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Rho-Associated Kinase Phosphorylates Desmin, the Myogenic Intermediate Filament Protein, at Unique Amino-Terminal Sites

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We obtained evidence that Rho-associated kinase (Rho-kinase) phosphorylates desmin, the myogenic intermediate filament protein, with approximately 2 mol phosphate per mole of desmin *in vitro*. Desmin phosphorylated by Rho-kinase lost the potential to form 10-nm filaments. Thr-16, Thr-75, and Thr-76 on desmin proved to be the major phosphorylation sites for Rho-kinase. All these sites are located within the head domain and are different from the reported phosphorylation sites of protein kinase A, protein kinase C, and cdc2 kinase. We are entertaining the notion that Rho-kinase may regulate filament structures of desmin by site-specific phosphorylation.

It is generally agreed that myosin light chain (MLC) phosphorylation plays an important role in initiating smooth muscle contraction. MLC is phosphorylated at serine 19 by a Ca^{2+}-calmodulin-dependent MLC kinase pathway, thereby increasing the actin-activated ATPase activity of myosin. Smooth muscle contraction is determined not only by the level of intracellular free Ca^{2+} but also by a GTP-dependent mechanism that enhances contraction at a fixed concentration of calcium. This GTP-dependent mechanism is known as GTP-induced increase in the force/Ca^{2+} ratio, so-called calcium sensitization (1–4). Recently, it was reported that Rho-kinase (also called ROK or $p160^{ROCK}$) is likely to be one of targets for Rho, a small GTP-binding protein (5–7). Rho-kinase can induce smooth muscle contraction, calcium-independently, by directly phosphorylating MLC on serine 19 and by phosphorylating and inhibiting the myosin binding subunit (MBS) of myosin phosphatase (8–11).

Intermediate filaments (IFs) are major components of the cytoskeleton and nuclear envelope, in most types eukaryotic cells. IF proteins undergo a dramatic reorganization of their structure by phosphorylation (12–14). Desmin, one of type III IF proteins, is restrictedly displayed in smooth, cardiac, and skeletal muscle cells. When Rho-kinase induces Ca^{2+}-independent contraction of smooth muscle, not only the phosphorylation of MLC but also phosphorylation of desmin occurs (15). We examined the effect of phosphorylation by this kinase on organization of desmin filaments. The evidence we obtained shows that Rho-kinase stoichiometrically phosphorylates desmin, in a GTP-Rho-dependent manner at unique amino-terminal sites and that functional alteration ensues *in vitro*.

MATERIALS AND METHODS

Proteins. Full-length human desmin cDNA was isolated from a human heart cDNA library (STRATAGENE) using PCR, and subcloned to expression vector pET3a. Recombinant human desmin expressed in *E. coli* was prepared as described (16). Rho-kinase was purified from bovine brain (5), GST–Rho A was purified and loaded with guanine nucleotides (17) and GST–Rho-kinase was purified from Sf9 cells as described (10).

Phosphorylation of desmin. Desmin (120 μg/ml) was phosphorylated in a 20-μl reaction mixture by incubation with 25 mM Tris–HCl (pH 7.5), 0.2% Chaps, 4 mM $MgCl_2$, 3.8 mM EDTA, 100 μM [γ-^{32}P]ATP, 0.1 μM calyculin A, and 0.5 μg/ml purified Rho-kinase in the presence of either GST, GDP · GST–RhoA, or GTPγS · GST–RhoA (each 1 μM) for 30 min at 25°C. The phosphorylation reaction for GST–Rho-kinase was performed in 20 μl of desmin, 25 mM Tris–HCl (pH 7.5), 0.2 mM $MgCl_2$, 100 μM [γ-^{32}P]ATP, 0.1 μM calyculin A, and 11 μg/ml GST–Rho-kinase at 25°C.

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Abbreviations used: MLC, myosin light chain; MBS, myosin binding subunit of myosin phosphatase; IF, intermediate filament; GST, glutathione *S*-transferase; GTPγS, guanosine 5′-(3-*O*-thio)-triphosphate; Chaps, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography.

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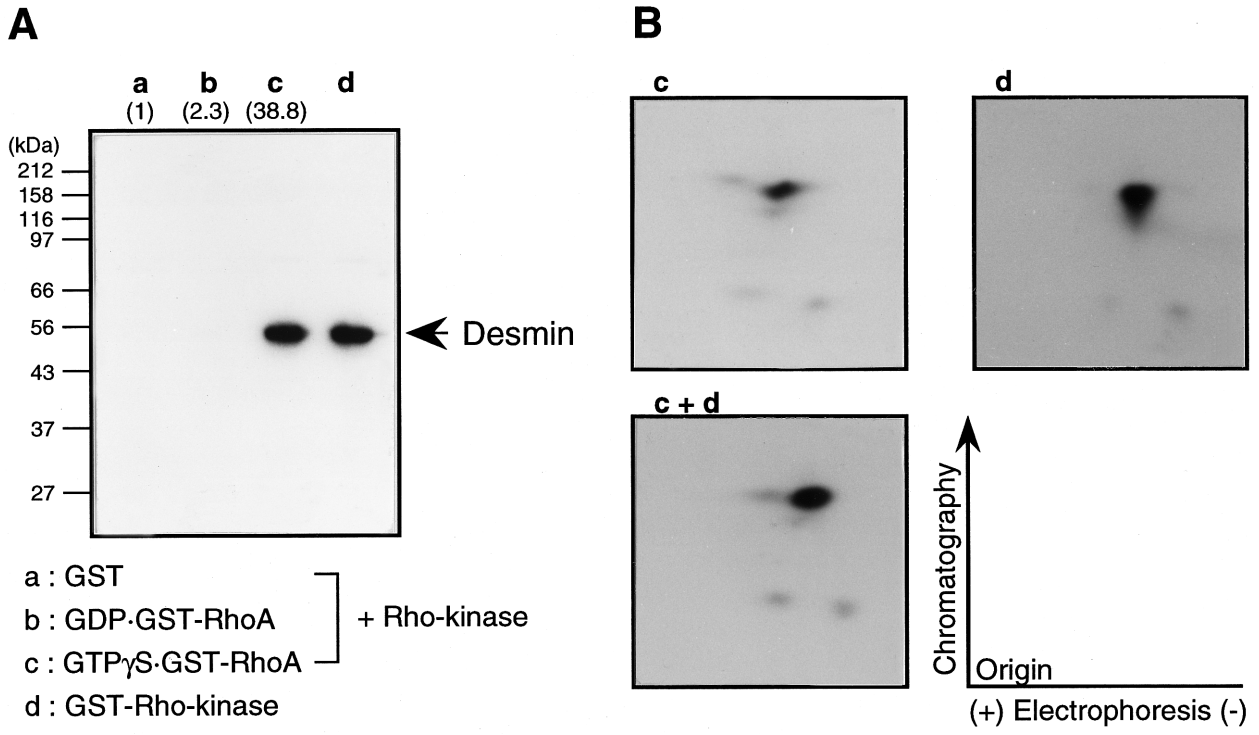

FIG. 1. Phosphorylation of desmin by Rho-kinase. (A) Phosphorylation of desmin by Rho-kinase and by constitutive active GST–Rho-kinase, *in vitro,* and as described under Materials and Methods. GST alone (a), GDP · GST–RhoA (b), GTPγ · GST–RhoA (c), and 11 μg/ml of GST–Rho-kinase (d). Each phosphorylated desmin was examined using autoradiography and radioactivity was measured in a ^{32}P Beckman liquid scintillation counter. (B) Phosphopeptide maps of desmin phosphorylated by Rho-kinase in the presence of GTPγ · GST–RhoA (c) and by GST–Rho-kinase (d). Two-dimensional peptide mapping was carried out as described under Materials and Methods.

Two-dimensional peptide mapping. Two-dimensional peptide mapping of radioactive tryptic phosphopeptides was carried out, as described (18). Desmin was phosphorylated by Rho-kinase in the presence of GTPγS · GST–RhoA and GST–Rho-kinase for 30 min at 25°C, as described above. The radioactive desmin was isolated by SDS–PAGE, eluted from the gel, and digested with trypsin. Radioactive tryptic phosphopeptides were then subjected to peptide mapping, using cellulose thin-layer plates. The first dimension was electrophoresis with acetic acid/formic acid/H_20 (78/25/897) at 1500 V. And the second was chromatography in *n*-butyl alcohol/pyridine/acetic acid/H_2O (15/10/3/12). Following the mapping, the plates were visualized using autoradiography.

Assembly of desmin and electron microscopy. Desmin was phosphorylated in the presence or absence of GST–Rho-kinase for 120 min at 25°C. Samples were then incubated with 100 mM NaCl for 60 min at 25°C, then were subjected to high speed centrifugation (12,000*g*) and supernatant(s) and precipitate(s) were subjected to SDS–PAGE. After incubation, the samples were placed directly on carbon film-coated specimen grids and stained with 2% uranyl acetate.

Purification of the phosphopeptide. After phosphorylation for 2 h at 25°C, desmin was digested overnight with endoproteinase Glu-C (Sigma) in 100 mM phosphate buffer containing 4 M urea, at 37°C. The phosphorylated peptides were purified by HPLC on a Zorbax C8 (0.46 × 25 cm) reverse-phase column. The purified fragments were dissolved in 50 mM Tris–HCl (pH 8.0) and treated for 4 hours with L-1-tosylamide-2-phenylethyl chloromethyl ketone-treated trypsin (Sigma, 1:50 (w/w) of desmin), at 37°C. Fresh trypsin was added and the mixture was then incubated overnight. Trypsin-digested peptides of phosphorylated desmin were separated by reverse-phase HPLC on Zorbax C8 column using 60 min linear gradient of 5–50% (v/v) 2-propanol/acetonitrile, followed by a further 20-min linear gradient at 50–80% (v/v) 2-propanol/acetonitrile.

Phosphoamino acid analysis and amino acid sequence analysis. Phosphoamino acid analyses of the radioactive peptides were performed as described (18). Amino acid sequences were determined using an ABI 476A gas-phase sequencer. The site of phosphorylation in the phosphopeptide was determined as described (19). After the sequencing reached cycles corresponding to the phosphorylated residue, the inorganic phosphate (Pi) and phosphopeptide were separated by electrophoresis at pH 3.5 on a cellulose thin-layer plate.

RESULTS AND DISCUSSION

We attempted to determine if desmin could be phosphorylated by the Rho-kinase purified from bovine brain, *in vitro.* The level of phosphorylation was enhanced about 2.3-fold by GDP · GST–RhoA, and by GTPγS · GST–RhoA about 38.8-fold relative to GST (Fig. 1A). GST–Rho-kinase, constitutively active Rho-kinase, could also phosphorylate desmin. When desmin was phosphorylated by Rho-kinase in the presence of GTPγS · GST–RhoA and GST–Rho-kinase, the same pattern was obtained following two-dimensional peptide mapping (Fig. 1B). Thus, we used GST–Rho-kinase for the following experiments.

The time course of phosphorylation of desmin by GST–Rho-kinase is shown in Fig. 2A. The level of phos-

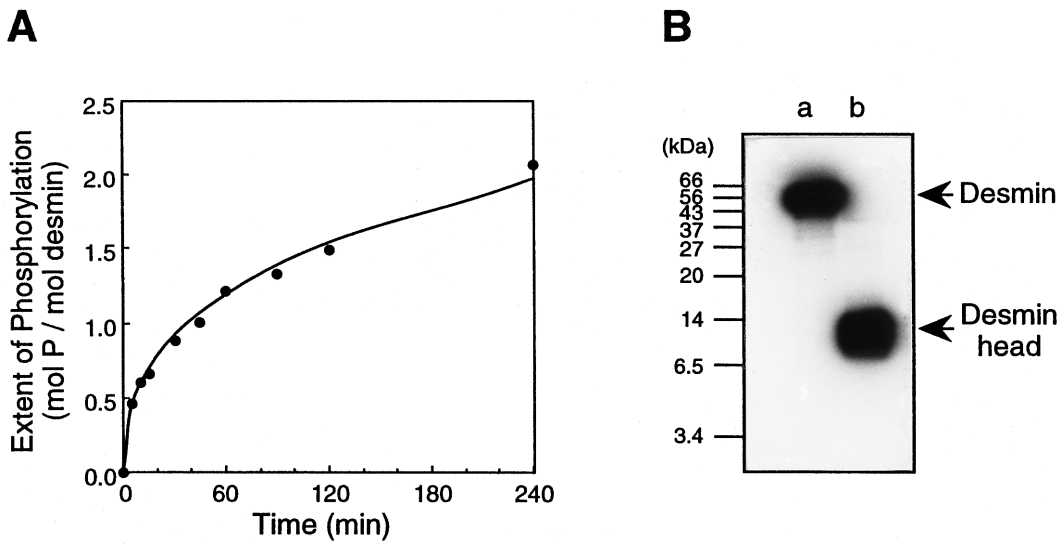

FIG. 2. Time course of domain-specific phosphorylation of desmin by GST–Rho-kinase. (A) Desmin was phosphorylated by GST–Rho-kinase for various times and the samples were subjected to SDS–PAGE. The radioactive desmin was counted for ^{32}P in a Beckman liquid scintillation counter. (B) Phosphorylated desmin was treated in the presence (b) or absence (a) of endoproteinase Glu-C, and samples were subjected to SDS–PAGE and autoradiography. Arrows indicate desmin and the head domain of desmin.

phorylation increased in a time-dependent manner. GST–Rho-kinase incorporated about 2 mol of phosphate into 1 mol of desmin molecule at the end of 4 h. We then determined which domain(s) of desmin were phosphorylated by GST–Rho-kinase. For this, radiolabeled desmin was digested with endoproteinase Glu-C.

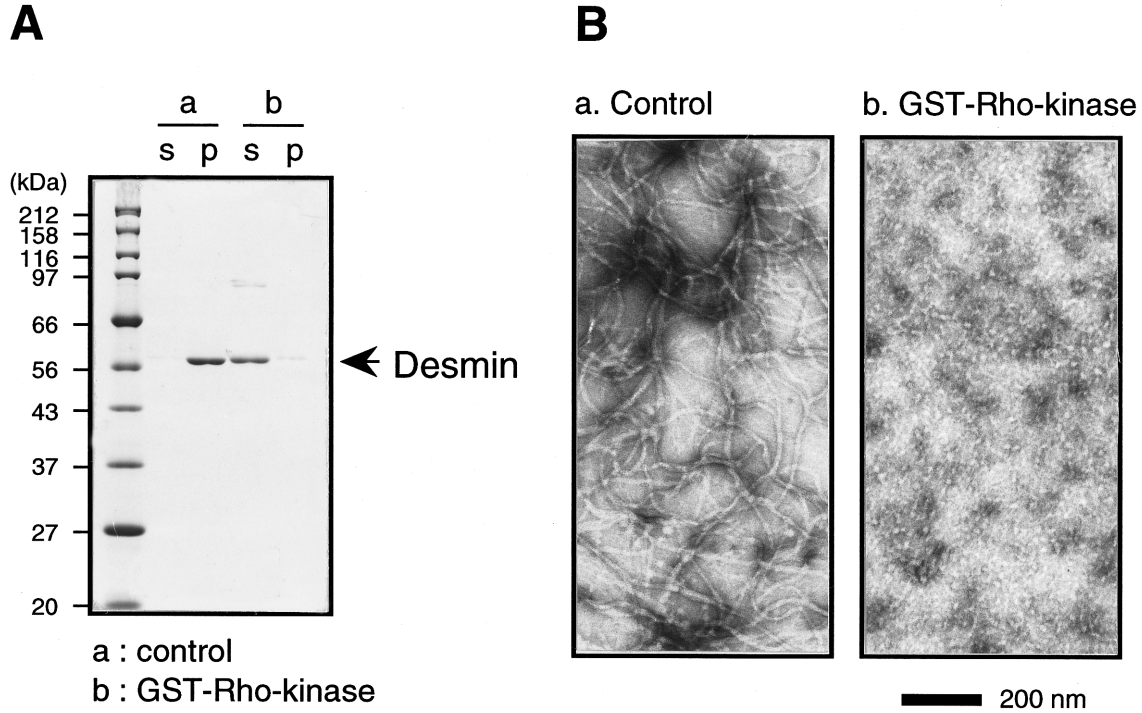

FIG. 3. Effect of phosphorylation by GST–Rho-kinase on the filament forming potential of desmin. (A) Fractionation of soluble desmin and desmin filament. After desmin was phosphorylated with (b) or without (a) GST–Rho-kinase for 120 min at 25°C, the samples incubated with 100 mM NaCl for further 60 min at 37°C and subjected to high speed centrifugation (12,000*g*). The supernatant (s) and the precipitate (p) were then subjected to SDS–PAGE. (B) Electron microscopy of negatively stained desmin samples was performed as described under Materials and Methods. Scale bar = 200 nm, magnification ×20,000.

SDS–PAGE analysis revealed that almost all the radioactivity in the phosphorylated desmin was recovered in the 8.8-kDa amino-terminal head domain (Fig. 2B), which means that the phosphorylation sites were restricted to only the head domain of desmin.

To examine the effect of phosphorylation by GST–Rho-kinase on desmin filament formation, soluble desmin was preincubated for 2 h with or without GST–Rho-kinase, then the samples were incubated under conditions of filament formation (100 mM NaCl at 25°C) for a further 1 h. Analyses of these samples by centrifugation (Fig 3A) and by electron microscopy (Fig. 3B) revealed that the phosphorylation of desmin by Rho-kinase dramatically inhibited filament formation.

To identify the phosphorylation sites, the radioactive head domain of desmin was completely digested with trypsin, and the phosphopeptides were purified by reverse-phase HPLC. Evidence for the major radioactive phosphopeptides, DR1 and DR2, is shown in Fig. 4A. Phosphoamino acid analysis of these phosphopeptides showed that these peptides were phosphorylated exclusively at threonine residues (Fig. 4B). The phosphopeptides, DR1 and DR2, were then analyzed for gas-phase Edman degradation. The amino acid sequence of DR1 was Leu-Gly-Thr-Thr-Arg, and that of DR2 was Thr-Phe-Gly-Gly-Ala-Pro-Gly-Phe-Pro-Leu-Gly-Ser-Pro-Leu-Ser-Ser-Pro-Val-Phe-Pro-Arg (Table 1). These phosphopeptides located at residues 73–77 (DR1), and 16–36 (DR2) of human desmin. The amount of phosphates at DR1 and DR2 accounted for about 54.1 and 18.8% of those on desmin phosphorylated by GST–Rho-kinase (Table 1). As the DR2 phosphopeptide contained only one threonine residue, we considered that Thr-16 at DR2 was phosphorylated by GST–Rho-kinase. On the other hand, DR1 contained two threonine residues, so we determined if Thr-75 or Thr-76 was phosphorylated by GST–Rho-kinase. When the N-terminal phosphoamino acid was cut out from the peptide on the PVDF membrane of the gas-phase sequencer, inorganic phosphate released from the phosphoamino acid was retained on the membrane (19). After extraction from the membrane, Pi and the partially degraded phosphopeptide were separated, and radioactivity was measured (Fig. 4C). If the Edman degradation reaction was completed at all steps, the Pi was released from Thr-75 at cycle 3 and from Thr-76 at cycle 4. The amount of Pi was 6551.8 cpm from cycle 3 and 16588.0 cpm from cycle 4, respectively. At least, Thr-75 could be phosphorylated by GST–Rho-kinase, because the radioactivity of Pi was dramatically increased between step 2 (129.0 cpm) and step 3 (6551.8 cpm). Although the release of Pi at step 4 might result from a carry-over of the Edman degradation, the total radioactivity of Pi from step 4 was approximately 2.5-fold relative to step 3, hence we considered that the total Pi at step 4 was released from

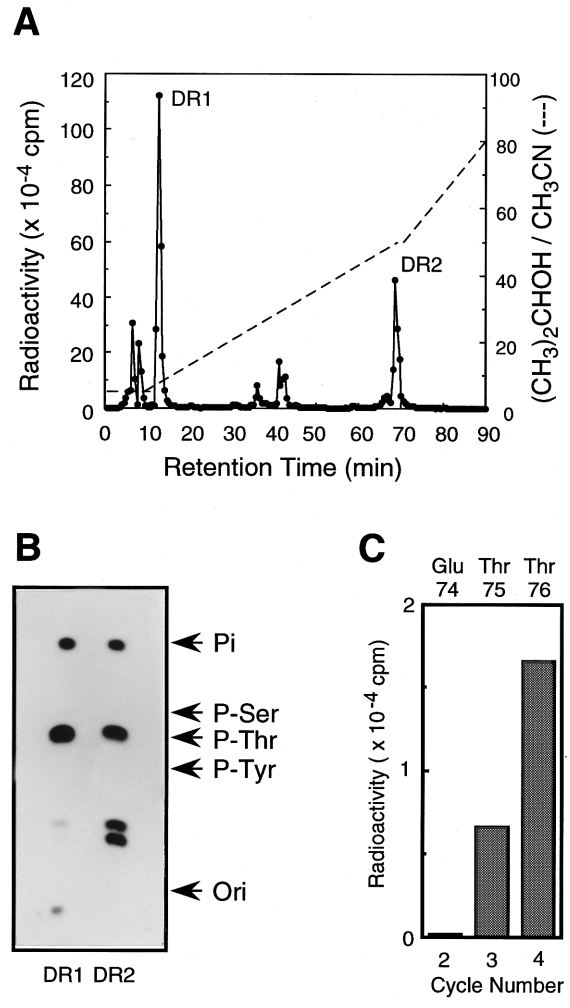

FIG. 4. Analysis of trypsin-digested fragments of phosphorylated desmin. (A) Purified amino-terminal head domain of phosphorylated desmin was treated with trypsin and generated fragments were separated by reverse-phase HPLC, as described under Materials and Methods. (B) Two radioactive peaks (DR1 and DR2 in A) were subjected to phosphoamino acid analysis. (C) Distribution of Pi into two threonine residues of the phosphopeptide (DR1, residues 73–77). The PVDF membrane was removed from the sequencer at cycles 2, 3, and 4. Pi and the degraded phosphopeptide were extracted from each membrane and separated by cellulose thin-layer gel plate electrophoresis. Radioactivity of Pi in each step was measured by BAS 2000 (Fuji). The amino acid released is given above each bar, Gly (residue 74) was released at cycle 2, Thr (residue 75) at cycle 3, and Thr (residue 76) at cycle 4 from DR1 (residues 73–77, sequence:LGTTR).

Thr-75 and Thr-76, and that Thr-76 could be phosphorylated by GST–Rho-kinase. Major phosphorylation sites of desmin by GST–Rho-kinase were Thr-16, Thr-75, and The-76.

Major sites phosphorylated by Rho-kinase located in the head domain, and all the sites differed from those for protein kinase A, protein kinase C and cdc2 kinase (20–22). The phosphorylation by each protein kinase led to disassembly of the desmin filaments (16, 22, and in this paper). Multi-site phosphorylation of desmin by

TABLE 1

Amino Acid Sequence of ^{32}P-Labeled Phosphopeptides of Desmin by GST–Rho–Kinase

Phosphopeptide	Amino acid sequence[a]	Phosphorylation site	Relative amount[b] of phosphate in peptide (% total)
DR 1	LG TT R (residues 73–77)	Thr-75, Thr-76	54.1
DR 2	T FGGAPGFPLGSPLSSPVFPR (residues 16–36)	Thr-16	18.8

Note. The phosphorylated residue(s) is underlined.
[a] Residue numbers in parentheses were determined from the amino terminal of human desmin.
[b] Determined from radioactivity in the HPLC analysis as described under Materials and Methods.

different protein kinases may increase the regulatory potential for organization of desmin filaments, in response to various physiological stimuli.

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