Communication

Phosphorylation of Glial Fibrillary Acidic Protein at the Same Sites by Cleavage Furrow Kinase and Rho-associated Kinase*

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Site- and phosphorylation state-specific antibodies are useful to analyze spatiotemporal distribution of site-specific phosphorylation of target proteins in vivo. Using several polyclonal and monoclonal antibodies that can specifically recognize four phosphorylated sites on glial fibrillary acidic protein (GFAP), we have previously reported that Thr-7, Ser-13, and Ser-34 on this intermediate filament protein are phosphorylated at the cleavage furrow during cytokinesis. This observation suggests that there exists a protein kinase named cleavage furrow kinase specifically activated at metaphase-anaphase transition (Matsuoka, Y., Nishizawa, K., Yano, T., Shibata, M., Ando, S., Takahashi, T., and Inagaki, M. (1992) EMBO J. 11, 2895–2902; Sekimata, M., Tsujimura, K., Tanaka, J., Takeuchi, Y., Inagaki, N., and Inagaki, M. (1996) J. Cell Biol. 132, 635–641). Here we report that GFAP is phosphorylated specifically at Thr-7, Ser-13, and Ser-34 by Rho-associated kinase (Rho-kinase), which binds to the small GTPase Rho in its GTP-bound active form. The kinase activity of Rho-kinase toward GFAP is dramatically stimulated by guanosine 5′-(3-O-thio)-triphosphate-bound RhoA. Furthermore, the phosphorylation of GFAP by Rho-kinase results in a nearly complete inhibition of its filament formation in vitro. The possibility that Rho-kinase is a candidate for cleavage furrow kinase is discussed.

Intermediate filaments (IFs), 1 major components of the cytoskeleton and the nuclear envelope in most eukaryotic cells, undergo dramatic reorganization of their structure during cell signaling and cell cycle (for review, see Refs. 1–3). This IF reorganization 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 (for review, see Ref. 4). Site- and phosphorylation state-specific antibodies that recognize a phosphorylated serine/threonine residue and its flanking sequence can visualize site-specific IF phosphorylation and thereby IF kinase activities in situ by immunocytochemistry (Ref. 5; for review, see Ref. 6). Recently, we reported two distinct types of mitotic phosphorylation of glial fibrillary acidic protein (GFAP), an IF protein expressed in the cytoplasm of astroglia, using antibodies that react with four distinct phosphorylated sites on GFAP (7, 8). One type is the phosphorylation of Ser-8 on GFAP, which appeared at G2-M phase transition in the entire cytoplasm. The other type is the phosphorylation of Thr-7, Ser-13, and Ser-34, which appeared at metaphase-anaphase transition at the cleavage furrow. This GFAP phosphorylation specifically localized at the cleavage furrow was observed not only in astroglial cells but also in other cultured cells transfected with GFAP cDNA (8). These findings suggested the existence of a protein kinase specifically activated at the cleavage furrow and its important role in cytokinesis. We tentatively termed this kinase cleavage furrow (CF) kinase (8). However, the molecular identity, regulation, and function of CF kinase remained to be examined.

The small GTP-binding protein Rho is implicated in the control of cytoskeletal structures, cell adhesions, and cell morphology (for review, see Ref. 9). Upon stimulation with certain signals, the GDP-bound inactive form of Rho may be converted to the GTP-bound active form, which binds to specific targets and thereby exerts its biological functions. We have identified three putative targets for Rho, p128 protein kinase N (10, 11), p138 myosin-binding subunit (MBS) of myosin phosphatase (12), and p164 Rho-kinase (13), which is also named ROK (14). Rho-kinase phosphorylates MBS and consequently inactivates myosin phosphatase (12). Rho-kinase also phosphorylates myosin light chain and thereby activates myosin ATPase (15). Other putative targets for Rho include rhophilin (11), p160 Rho-associated coiled-coil containing protein kinase (16), and citron (17).

Recently, Rho was shown to play a critical role in cytokinesis by inducing and maintaining the contractile ring, an actin-based cytoskeletal structure (18, 19). In addition, Rho was reported to be translocated from the cytosol to the cleavage furrow during cytokinesis (20). These results raise the possibility that Rho may also be implicated in the regulation of CF kinase and thereby in the efficient separation of IFs to daughter cells. As a first step toward defining this possibility, we have examined whether protein kinase N and/or Rho-kinase can phosphorylate GFAP at the same sites as CF kinase. Protein kinase N was found to phosphorylate GFAP mainly at Ser-8, a site that is not phosphorylated by CF kinase. 2

1 The abbreviations used are: IF, intermediate filament; GFAP, glial fibrillary acidic protein; CF, cleavage furrow; MBS, myosin-binding subunit; GST, glutathione S-transferase; GTPyS, guanosine 5′-(3-O-thio)-triphosphate; CHAPS, 3-[3-cholamidopropyl]dimethylammonio-1-propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; Rho-kinase, Rho-associated kinase.

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In this report, we show that GFAP can serve as an excellent substrate for Rho-kinase and that the GFAP phosphorylation by Rho-kinase prevents its filament formation in vitro. Furthermore, we present evidence that Rho-kinase phosphorylates GFAP at Thr-7, Ser-13, and Ser-34 in vitro, the same sites that are phosphorylated by CF kinase in vivo.

EXPERIMENTAL PROCEDURES

Materials—Recombinant human GFAP was prepared from Escherichia coli as described previously (8). Mouse monoclonal antibodies YC10, KT13, KT34, and MD389 were prepared as described previously (8, 21). GST-RhoA was purified and loaded with guanine nucleotides (22). Rho-kinase was purified from bovine brain (13). Constitutively active GST-Rho-kinase, a GST fusion protein of the catalytic fragment of Rho-kinase, was purified from Sf9 cells as described previously (15). The catalytic subunit of cAMP-dependent protein kinase (protein kinase A) was prepared in Sf9 cells by recombinant baculovirus infection (23). Cdc2 kinase was prepared from FM3A cells by the method of Beavo et al. (24). Protein concentrations were measured according to Bradford (25) using bovine serum albumin as a standard.

Phosphorylation of GFAP—The phosphorylation reaction for Rho-kinase was performed for 30 min at 25 °C in 20 μl of 25 mM Tris-Cl (pH 7.5), 0.2 mM CHAPS, 0.5 mM EDTA, 100 μM γ-[32P]ATP (5 μCi), 1 μg calyculin A, 130 μg/ml GFAP, and 0.5 μg/ml purified Rho-kinase in the presence of GST, GDP-GST-RhoA, or GTP-S-GST-RhoA (each 1 μl). The phosphorylation reaction for GST-Rho-kinase, protein kinase A, or Cdc2 kinase was performed for 30 min at 25 °C in 100 μl of the reaction mixture (25 mM Tris-Cl (pH 7.5), 0.4 mM MgCl2, 100 μM ATP, 0.1 μM calyculin A, and 130 μg/ml GFAP) in the presence of 5.5 μg/ml GST-RhoA, 5 μg/ml protein kinase A, or 0.5 μg/ml Cdc2 kinase. The reaction was stopped by the addition of Laemmli’s sample buffer and boiling.

Fragmentation of Phosphorylated GFAP—GFAP (130 μg) was incubated with GST-Rho-kinase (8.5 μg) and γ-[32P]ATP (50 μCi) for 120 min at 25 °C in 1 ml of the reaction mixture described above. The phosphorylated GFAP was precipitated with 10% trichloroacetic acid. Elution was carried out with a 60-min linear gradient of 5–50% 2-propanol/acetonitrile followed by a further elution with 0.1% trifluoroacetic acid. Immunoreactivity of the anti-GFAP antibody (MO389) and the anti-phosphoGFAP antibodies (YC10, KT13, and KT34) was observed specifically between the daughter nuclei and at the cleavage furrow of anaphase cells (Fig. 1A). Immunocytochemical studies with KT13 (Fig. 1B) and KT34 (data not shown) using confocal laser scanning microscopy revealed that GFAP phosphorylated at Ser-13 and Ser-34 is associated with the cleavage furrow to form a ring-like structure but not a disc-like structure, such as the telophase disc.

RESULTS AND DISCUSSION

We recently developed four monoclonal antibodies, YC10 (21), KT13, KT34, and MO389 (8) against four distinct phosphopeptides corresponding to the partial amino acid sequences of porcine GFAP. YC10, KT13, and KT34 react with GFAP phosphorylated at Ser-8, Ser-13, and Ser-34, respectively. MO389 reacts with both the phosphorylated and unphosphorylated GFAPs and stains filamentous structures in both mitotic and interphase cells. MO389 immunostained the interphase of the entire cytoplasm of both metaphase and anaphase cells, but YC10 stained filamentous structure throughout the cytoplasm of metaphase but not anaphase cells (Fig. 1A). In contrast, the immunoreactivities of KT13 and KT34 were observed specifically between the daughter nuclei and at the cleavage furrow of anaphase cells (Fig. 1A). Immunocytocchemical studies with KT13 (Fig. 1B) and KT34 (data not shown) using confocal laser scanning microscopy revealed that GFAP phosphorylated at Ser-13 and Ser-34 is associated with the cleavage furrow to form a ring-like structure but not a disc-like structure, such as the telophase disc.

Fig. 1. Immunoreactivity of the anti-GFAP antibody (MO389) and the anti-phosphoGFAP antibodies (YC10, KT13, and KT34) in U251 human astrocytoma cells. A, fluorescent photomicrographs of mitotic U251 cells stained with the antibody MO389, YC10, KT13, or KT34 (green). Chromosomes were stained with propidium iodide (red). The bar represents 10 μm. B, confocal images on nine serial focal planes of an anaphase U251 cell stained with KT13 and propidium iodide were obtained by confocal laser scanning microscopy (Olympus GB200).

To search for the putative CF kinase responsible for the cleavage furrow-specific phosphorylation described above, we examined whether Rho-kinase purified from bovine brain can phosphorylate GFAP in vitro. The results indicated clearly that Rho-kinase phosphorylated GFAP in a GST-RhoA-dependent manner (Fig. 2A). GDP-bound GST-RhoA enhanced the phosphorylation of GFAP by Rho-kinase 13-fold, and GTP-S-bound GST-RhoA enhanced it 291-fold (Fig. 2A). We then examined the phosphorylation sites on GFAP by Rho-kinase using the anti-phosphoGFAP antibodies described above. After the phosphorylation reaction, samples were resolved by SDS-PAGE and immunoblotted with MO389, YC10, KT13, or KT34. As shown in Fig. 2B, Rho-kinase phosphorylated GFAP at Ser-13 and Ser-34 but not at Ser-8 in a GTP-S-GST-RhoA-dependent manner.

We also used the constitutively active GST-Rho-kinase, a fusion protein between GST and the catalytic fragment of Rho-kinase produced in Sf9 cells by recombinant baculovirus infection. Analyses with the anti-phosphoGFAP antibodies revealed that GST-Rho-kinase also phosphorylated GFAP at Ser-13 and Ser-34 but not at Ser-8 (Fig. 2C). These results suggest that catalytic characteristics of GST-Rho-kinase are similar to those of native Rho-kinase activated by GTP-S-GST-RhoA. In contrast, the catalytic subunit of cAMP-dependent protein kinase phosphorylated all three serine residues, and Cdc2 kinase weakly phosphorylated only Ser-8 (Fig. 2C).

To confirm phosphorylation sites on GFAP by Rho-kinase, GFAP (130 μg) was phosphorylated by GST-Rho-kinase in the presence of [γ-32P]ATP to approximately 2.7 mol of phosphate/mole of GFAP (Fig. 3A). The radioactive GFAP was then digested with lysyl endopeptidase to generate about a 6.5-kDa
fragment consisting mainly of the amino-terminal head domain. Tricine-SDS-PAGE analysis (30) revealed that all radioactivity associated with GFAP was retained in this 6.5-kDa head domain (Fig. 3B). This phosphorylated head domain was isolated by reverse-phase HPLC, digested with trypsin, and then again subjected to reverse-phase HPLC. As shown in Fig. 4A, three major radioactive peaks, R1 to R3, were obtained. Phosphoamino acid analysis showed the presence of phosphothreonine in R1 and phosphoserine in both R2 and R3 (Fig. 4B). Amino acid sequence analysis revealed that R1 was the peptide containing Thr-7, R2 was the peptide containing Ser-34, and R3 was the peptide containing Ser-13 (Fig. 4A). Ethanethiol treatment of R2 and R3, a procedure that converts specifically phosphoserine into \( S -\)ethylcysteine (31), suggested that phosphates were located on Ser-34 and Ser-13, respectively (data not shown). Therefore, GFAP was shown to be phosphorylated at Thr-7, Ser-13, and Ser-34 by GST-Rho-kinase. By using a rabbit polyclonal antibody recognizing phosphorylated Thr-7, we have previously reported that Thr-7 is also phosphorylated at the cleavage furrow (7).

We then examined the effect of phosphorylation of GFAP by Rho-kinase on the filament forming ability of GFAP. Soluble GFAP was preincubated with or without GST-Rho-kinase for 30 min, and the samples were incubated under conditions of polymerization (25 mM imidazole-HCl, pH 6.75, and 100 mM NaCl at 37 °C) (28) for a further hour. Then the NaCl- and pH-dependent filament formation of GFAP in these samples was analyzed by centrifugation (Fig. 5A) and electron microscopy (Fig. 5B). As shown in Fig. 5, the phosphorylation of GFAP by GST-Rho-kinase resulted in a nearly complete inhibition of its filament formation. These results increase the possibility...
that GFAP phosphorylation at Thr-7, Ser-13, and Ser-34 during cytokinesis may induce the fragmentation of glial filaments at the cleavage furrow.

In the present study, we obtained evidence that GFAP can serve as an excellent substrate for Rho-kinase in a GTP-Rho-dependent manner. So far, MBS (12) and myosin (15) were the only preferred substrates for Rho-kinase. The phosphorylated GFAP lost its ability to form filaments in vitro. The in vitro phosphorylation sites on GFAP by Rho-kinase were Thr-7, Ser-13, and Ser-34, which are the same sites that CF kinase phosphorylates at the cleavage furrow during cytokinesis. We are considering that Rho-kinase may be CF kinase itself, and if so it may play an important role in the cleavage furrow-specific reorganization of IFs during cytokinesis.

Because Rho-kinase was recently reported to act downstream of Rho in the regulation of myosin phosphorylation (12, 15) and the formation of stress fibers and focal adhesion complexes (32, 33), Rho-kinase may also mediate the regulation of cytokinesis by Rho (18, 19). Whether Rho-kinase is activated during cytokinesis is the subject of ongoing studies. Because Rho-kinase belongs to a family of related serine/threonine kinases including myotonic dystrophy kinase, these kinases may phosphorylate the similar sites on GFAP. Further investigations are necessary to elucidate the relationship between CF kinase and Rho-kinase or its family members.

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REFERENCES
1. Steinert, P. M., and Roop, D. R. (1988) Annu. Rev. Biochem. 57, 593–625.
2. Eriksson, J. E., Opal, P., and Goldberg, R. D. (1992) Curric. Opin. Cell Biol. 4, 99–104.
3. Fucks, E., and Weber, K. (1994) Annu. Rev. Biochem. 63, 345–382.
4. Amano, M., Matsuzuka, Y., Tsujimura, K., Ando, S., Tokuji, T., Takahashi, T., and Inagaki, N. (1996) Bioessays 18, 481–487.
5. Nishizawa, K., Yano, T., Shibata, M., Ando, S., Sagara, S., Takahashi, T., and Inagaki, M. (1991) J. Biol. Chem. 266, 3073–3078.
6. Inagaki, M., Nishizawa, K., Takahashi, T., and Takai, Y. (1997) J. Biochem. (Tokyo) 121, 407–417.
7. Matsuoka, Y., Nishizawa, K., Yano, T., Shibata, M., Ando, S., Takahashi, T., and Inagaki, M. (1992) EMBO J. 11, 2985–2992.
8. Sekimoto, M., Tsujimura, K., Tanaka, J., Takeuchi, Y., Inagaki, N., and Inagaki, M. (1996) J. Cell Biol. 132, 635–641.
9. Machelsky, L. M., and Hall, A. (1990) Trends Cell Biol. 6, 304–310.
10. Amano, M., Mukai, H., Ono, Y., Chihara, K., Matsui, T., Hamajima, Y., Okawa, K., Iwamatsu, A., and Kaibuchi, K. (1996) Science 271, 648–650.
11. Watanabe, G., Saito, Y., Maddalena, P., Ishizaki, T., Fujisawa, K., Morii, N., Mukai, H., Ono, Y., Kakizuka, A., and Narumiya, S. (1996) Science 271, 645–648.
12. Kinuura, K., Itto, M., Amano, M., Chihara, K., Fukuta, Y., Nakafuku, M., Yamamori, N., and Inagaki, N. (1991) EMBO J. 15, 2208–2216.
13. Leung, T., Manser, E., Tan, L., and Lim, L. (1995) J. Biol. Chem. 270, 29051–29054.
14. Amano, M., Itto, M., Kinuura, K., Fukuta, Y., Chihara, K., Nakano, T., Matsuura, Y., and Kaibuchi, K. (1996) J. Biol. Chem. 271, 20246–20249.
15. Ishizaki, T., Maekawa, M., Fujisawa, K., Okawa, K., Iwamatsu, A., Fujita, A., Watanabe, N., Saito, Y., Kakizuka, A., Morii, N., and Narumiya, S. (1996) EMBO J. 15, 1885–1893.
16. Ishizaki, T., Kamen, K., Ito, M., Takeuchi, Y., Inagaki, N., and Inagaki, M. (1993) J. Biol. Chem. 268, 245–249.
17. Kishii, K., Suzuki, K., Ito, M., and Inagaki, M. (1991) J. Biol. Chem. 266, 11387–11195.
18. Mabuchi, I., Hamaguchi, Y., Fujimoto, H., Morii, N., Mishima, M., and Narumiya, S. (1993) J. Biol. Chem. 268, 3323–3331.
19. Takaiishi, K., Sasaki, T., Kameyama, T., Tsukita, S., Tsukita, S., and Takai, Y. (1995) Oncogene 11, 39–48.
20. Yan, T., Taura, C., Shibata, M., Hirota, Y., Ando, S., Kusuhara, M., Takahashi, T., and Inagaki, M. (1991) Biochem. Biophys. Res. Commun. 175, 1144–1151.
21. Shimizu, K., Kuroda, S., Yamamori, B., Matsuda, S., Kaibuchi, K., Yamauchi, T., Isobe, T., Irie, K., Matsumoto, K., and Takai, Y. (1994) J. Biol. Chem. 269, 22917–22920.
22. Beavo, J. A., Bechtel, P. J., and Krebs, E. G. (1974) Methods Enzymol. 38, 299–308.
23. Kusuhara, T., Tokuji, T., Matsuzuka, Y., Okumura, E., Takahama, K., Hisanaga, S., Kimishima, T., Yasuda, H., Kamijo, M., Ohba, Y., Tsujimura, K., and Yamanaka, K. (1995) FEBS Lett. 377, 243–248.
24. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254.
25. Tsujimura, K., Takai, Y., Ando, S., Matsuoka, Y., Kusuhara, M., Sugiura, H., Yamauchi, T., and Inagaki, M. (1994) J. Biochem. (Tokyo) 116, 426–434.
26. Boyle, W. J., van der Geer, P., and Hunter, T. (1991) Methods Enzymol. 201, 110–149.
27. Inagaki, M., Gonda, Y., Nishizawa, K., Kitamura, S., Sato, C., Ando, S., Tanabe, K., Kikuchi, K., Tsukita, S., and Nishi, Y. (1990) J. Biol. Chem. 265, 4722–4729.
28. Andreassen, P. R., Palmer, D. K., Wener, M. H., and Margolis, R. L. (1991) J. Cell Sci. 99, 523–534.
29. Schagger, H., and Von Jagow, G. (1987) Anal. Biochem. 166, 368–379.
30. Meyer, H. E., Hoffmann-Porsorke, E., Korte, H., and Heilmeyer, L. M. G. (1986) FEBS Lett. 204, 61–66.
31. Leung, T., Chen, X-Q., Manser, E., and Lim, L. (1996) Mol. Cell. Biol. 16, 5313–5327.
32. Amano, M., Chihara, K., Kinuura, K., Fukuta, Y., Nakamura, N., Matsuura, Y., and Kaibuchi, K. (1997) Science 275, 1308–1311.
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