic process, we asked if phosphorylation of GFAP at Thr-7, Ser-13, and Ser-38 by Rho-kinase is responsible for the in vivo disassembly of glial filaments. Since the introduction of dominant-negative Rho-kinase into *Xenopus* embryos or EL cells did not provide the direct link between Rho-kinase and GFAP function, we made use of mutational analyses. Mutations in the Rho-kinase phosphorylation sites of GFAP (Thr-7, Ser-13, and Ser-38) resulted in impaired segregation of glial filaments in cytokinetic cells, thereby indicating that Rho-kinase has an essential role in proper segregation of glial filaments. To further characterize the significance of Rho-kinase–dependent phosphorylation of GFAP in cytokinetic segregation of glial filaments, we introduced a single or double mutation and found that Ser-38 is essential for the long bridge-like cytoplasmic structure. When compared with nonsite-specific mutants such as m(8,16,35), a single point mutation at Thr-7 or Ser-13 had a weak but consistent effect on GFAP bridge-like structure. It must be noted that an additional single point mutation at Thr-7 or Ser-13 to Ser-38 mutation has synergistic effects, although the full effect was observed only when Thr-7, Ser-13, and Ser-38 were all mutated. These data suggest that triphosphorylation at these sites is required for efficient segregation of glial filaments. These results with the mutants indicate that phosphorylation of GFAP at Thr-7, Ser-13, and Ser-38 facilitates disassembly of glial filaments at cleavage furrow as expected by the in vitro experiments (Kosako et al., 1997). However, it is notable that actual in vivo phosphorylation level at these three sites is not determined, and it may be possible that GFAP molecules are variously phosphorylated at cleavage furrow of living cells. This interpretation is consistent with the observation that GFAP phosphorylated by Rho-kinase appears to be localized at the furrow region (Fig. 3 *B*, *bottom right panel*) and is not totally solubilized as observed in in vitro study (Kosako et al., 1997). Taken together, the results presented here clarified that Rho-kinase is involved in cytokinesis by not only regulating the actomyosin cytoskeleton but also by regulating the IF cytoskeleton.

In the present study, we used T24 cells, which do not contain Type III IF, to analyze the biological effects of GFAP mutants on cytokinesis. One would argue that, in T24 cells, type III IF has no physiological role in cytokinesis since T24 cells can complete cytokinesis without type III IFs. The reason why we use T24 cells in the present study is to show the physiological importance of GFAP phosphorylation at Thr-7, Ser-13, and Ser-38 under the conditions where any artifact by endogenous type III IF(s) is excluded. Consequently, we could show that m(7,13,38) mutant impairs completion of cytokinesis even if endogenous IF in T24 cells segregates properly.

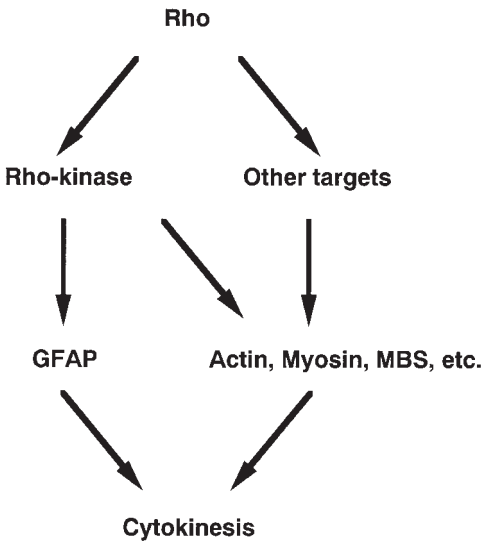

Figure 8. Signaling pathways from Rho for cytokinesis.

Rho-kinase is expressed not only in glial cells but also in other types of cells (Leung et al., 1996; Matsui et al., 1996; Ishizaki et al., 1997). It also phosphorylates another IF protein vimentin at the cleavage furrow of cytokinetic cells (Goto et al., 1998). Therefore, Rho-kinase may be sufficient for the separation of other IFs during cytokinesis.

In summary, we observed functional effects of Rho-kinase–dependent phosphorylation of GFAP with a variety of approaches; microinjection of dominant-negative Rho-kinase (RB/PH [TT]) into *Xenopus* embryos, expression of it in EL cells, and expression of various GFAPs with mutations at Rho-kinase phosphorylation sites in T24 cells. Like C3 enzyme, RB/PH (TT) blocked cleavage furrow formation, suggesting that Rho-kinase is involved in contractile ring formation, under the control of Rho. It is likely that Rho-kinase plays at least two roles in the regulation of cytokinesis. One role is to control the contractility of actin-myosin–based contractile ring, to proceed cleavage furrow formation of the cell. The other is to phosphorylate IF proteins and depolymerize IFs at the cleavage furrow to accomplish cytokinesis (Fig. 8). Rho is thought to regulate Rho-kinase and other targets simultaneously, so phenotype by RB/PH (TT) might be milder than that of C3. The Rho-kinase phosphorylation site-specific mutation demonstrated a phenotype, which fails to segregate GFAP filaments and consequently possesses unusually long bridge-like cytoplasmic structures. Analysis with single and double mutations at Rho-kinase phosphorylation sites revealed that phosphorylation at Ser-38 is the most important for segregation of glial filaments. Based on these findings we conclude that GFAP phosphorylation has definitive roles in governing regulatory processes in assembly-disassembly of glial filaments and turnover of GFAP not only in vitro but also in vivo. This is the first in vivo data that Rho-kinase regulates cytokinesis through

cleavage furrow formation and segregation of glial filaments.

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