We found that vimentin, the most widely expressed intermediate filament protein, served as an excellent substrate for Rho-associated kinase (Rho-kinase) and that vimentin phosphorylated by Rho-kinase lost its ability to form filaments in vitro. Two amino-terminal sites on vimentin, Ser38 and Ser71, were identified as the major phosphorylation sites for Rho-kinase, and Ser71 was the most favored and unique phosphorylation site for Rho-kinase in vitro. To analyze the vimentin phosphorylation by Rho-kinase in vitro, we prepared an antibody (GK71) that specifically recognizes the phosphorylation of vimentin-Ser71. Ectopic expression of constitutively active Rho-kinase in COS-7 cells induced phosphorylation of vimentin at Ser71, followed by the reorganization of vimentin filament networks. During the cell cycle, the phosphorylation of vimentin-Ser71 occurred only at the cleavage furrow in late mitotic cells but not in interphase or early mitotic cells. This cleavage furrow-specific phosphorylation of vimentin-Ser71 was observed in the various types of cells we examined. All these accumulating observations increase the possibility that Rho-kinase may have a definite role in governing regulatory processes in assembly-disassembly and turnover of vimentin filaments at the cleavage furrow during cytokinesis.

Intermediate filaments (IFs) constitute one of the three major cytoskeletal elements in eukaryotic cells. An important feature of IF proteins is their tissue preferential expression. For example, glial fibrillary acidic protein (GFAP) is expressed specifically in astroglia. On the other hand, vimentin is the most common IF protein and is expressed during development in a wide range of cells, in mesenchymal cells and in a variety of cultured cells and tumors. Previous studies have demonstrated that IFs can undergo dynamic changes in their organization during different stages of the cell cycle or during cell signaling (for review see Refs. 1–3). The reorganization of IFs is thought to be regulated by site-specific phosphorylation of IF proteins at serine and threonine residues, and several protein kinases have been shown to act as IF kinases in vivo (Ref. 4; for review see Ref. 5). Site- and phosphorylation state-specific antibodies that can recognize a phosphorylated serine/threonine residue and its flanking sequence are powerful tools to visualize site-specific IF phosphorylation in cells and to identify in vivo IF kinases (Refs. 6 and 7; for review see Ref. 8). By using several types of such antibodies, we previously detected a protein kinase activity that phosphorylates GFAP at Thr7, Ser13, and Ser24 specifically at the cleavage furrow during cytokinesis (6, 9). This kinase activity, tentatively named cleavage furrow (CF) kinase activity, was observed not only in astroglial cells but also in other cultured cells in which GFAP was ectopically expressed (10). These findings indicate that the activation of CF kinase occurs in a wide range of cell types, suggesting its important role in cytokinesis. Using a series of monoclonal antibodies (MO6, YT33, TM50, 4A4, and MO82) which specifically recognize the phosphorylation of vimentin at Ser6, Ser33, Ser50, Ser55, and Ser82, respectively, we visualized in vivo vimentin kinase activities in cell signaling or mitosis (11–14), but we detected no CF kinase-like activity that phosphorylates vimentin during cytokinesis.

The small GTP-binding protein Rho is implicated in the formation of stress fibers and focal adhesion complexes (15, 16) and in the regulation of cell morphology (17), cell aggregation (18), cell motility (19), smooth muscle contraction (20, 21), and cytokinesis (Refs. 22–24; for review see Refs. 25 and 26). Upon stimulation with certain signals, the GDP-bound inactive form of Rho may be converted to GTP-bound active form, which presumably binds to specific targets and thereby exerts its biological functions. The putative target proteins for Rho include protein kinase N (27, 28), rhophilin (28), citron (29), rhoetkin (30), the myosin binding subunit of myosin phosphatase (31), p140mDia (32), and Rho-kinase (33) (also called ROK34 or ROCK (35)) (for review see Ref. 36). Recently, we have reported that Rho-kinase phosphorylates GFAP at Thr7, Ser13, and Ser24 in vitro, the same sites that are phosphorylated by CF kinase in vivo (37). These observations raise the possibility that Rho-kinase may act downstream of Rho in the regulation of cleavage furrow-specific phosphorylation of GFAP during cytokinesis. However, one could not rule out the possibility that other unknown kinase(s) are responsible for the CF kinase.
activity because these three phosphorylation sites are phosphorylated by several kinases in vitro.

In this report, we showed that vimentin was phosphorylated by Rho-kinase in a GTP-Rho-dependent manner and that vimentin phosphorylation by Rho-kinase resulted in a nearly complete inhibition of its filament formation in vitro. We then identified Ser71 on vimentin as the most favored and unique phosphorylation site for Rho-kinase in vitro. By producing a site- and phosphorylation state-specific antibody for this site, we demonstrated that vimentin-Ser71 was phosphorylated specifically at the cleavage furrow during cytokinesis in various types of cells.

**EXPERIMENTAL PROCEDURES**

**Preparation of Proteins**—Recombinant mouse vimentin was prepared from Escherichia coli as described previously (12). Vimentin phosphorylated by the catalytic subunit of cAMP-dependent protein kinase A, Ca2+-calmodulin-dependent protein kinase II (CaM kinase II), protein kinase C, and Cdc2 kinase were prepared as described previously (11). Antibodies against PV71 were prepared by injecting mice with PV71 protein electrophoresis with formic acid/acetic acid/H2O (25:78:897) at 1500 V for 40 min; vertical dimension, ascending chromatography in 2-propanol/acetonitrile (7:3) containing 0.1% trifluoroacetic acid. All radioactivity loaded was recovered in a single peptide (a 12-kDa fragment from the amino-terminal domain of vimentin) by the retention time of 65–68 min. This phosphorylated head domain was vacuum-dried, resuspended in 50 mM Tris-Cl (pH 7.5), and treated with 1.50 (w/v) 1,1-tosylamide-2-phenylethyl chloromethyl ketone-treated trypsin (Sigma) at 37 °C for 8 h. The samples were treated identically for an additional 8 h. The obtained peptides were fractionated by HPLC on the Zorbax C8 column as described above. All the chromatographies were performed at room temperature with a flow rate of 0.8 ml/min and a fraction size of 0.8 ml.

**Amino Acid Sequence Analysis and Phosphoamino Acid Analysis of Tryptic Peptides**—Amino acid sequences of each phosphopeptide were analyzed using an ABI 476A gas-phase sequencer. To determine at which position on vimentin each peptide exists, the sequences were then compared with the published amino acid sequence predicted from mouse vimentin cDNA (40).

**Peptide Synthesis and Production of Anti-PV71 Antibody (GK71)**—Vimentin peptides PV71 (Cys-Ala-Val-Arg-Leu-Arg-phospho-Ser71-Ser-Val-Pro-Gly-Val), PV6 (Cys-Ser-Thr-Arg-Val-Arg-phospho-Ser71-Ser-Val-Pro-Gly-Val), PV24 (Cys-Ser-Thr-Arg-Val-Arg-phospho-Ser46-Ser-Asn-Arg-Ser-Tyr), PV33 (Cys-Ser-Thr-Arg-Val-Arg-phospho-Ser46-Ser-Asn-Arg-Ser-Tyr), PV38 (Cys-Ser-Thr-Arg-Val-Arg-phospho-Ser46-Ser-Asn-Arg-Ser-Tyr), PV41 (Cys-Thr-Tyr-Arg-Leu-Val-Arg-phospho-Ser41-Ser-Val-Pro-Gly-Val), PV46 (Cys-Ala-Val-Arg-Leu-Arg-phospho-Ser71-Ser-Val-Pro-Gly-Val), PV50 (Cys-Pro-Ser-Thr-Arg-Val-Arg-phospho-Ser46-Ser-Asn-Arg-Ser-Leu), PV55 (Ser-Leu-Tyr-Ser-Arg-phospho-Ser82-Val-Asp-Ser-Leu), and PV82 (Cys-Ala-Val-Leu-Gln-Asp-phospho-Ser82-Val-Asp-Phe-Ser-Leu) were synthesized and purified as described previously (11). Antibodies against PV71 were prepared by injecting four rabbits with PV71 coupled to keyhole limpet hemocyanin. Monospecific antibodies against PV71 were purified from the obtained serum by two-step chromatography: affinity chromatography on PV71-coupled Cellulose (Seikagaku Corp.) and then absorption in V71-coupled Cel-

**FIG. 1. Phosphorylation of vimentin by Rho-kinase.** A, vimentin was phosphorylated by Rho-kinase (0.5 μg/ml) in the presence of either GST (a), GDP-GST-RhoA (b), or GTPyS-GST-RhoA (c), or by GST-Rho-kinase (1.0 μg/ml) (d). Radiolabeled bands were visualized by autoradiography. After staining with Coomassie Brilliant Blue, each band of vimentin was cut from the gel, and each radioactivity was measured in 22P Beckman liquid scintillation counter. The values in parentheses represent fold stimulation relative to Rho-kinase in the presence of GST. The position of vimentin is indicated (arrowhead). The positions of molecular size standards (in kilodaltons) are indicated on the left. B, tryptic phosphopeptide mapping of vimentin phosphorylated by either Rho-kinase in the presence of GTPyS-GST-RhoA (c) or GST-Rho-kinase (d). Each radioactive vimentin was eluted from the gel strip described above (a or a-d), precipitated with trichloroacetic acid, oxidized with performic acid, and digested with trypsin as described previously (41). Each trypsic peptide was loaded onto a thin layer cellulose gel plate (horizontal dimension, electrophoresis with formic acid/acetic acid/H2O (25:78:897) at 1500 V for 40 min; vertical dimension, ascending chromatography in 2-propanol/acetonitrile (7:3) containing 0.1% trifluoroacetic acid. The peptides were eluted with a 60-min linear gradient of 5–50% (v/v) 2-propanol/acetonitrile, followed by a further 10-min linear gradient of 50–80% (v/v) 2-propanol/acetonitrile. All radioactivity loaded was recovered in a single peptide (a 12-kDa fragment from the amino-terminal domain of vimentin) by the retention time of 65–68 min. This phosphorylated head domain was vacuum-dried, resuspended in 50 mM Tris-Cl (pH 7.5), and treated with 1.50 (w/v) 1,1-tosylamide-2-phenylethyl chloromethyl ketone-treated trypsin (Sigma) at 37 °C for 8 h. The samples were treated identically for an additional 8 h. The obtained peptides were fractionated by HPLC on the Zorbax C8 column as described above. All the chromatographies were performed at room temperature with a flow rate of 0.8 ml/min and a fraction size of 0.8 ml.
Phosphorylation of Vimentin by Rho-kinase

**Fig. 2**. The effect of domain-specific phosphorylation by GST-Rho-kinase on the formation of vimentin filament. A, time course of phosphorylation of vimentin by GST-Rho-kinase. B, vimentin (Vim.) phosphorylated by GST-Rho-kinase was incubated in the presence (a) or absence (b) of lysyl-endopeptidase and subjected to SDS-PAGE. Radiolabeled bands were visualized by autoradiography. The positions of vimentin and the head domain of vimentin are indicated (arrowheads). The phosphorylation reaction was performed in the absence (control) or the presence (GST-Rho-kinase) of GST-Rho-kinase. The samples were then incubated with 100 mM NaCl at 37 °C for further 60 min. C, after the incubation, the samples were subjected to high speed centrifugation (12,000 × g). The supernatant (s) and the precipitate (p) were subjected to SDS-PAGE, and the gel was stained with Coomassie Brilliant Blue. D, after the incubation, the samples were placed directly on carbon film-coated specimen grids, stained with 2% uranyl acetate, and subjected to the electron microscopy. Bar, 200 nm.

Protein concentrations were determined by absorbance at 280 nm using rabbit IgG (Sigma) as a standard. This anti-PV71 antibody (referred to as GK71) obtained from one of the rabbits was used for the following experiments. Immunoblotting was performed as described previously (6), using horseradish peroxidase-conjugated secondary antibodies and the ECL Western blotting detection system (Amersham Pharmacia Biotech).

**Transfection**—The pEF-BOS-myc mammalian expression plasmids encoding the catalytic domain of bovine Rho-kinase (CAT; amino acids 6–553) and the catalytic domain mutated at the ATP-binding site (CAT-KD; Lys121 → Gly) were constructed as described previously (43). COS-7 cells (obtained from RIKEN Cell Bank) were plated at a density of 1.5 × 10⁵ cells per 35-mm dish. After culturing for 1 day, cells were transfected with 2 μg of the plasmid DNA by the application of SuperFect-DNA complexes (Qiagen). At 2 h, Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum was added, and the cells were cultured for another 24 h. These cells were used for immunoblotting and immunofluorescence studies.

**Immunofluorescence Microscopy**—Cells growing on glass coverslips were fixed with 3.7% formaldehyde in ice-cold PBS for 10 min and then treated with methanol at −20 °C for 10 min. For double immunostaining with GK71 and DM1A, cells were fixed with 3.7% formaldehyde in PBS for 10 min at 37 °C and then permeabilized with 0.1% Triton X-100 in PBS for 10 min at room temperature. Incubation with primary antibodies diluted in PBS containing 1% sucrose and 1% bovine serum albumin was for 2 h at 37 °C. After three washes with PBS, cells were incubated for 1 h with appropriate secondary antibodies diluted 1:100 and subsequently washed with PBS. Then DNAs were stained with 0.5 μg/ml propidium iodide (Sigma) or 0.5 μg/ml DAPI (Boehringer Mannheim) for 10 min at room temperature.

The following antibodies were used for indirect immunofluorescence microscopy: GK71 (anti-phospho-Ser⁷¹ of vimentin) rabbit polyclonal antibody diluted 1:100; 1B8 (anti-vimentin) mouse mAb (11) diluted 1:2; 9E10 (anti-Myc, from Babco) mouse mAb diluted 1:200; DM1A (anti-a-tubulin, from Sigma) mouse mAb diluted 1:500; fluorescein isothiocyanate-conjugated goat anti-rabbit or anti-mouse immunoglobulins (BioSource International, Camarillo, CA); and Texas Red-conjugated sheep anti-mouse immunoglobulins (Amersham Pharmacia Biotech).

Fluorescently labeled cells were examined either with an Olympus BH2-RFCA microscope or an Olympus LSM-GB200 confocal microscope.

Preparation of Interphase, Early Mitotic, and Late Mitotic Cell Ly-
Phosphorylation of Vimentin by Rho-kinase

**RESULTS**

Phosphorylation of Vimentin by Rho-kinase—We examined whether Rho-kinase purified from bovine brain can phosphorylate vimentin in a GTP-Rho-dependent manner. GTPγS-GST-RhoA enhanced the phosphorylation of vimentin by Rho-kinase about 58-fold relative to GST, about 26-fold relative to GDP-GST-RhoA (Fig. 1A). The recombinant Rho-kinase (GST-Rho-kinase), which is constitutively active, also phosphorylated vimentin (Fig. 1A). The pattern of tryptic phosphopeptide mapping of vimentin phosphorylated by GST-Rho-kinase was identical to that by purified Rho-kinase in the presence of GTPγS-GST-RhoA (Fig. 1B). This result suggests that catalytic characteristics of GST-Rho-kinase are similar to those of native Rho-kinase activated by GTPγS-bound RhoA.

The level of the vimentin phosphorylation by GST-Rho-kinase increased in a time-dependent manner and was approximately 2.5 mol of phosphate/mol of protein at 2 h (Fig. 2A). To investigate which structural domain of vimentin is phosphorylated, the radiolabeled protein was digested with lysyl-endopeptidase. SDS-PAGE analysis revealed that almost all the radioactive head domain of vimentin was obtained by the treatment with lysyl-endopeptidase. This head domain purified by reverse-phase HPLC was digested with trypsin, and the resulting peptides were again separated by reverse-phase HPLC. As shown in Fig. 3A, three major radioactive peptides, RV1 to RV3, were obtained. All of these peptides were phosphorylated at serine residues, as determined by two-dimensional phosphoamino acid analysis (Fig. 3B). The phosphoserine-containing peptides were then sequenced after ethanethiol treatment which specifically converts phosphoserine into S-ethylcysteine. The generation of S-ethylcysteine at a particular Edman degradation cycle where serine is predicted provides a definitive way to locate the phosphoserine residue(s) on each peptide. The lack of generation of S-ethylcysteine indicates that phosphoserine is located in the amino-terminal serine residue as there is no conversion of the amino-terminal phosphoserine to S-ethylcysteine (42). Fig. 3C shows that phosphates of RV1, RV2, and RV3 peptides were located on Ser71, Ser71, and Ser38, respectively. As shown in Fig. 3C and Table I, RV1 peptide was the complete digestion product of RV2 peptide. Phosphates at Ser71 and Ser38 accounted for about 41.7 and 23.3% of those on vimentin phosphorylated by GST-Rho-kinase, respectively (Table I). As shown in Fig. 4A and Table I, vimentin-Ser71 was the most favored and unique phosphorylation site for Rho-kinase.

Production and Characterization of the Site- and Phosphorylation State-specific Antibody for Vimentin-Ser71—Since vimentin-Ser71 is the phosphorylation site specific to Rho-kinase among known vimentin kinases in vitro (Fig. 4A), this residue can serve as a pertinent indicator to study in vivo vimentin phosphorylation by Rho-kinase. Thus we prepared a rabbit polyclonal antibody (referred to as GK71), raised against the synthetic phosphopeptide PV71 (phosphovimentin-Ser71; Cys-Ala-Val-Arg-Leu-Arg-phospho-Ser71-Val-Prol-Gly-Val) (Fig. 4A). In Fig. 4, B and C, the specificity of GK71 was examined by Western blotting. GK71 reacted with vimentin phosphorylated by Rho-kinase but not with nonphosphorylated vimentin or vimentin phosphorylated by protein kinase A, protein kinase C, Cdk2 kinase, or CaM kinase II (Fig. 4B). As shown in Fig. 4C, the immunoreactivity of GK71 for vimentin phosphorylated by Rho-kinase was neutralized by preincubation with the phosphopeptide PV71 but not with the nonphosphopeptide V71 (Cys-Ala-Val-Arg-Leu-Arg-Ser71-Ser-Val-Pro-Gly-Val) or other phosphopeptides such as PV6, PV24, PV33, PV38, PV41, PV46, PV50, PV55, PV65, and PV82 (which were designed to represent vimentin phosphorylated at other sites, Ser6, Ser24, Ser33, Ser38, Ser41, Ser46, Ser50, Ser55, Ser65, and Ser82, respectively). These results indicate that GK71 specifically recognizes the phosphorylation of vimentin at Ser71 by Rho-kinase.

**Phosphorylation of Vimentin-Ser71 by Ectopic Expression of Active Rho-kinase in COS-7 Cells**—To examine whether Rho-kinase can phosphorylate vimentin at Ser71 in cells, we next analyzed the phosphorylation of vimentin-Ser71 in monkey kidney epithelial (COS-7) cells which ectopically express the active form of Rho-kinase. Cells were transfected with the pEF-BOS vector, or the pEF-BOS-myc plasmid carrying the catalytic domain of Rho-kinase (CAT), or the catalytic domain mutated at the ATP-binding site (CAT-KD). Expression levels of the
Myc epitope-tagged CAT and CAT-KD were almost the same, and endogenous vimentin was equally expressed in the three types of transfected cells (Fig. 5A). However, immunoblot analysis using GK71 revealed that the phosphorylation of vimentin-Ser\(^{71}\) occurred only in lysates from CAT-expressing cells (Fig. 5A). Immunofluorescence analysis of the transfected cells is shown in Fig. 5B. The phosphorylation of vimentin-Ser\(^{71}\) was observed in cells expressing Myc-tagged CAT but not in cells expressing Myc-tagged CAT-KD. These results demonstrate that constitutively active Rho-kinase can phosphorylate vimentin at Ser\(^{71}\) in intact cells.

We often observed abnormal vimentin filament networks in COS-7 cells which ectopically expressed CAT (Fig. 5C). In some cases, fiber bundles or granulates of phospho-vimentin were observed in these cells. Together with the data shown in Fig. 2, C and D, these results may suggest that the phosphorylation by Rho-kinase induces dynamic changes in vimentin-IF organization.

Specific Phosphorylation of Vimentin-Ser\(^{71}\) at the Cleavage Furrow during Cytokinesis—Fig. 6 shows U251 glioma cells immunostained with 1B8 or GK71. 1B8, which reacts with both the phosphorylated and unphosphorylated forms of vimentin...
stained with 1B8 and GK71. Bars

contracepted during cytokinesis, Western blot analysis of U251 cell
lysates was carried out. As shown in Fig. 8, GK71-immunoactive band at 57 kDa corresponding to the position of vimentin was detected only in the late mitotic cell lysate. No GK71-immunoreactive band was detected in the lysate of interphase or metaphase cells. These results strongly suggest that the immunostaining with the antibody GK71 during late mitotic phase represents the presence of phospho-Ser71 of vimentin specifically at the cleavage furrow.

We then examined the spatial distribution of vimentin-Ser71 phosphorylation at the cleavage furrow. Immunocytochemical analysis with GK71 using confocal laser scanning microscopy revealed that vimentin phosphorylated at Ser71 was associated with the cleavage furrow to form a ring-like structure (Fig. 9A) and was localized at the outside of spindle microtubules in the cleavage furrow (Fig. 9B).

To examine whether or not phosphorylation of vimentin-Ser71 is generally observed at the cleavage furrow during cytokinesis, various cell lines were stained with the antibody GK71, as shown in Fig. 10 (A, Ltk− mouse fibroblastic cell; B, Swiss 3T3 mouse fibroblastic cell; C, Madin-Darby canine kidney epithelial cell; D, COS-7 monkey kidney epithelial cell). GK71 reacted with vimentin only at the cleavage furrow during cytokinesis in these cells. Therefore, we believe that vimentin-Ser71 phosphorylation at the cleavage furrow during cytokinesis is a general feature in vimentin-expressing cells.

**DISCUSSION**

In the present study, we obtained evidence that Rho-kinase phosphorylates vimentin in a GTP-Rho-dependent manner and that the phosphorylation of vimentin by Rho-kinase prevents its filament formation in vitro. Vimentin-Ser71, which was identified here as the phosphorylation site specific to Rho-kinase in vitro, was shown to be specifically phosphorylated at the cleavage furrow during cytokinesis.

One of the dynamic changes in cellular morphology during mitosis is the reorganization of three major cytoskeletal structures, microfilaments (actin filaments), microtubules, and intermediate filaments (IFs). Two distinct cytoskeletal structures, a bipolar mitotic spindle and a contractile ring, appear transiently and play active roles in the mitotic phase of animal cells (for review, see Refs. 44 and 45). 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 (for review, see Refs. 46 and 47).

Unlike microtubules and actin filaments which are largely reorganized for the specific mitotic functions described above, the behavior of IFs during mitosis differs depending on cell types. Rosevear et al. (48) reported changes of IF network in baby hamster kidney (BHK-21) cells during different stages of mitosis. During prometaphase/metaphase, the typical network of long 10-nm diameter IFs characteristic of interphase cells disassembled into aggregates containing short 4–6-nm filaments. During anaphase/telophase, arrays of short IFs appeared throughout cytoplasm, and in cytokinesis, the majority of IFs was longer and concentrated mainly in a juxtanuclear cap. Franke et al. (49, 50) observed punctate or granular structures of IFs even during cytokinesis in some types of cells. However, IFs of many types of cells appeared to be interpreted as intact bundles in the plane of the cleavage furrow during cytokinesis (51–54). There seems to be a mechanism that accounts for the locally controlled breakdown of the filaments before the final separation of daughter cells. In vitro studies revealed that the site-specific phosphorylation of IF proteins by several kinases induced disassembly of the filament structure (for review, see Ref. 5). Protein kinase A, protein kinase C, CaM

![Fig. 5. Phosphorylation of vimentin-Ser71 in COS-7 cells overexpressing Rho-kinase mutants.](http://www.jbc.org/)
kinase II, and Cdc2 kinase have been known as such kinases. In a previous study (37) and in the present study, we demonstrated that Rho-kinase also acts as an in vitro IF kinase which induces alterations in the filament structure.

Identifying protein kinases responsible for in vivo IF phosphorylation is of great importance in order to understand how cellular IF reorganization is regulated. As a method for the identification of in vivo IF kinases, we have utilized site- and phosphorylation state-specific antibodies (for review, see Ref. 8). Among the in vitro phosphorylation sites, there are sites specifically phosphorylated by a single kinase. For example, Ser33, Ser55, and Ser82 on vimentin are site-specific for protein kinase C, Cdc2 kinase, and CaM kinase II, respectively (Fig. 4A). These specific sites can serve as a pertinent indicator for the detection of in vivo IF phosphorylation by the kinase. To determine whether Cdc2 kinase phosphorylates vimentin in vivo, a monoclonal antibody 4A4 that recognizes the phosphorylation of Ser55 on vimentin was produced (11). Ser 55 was phosphorylated in various types of cells only during early mitotic phase, and the chromatographic analysis of mitotic cell lysates revealed a single peak of Ser55 kinase activity that is identical to Cdc2 kinase. These data together with data obtained by tryptic phosphopeptide analysis (55) strongly suggest that Cdc2 kinase directly phosphorylates vimentin in mitotic cells. By using antibodies recognizing the phosphorylation of the distinct specific sites on vimentin, we further identified protein kinase C and CaM kinase II as in vivo vimentin kinases that act during cell cycle and cell signaling, respectively (12–14). Here, we have identified Ser71 on vimentin as a unique site for Rho-kinase in vitro. By producing and utilizing the site- and phosphorylation state-specific antibody GK71 which recognizes the phosphorylation of Ser71, we have demonstrated that vimentin-Ser71 is specifically phosphorylated at the cleavage furrow during cytokinesis. This observation suggests the possibility that Rho-kinase may be responsible for the cleavage furrow-specific phosphorylation of vimentin.

Rho was reported to be translocated from the cytosol to the cleavage furrow (56) and to play a critical role in inducing and maintaining the contractile ring during cytokinesis (22–24). We recently found that Rho-kinase is also translocated to the
These accumulating observations allow us to speculate on the possible mechanism regulating cytokinesis. The GTP-bound active form of Rho concentrated at the cleavage furrow may bind to and activate its specific targets around the cleavage furrow. Rho-kinase may phosphorylate several proteins including vimentin specifically at the cleavage furrow. Since phosphorylation of GFAP at Thr7, Ser13, and Ser34 was also observed at the cleavage furrow (9, 10) and these three sites were phosphorylated by Rho-kinase in vitro (37), Rho-kinase might also phosphorylate GFAP during cytokinesis. The cleavage furrow-specific phosphorylation of vimentin and GFAP might contribute to the efficient separation of these IF structures and allow the cleavage furrow to contract unencumbered by continuous filaments. So far, myosin binding subunit of myosin phosphatase (31) and myosin light chain (39) have been identified as other putative substrates for Rho-kinase. Phosphorylation of these proteins by Rho-kinase resulted in activation of myosin ATPase by actin (31, 39). Therefore, there is a possibility that Rho-kinase may also play an important role in the contraction and the formation of the contractile ring through the phosphorylation of these proteins.

Rho is known to regulate the assembly of focal adhesions and actin stress fibers in response to extracellular signals, such as lysophosphatidic acid (15). Rho-kinase was recently reported to act downstream of Rho in the regulation of the formation of stress fibers and focal adhesion complexes (43, 57, 58). These studies suggested that Rho may activate Rho-kinase to control actin filament reorganization in response to extracellular signals during interphase. However, vimentin phosphorylation at Ser71 did not occur in interphase cells cultured in the presence of the serum, which contains lysophosphatidic acid (Fig. 7). Furthermore, the phosphorylation of vimentin-Ser71 was not observed when quiescent serum-starved Swiss 3T3 cells were stimulated by lysophosphatidic acid. Why was vimentin phosphorylated by constitutively active Rho-kinase in COS-7 cells but not by endogenous activated Rho-kinase in interphase cells? One possible explanation is the compartmentalized distribution of activated Rho-kinase in the cell. Since Rho-kinase in the cytoplasm is thought to be translocated to membranes by forming a complex with GTP-bound Rho (33, 34), endogenous activated Rho-kinase might be kept apart from cytoplasmic vimentin-IFs and unable to phosphorylate vimentin in interphase cells. Definitive mechanism governing the cleavage furrow-specific phosphorylation of vimentin at Ser71 remains unclear, but the contractile force might partly contribute to the interaction between cytoplasmic vimentin-IFs and membrane-bound active Rho-kinase at the cleavage furrow.

Since Rho-kinase belongs to a family of related serine/threonine kinases including myotonic dystrophy kinase, these kinases may phosphorylate the similar sites on vimentin and GFAP. Further investigations are necessary to elucidate the relationship between IF phosphorylation at the cleavage furrow and Rho-kinase or its family members during cytokinesis.

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2 H. Kosako and M. Inagaki, unpublished observations.

3 H. Goto, H. Kosako, and M. Inagaki, unpublished observations.
Phosphorylation of Vimentin by Rho-associated Kinase at a Unique Amino-terminal Site That Is Specifically Phosphorylated during Cytokinesis
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