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Elongation Factor-1α Is a Novel Substrate of Rho-Associated Kinase[1]

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Rho-associated kinase (Rho-kinase), which is activated by the Rho small GTPase, phosphorylates the myosin-binding subunit (MBS) of myosin phosphatase, myosin light chain (MLC), the ERM family proteins, and adducin, thereby regulating the formation of stress fibers, focal adhesions, microvillus formation, and cell motility. Here, to further understand the role of Rho-kinase in the regulation of the numerous cellular processes by Rho, we purified a novel substrate of Rho-kinase having a molecular mass of 48 kDa (p48) from a rat liver cytosol extract. Mass spectral analysis revealed p48 to be elongation factor-1 alpha (EF-1α), which is known as an actin-binding protein besides a cofactor of polypeptide elongation. Rho-kinase directly phosphorylated recombinant EF-1α *in vitro*. A high- speed cosedimentation assay revealed that phosphorylation of EF-1α by Rho-kinase decreased the binding activity of EF-1α to filamentous actin (F-actin). A low-speed sedimentation assay revealed that phosphorylation of EF-1α by Rho-kinase decreased the F-actin-bundling activity. In addition, EF-1α bound to MBS of myosin phosphatase, suggesting that both Rho-kinase and myosin phosphatase regulate the phosphorylation state of EF-1α downstream of Rho as other substrates of Rho-kinase, i.e., MLC, adducin, and the ERM family. These results suggest that the Rho/Rho-kinase pathway regulates the organization of actin cytoskeleton via the phosphorylation of EF-1α.

Key Words: **Rho; Rho-kinase; myosin-binding subunit of myosin phosphatase; EF-1α; F-actin.**

The small GTPase Rho, together with Rac, Cdc42, and TC10, belongs to the Rho family. The activity of Rho in cells is regulated cyclically between its inactive GDP- and active GTP-bound forms in response to extracellular signals (1). The latter form of Rho interacts with specific effectors to exert cellular functions. Rho participates in signaling pathways that regulate actin cytoskeletal structures such as stress fibers, and cell-substratum adhesions such as focal adhesions in fibroblasts (reviewed in 2–4). Rho is also involved in the regulation of cell morphology, cell aggregation, cadherin-mediated cell–cell adhesion, cell motility, cytokinesis, membrane ruffling, neurite retraction, microvillus formation, and smooth muscle contraction (reviewed in 2–4). Rho-associated kinase (Rho-kinase)/ROK/ROCK, which is a serine/threonine kinase identified as a specific effector of Rho (5–7), has been most intensively studied. Rho-kinase regulates the phosphorylation state of the myosin light chain (MLC) by the direct phosphorylation of MLC (8) and by the inactivation of myosin phosphatase through the phosphorylation of the myosin-binding subunit (MBS) of myosin phosphatase (9). Thus, Rho-kinase regulates contractility of actin-myosin filaments and thereby induces the formation of stress fibers and focal adhesions (10–12), smooth muscle contraction (13, 14), and neurite retraction (15–17) via the MLC phosphorylation. In addition to MLC, the ezrin/radixin/moesin (ERM) family of proteins (18, 19) and adducin (20) were found

Abbreviations used: EF-1α, elongation factor-1alpha; Rho-kinase, Rho-associated kinase; GST, glutathione *S*-transferase; aa, amino acids; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; filamentous actin, F-actin; MLC, myosin light chain; MBS, myosin-binding subunit.

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to be substrates of both Rho-kinase and myosin phosphatase. The phosphorylation of MBS by Rho-kinase inhibits the phosphatase activity of myosin phosphatase toward these substrates (reviewed in 4). Rho-kinase and myosin phosphatase are thought to cooperatively control the phosphorylation level of a subset of substrates and to regulate various cellular functions, such as microvillus formation and membrane ruffling (4, 21, 22).

In the present study, a novel substrate of Rho-kinase with a molecular mass of 48 kDa (p48) was purified from rat liver extract. Mass spectral analysis revealed that p48 was identical to EF-1α, which is a cofactor of eukaryotic protein synthesis responsible for binding aminoacyl-tRNA to the ribosome during polypeptide elongation (23). EF-1α also has the filamentous actin (F-actin)-binding and -bundling activities (24–26) and microtubule-severing activity (27), and is colocalized with F-actin (28). We also found that Rho-kinase directly phosphorylated recombinant EF-1α *in vitro* and that the phosphorylation of EF-1α by Rho-kinase resulted in a reduction in its F-actin-binding and -bundling activities. Furthermore, EF-1α bound to MBS of myosin phosphatase, suggesting that Rho-kinase and myosin phosphatase regulate the state of phosphorylation of EF-1α to regulate the actin cytoskeleton as described for other substrates of Rho-kinase.

MATERIALS AND METHODS

Materials and chemicals. pET16b-EF-1α was kindly provided by Dr. E. Nishida (Kyoto University), and HisX10-tagged EF-1α (His-EF-1α) was purified as described (27). Glutathione-*S*-transferase (GST)-Rho-kinase [6–553 amino acids (aa)] was prepared as previously described (8). Native Rho-kinase was purified from bovine brain as previously reported (6). GST-RhoA was purified from *Escherichia coli* and loaded with guanine nucleotides as described earlier (29). GST-MBS-ankyrin repeat domain (ANK) (39–295 aa of rat3 MBS) and GST-MBS-C-terminal fragment (CT) (758–1032 aa of rat3 MBS) were prepared as previously reported (20, 30). F-actin was purified from an acetone powder prepared from rabbit skeletal muscle (31). Anti-EF-1α antibody was purchased from Upstate Biotechnology Incorporated. Other materials and chemicals were obtained from commercial sources.

Purification of a Rho-kinase substrate, p48. Rat livers (from retired Sprague–Dawley rats) were excised and homogenized in a threefold volume of Buffer A (25 mM Tris–HCl at pH 7.5, 1 mM EDTA, 1 mM dithiothreitol [DTT], 5 mM $MgCl_2$, 8.6% sucrose, 10 μg/ml leupeptin, and 100 μg/ml phenylmethylsulfonyl fluoride), and the homogenate was then centrifuged at 10,000*g* for 1 h. The obtained supernatant was further centrifuged at 100,000*g* for 1 h. The obtained cytosol fraction (35 mg of protein) was loaded onto a Mono Q HR5/5 column (Pharmacia Biotech Inc., Grand Island, NY) preequilibrated with 10 ml of Buffer B (20 mM Tris–HCl at pH 7.5, 1 mM EDTA, 1 mM DTT, and 5 mM $MgCl_2$). After having been washed with Buffer B, the bound proteins were eluted with a linear gradient of NaCl (0–0.5 M). Fractions (0.5 ml each) were collected, and an aliquot of each fraction (10 μl) was subjected to the phosphorylation assay as described below. The peak fractions containing the major phosphorylated protein, p48, were diluted fivefold with Buffer B containing 0.5% cholate, and further loaded onto a second Mono Q HR5/5 column preequilibrated with Buffer B containing 0.5% cholate. Proteins were eluted with a linear gradient of NaCl (0–0.5 M). Each fraction (10 μl) was subjected to the phosphorylation assay, and p48 was determined.

Phosphorylation assay. The kinase reaction with GST-Rho-kinase or native Rho-kinase was carried as previously reported (8, 32).

Mass spectral analysis. Identification of p48 obtained from the second Mono Q column was performed by mass spectral analysis using a PerSeptive Biosystem Voyager-DE/RP [Matrix-assisted Laser Desorption/Ionization time-of-flight (MALDI-TOF) mass spectrometer] as reported (33–35).

High-speed and low-speed sedimentation assay. His-EF-1α (1.0 μM) was incubated in the presence of GST-Rho-kinase (0.2 μM) with or without 100 μM ATP for 1 h at 30°C. Polymerized F-actin (3.3 μM) was mixed with His-EF-1α (0.6 μM) and incubated for 120 min at 4°C in 20 mM Pipes at pH 6.5, containing 50 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA, 0.5 mM ATP, 0.2 mM DTT, 0.1 mg/ml bovine serum albumin, and 10% (w/v) sucrose. After the incubation, 50 μl of each reaction mixture was layered onto a 100-μl sucrose barrier (20% (w/v) sucrose) and centrifuged at 100,000*g* for 1 h at 4°C. The supernatants and pellets were separated and subjected to SDS–PAGE. In the low-speed assay, after the incubation each mixture was centrifuged at 10,000*g* for 20 min at 4°C. The supernatants and pellets were carefully separated and subjected to SDS–PAGE.

Affinity column chromatography. The bovine brain membrane extract saturated with 40–80% ammonium sulfate was prepared as described (36). Under the conditions, the membrane extract contained a greater amount of EF-1α compared to the cytosol extract or the membrane extract saturated with 40% ammonium sulfate (Fukata *et al.*, unpublished data). The membrane extract was applied to affinity columns bearing immobilized GST, GST-MBS-ANK, or GST-MBS-C as previously described (19, 20), and the proteins bound to each column were eluted by high-ionic strength and evaluated by silver staining or immunoblot analysis using antibody against EF-1α.

RESULTS

To search for novel substrates of Rho-kinase, we separated rat liver cytosol proteins by Mono Q column chromatography and subjected the obtained fractions to a phosphorylation assay using a constitutively active form of Rho-kinase (GST-Rho-kinase). Several proteins were phosphorylated with [γ-^{32}P]ATP in the absence of exogenous GST-Rho-kinase (Fig. 1A, indicated by asterisks). One protein with a mass of about 48 kDa (p48) was recognized as a major protein phosphorylated in a GST-Rho-kinase-dependent manner (Fig. 1A). p48 was eluted at around 200 mM NaCl. p48 from this Mono Q column chromatography was further purified by chromatography on a second Mono Q column in the presence of cholate (Fig. 1B). p48 was eluted at around 300 mM NaCl (data not shown). p48 from the second Mono Q column was phosphorylated by GST-Rho-kinase again, but the efficiency of phosphorylation was not so high (Fig. 1B). This finding is consistent with our result that the activity of GST-Rho-kinase was considerably inhibited in the presence of cholate *in vitro* (Fukata *et al.*, unpublished data). The purified protein was subjected to mass spectrometry. The molecular

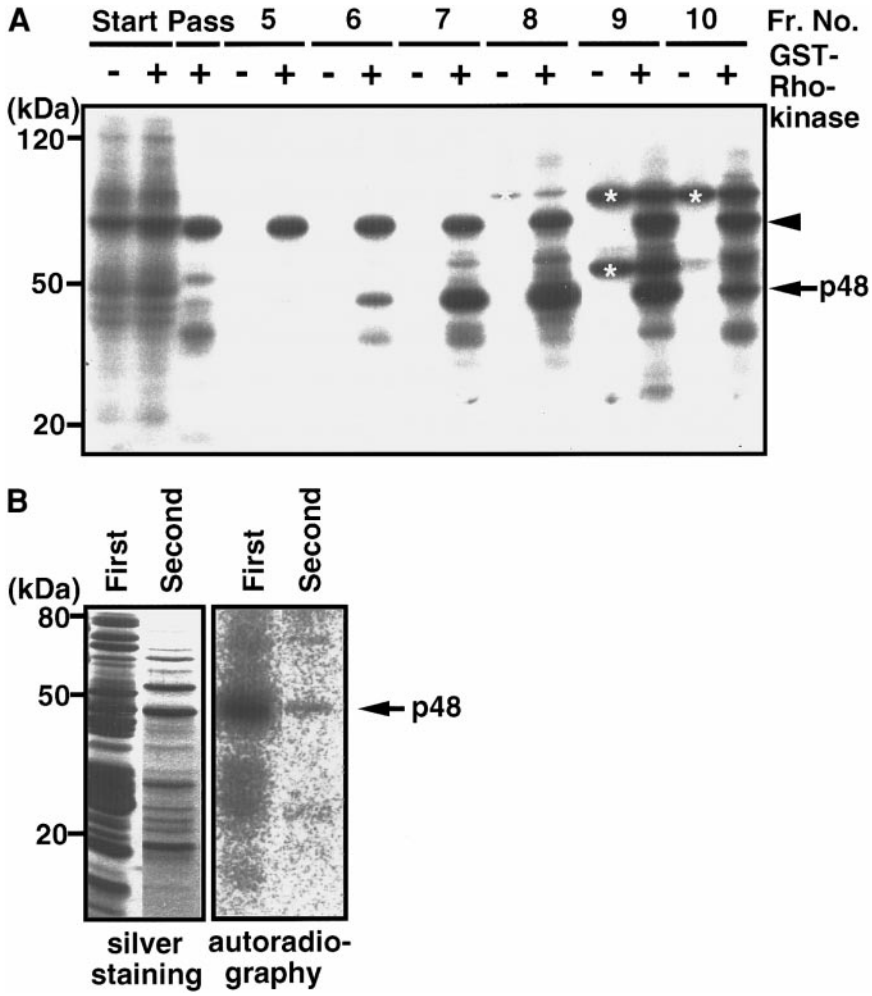

FIG. 1. Screening for Rho-kinase substrate from rat liver. (A) Rat liver cytosol fraction was loaded onto a first Mono Q column. Each fraction eluted with the linear gradient of NaCl was subjected to the phosphorylation assay with (+) or without (−) GST-Rho-kinase. The reaction mixture was subjected to SDS–PAGE, and the radioactive proteins were detected by autoradiography. p48 was detected as a major protein phosphorylated in a GST-Rho-kinase-dependent manner (indicated by arrow, fractions. 6–10). Asterisks indicate representative of proteins phosphorylated in a GST-Rho-kinase independent manner. Autophosphorylated GST-Rho-kinase was detected at around the 90-kDa position (indicated by arrowhead). (B) Fractions No. 6–10 obtained from this column were further purified by a second Mono Q column chromatography in the presence of cholate. The fractions containing p48 were determined by phosphorylation assay with GST-Rho-kinase. The fractions containing p48 from the first and second Mono Q columns were mixed separately and subjected to silver staining and phosphorylation assay.

weights of peptides derived from p48 were determined, and found to be identical to those from rat EF-1α (Fig. 2A). An immunoblot analysis with anti-EF-1α antibody confirmed that p48 was identical with EF-1α (Fig. 2B).

Next, we examined whether Rho-kinase could directly phosphorylate EF-1α *in vitro*. GST-Rho-kinase phosphorylated purified recombinant His-tagged EF-1α (His-EF-1α), and the amount of phosphate incorporated into His-EF-1α was approximately 1.0 mol per 1.0 mol of protein under the conditions used (Fig. 3A). Native Rho-kinase, which was purified from bovine brain, phosphorylated His-EF-1α in a GTPγS (a nonhydrolyzable GTP analog) · GST-RhoA-dependent manner *in vitro* (Fig. 3B). These results indicate that EF-1α is a putative substrate of Rho-kinase.

EF-1α has an F-actin-binding property (24, 25), and F-actin-bundling property, i.e., the ability to cross-link F-actin into bundles (26). Given that the Rho/Rho-kinase pathway participates in the reorganization of actin cytoskeleton (4), we next examined whether the phosphorylation of EF-1α by Rho-kinase would affect the F-actin-binding and -bundling activities of EF-1α. To test the F-actin-binding activity, we performed a high-speed cosedimentation assay by ultracentrifugation using His-EF-1α and F-actin. Nonphosphorylated His-EF-1α sedimented in high-speed precipitate in an F-actin-dependent manner (Fig. 4A). His-EF-1α phosphorylated by GST-Rho-kinase, which showed the slower mobility on SDS–PAGE gel (indicated by asterisk), hardly cosedimented with F-actin (Fig. 4A). The

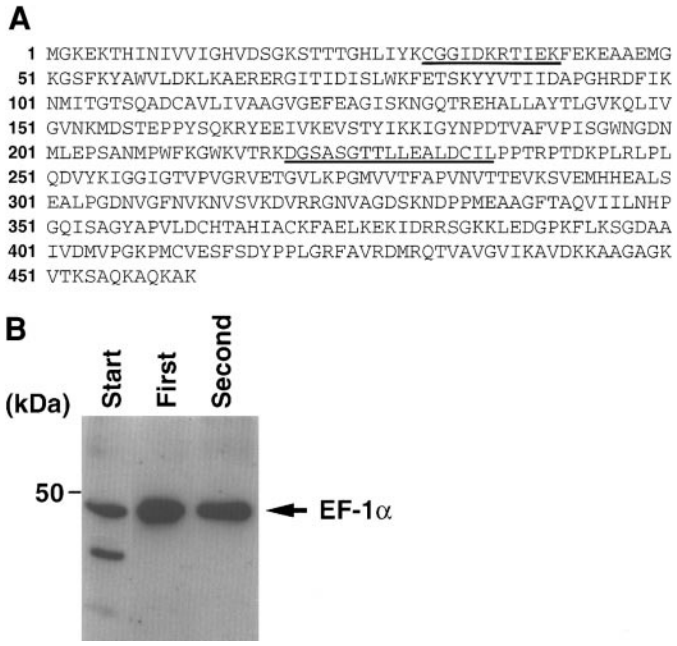

FIG. 2. Identification of EF-1α as a substrate of Rho-kinase. (A) The molecular weights of peptides derived from p48 were determined by mass spectral analysis and found to be identical to those from rat EF-1α, the sequence of which is shown. The estimated molecular weight of rat EF-1α (50 kDa) was consistent with that of p48. The peptides obtained from p48 are indicated by underlines (the sixth amino acid in the former peptide was di- (or tri)-methyl lysine). (B) p48 from the first or second Mono Q column was specifically recognized by anti-EF-1α antibody. Rat liver cytosol fraction (20 μg of protein) was also subjected to the immunoblot analysis (start).

binding of EF-1α to F-actin is sensitive to pH; as the pH increases, the F-actin-binding and –bundling activities decrease (37). The phosphorylation of His-EF-1α by Rho-kinase showed an essentially similar effect, i.e., reduction in F-actin-binding activity of His-EF-1α, at both pH 6.5 (Fig. 4) and pH 7.5 (data not shown). Furthermore, we examined the effect of the phosphorylation by Rho-kinase on the F-actin-bundling activity of EF-1α by using a low-speed sedimentation assay. In the absence of His-EF-1α, F-actin did not precipitate by low-speed centrifugation (Fig. 4B) as previously described (38). F-actin efficiently precipitated in the presence of non-phosphorylated His-EF-1α by the low-speed centrifugation, indicating that F-actin was cross-linked by His-EF-1α, as previously reported (37); whereas F-actin did not precipitate when His-EF-1α phosphorylated by GST-Rho-kinase was used (Fig. 4B). These results indicate that the phosphorylation of EF-1α by Rho-kinase inhibited both the F-actin-binding and -bundling activities of EF-1α *in vitro*.

Several substrates of Rho-kinase, such as MLC, the ERM family, and adducin bind to MBS of myosin phosphatase and are also substrates of myosin phosphatase (4). Because the phosphatase activity of myosin phosphatase toward these substrates is inhibited by the phosphorylation of MBS by Rho-kinase, it has been proposed that the phosphorylation level of these substrates is elevated by the direct phosphorylation by Rho-kinase and the inactivation of myosin phosphatase downstream of Rho (4, 9, 19, 20). It is tempting to speculate that EF-1α, like other substrates, forms a complex with MBS. MBS has an ankyrin repeat domain and a leucine-zipper-like motif, both of which are involved in the protein–protein interaction. It has been reported that MLC (39) and adducin (20) bind to the ankyrin repeat of MBS, and that the ERM family (19) and Rho (9) bind to the COOH (C)-terminal fragment of MBS containing the leucine-zipper-like motif. To examine the interaction of EF-1α with MBS, we loaded a bovine brain membrane extract onto a glutathione–Sepharose affinity column on which GST, GST-MBS-ankyrin repeat domain (ANK) or GST-MBS-C-terminal fragment (CT) had been immobilized. The proteins that bound to the affinity columns were eluted with a buffer containing 0.2 M NaCl. Protein with molecular mass of about 48 kDa was detected in the eluate from the GST-MBS-ANK affinity column but not in that from the GST or the GST-MBS-CT affinity column (Fig. 5A). p48 from the GST-MBS-ANK affinity

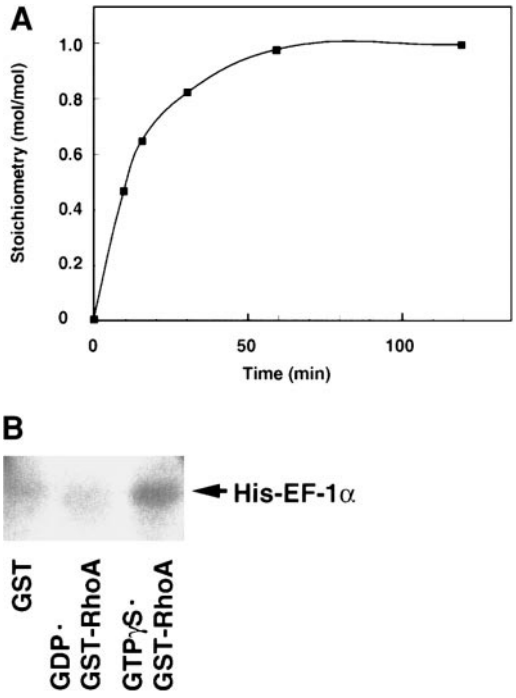

FIG. 3. Phosphorylation of recombinant EF-1α by Rho-kinase. (A) Recombinant His-EF-1α was phosphorylated by GST-Rho-kinase for various periods. The phosphorylated protein was resolved by SDS–PAGE and quantified by an image analyzer. About 1.0 mol of phosphate was maximally incorporated into 1.0 mol of His-EF-1α by GST-Rho-kinase. (B) Purified His-EF-1α was subjected to a phosphorylation assay with native Rho-kinase in the presence of GST, GDP · GST-RhoA, or GTPγS · GST-RhoA. The arrow denotes the position of His-EF-1α. The results shown are representative of three independent experiments.

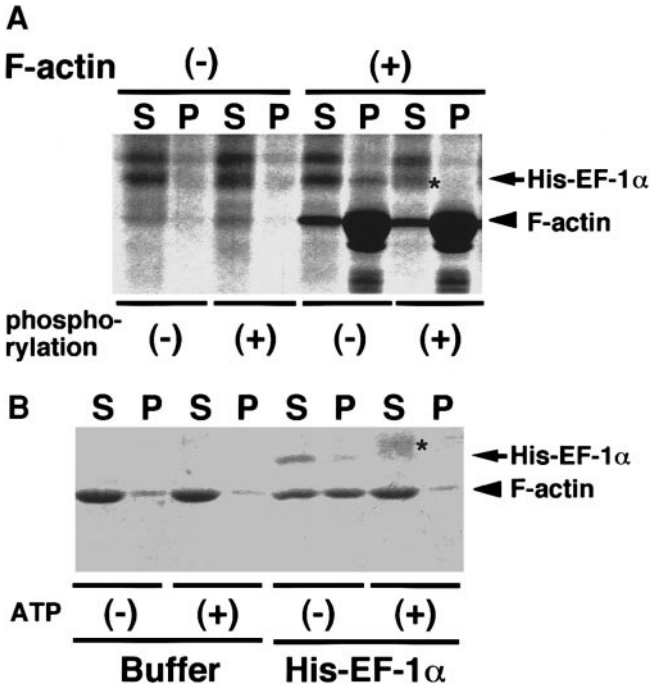

FIG. 4. Effects of phosphorylation of EF-1α by Rho-kinase on the F-actin-binding and -bundling activities of EF-1α. (A) High-speed cosedimentation assay. His-EF-1α was incubated with GST-Rho-kinase in the absence or presence of ATP (indicated by phosphorylation (−) or (+), respectively), and was then incubated for 120 min with or without F-actin (3.3 μM). After the incubation, each reaction mixture was separated into supernatant (S) and pellet (P) fractions by ultracentrifugation (at 100,000*g*). Each fraction was subjected to SDS–PAGE and silver staining. Arrow and arrowhead denote the positions of His-EF-1α and F-actin, respectively. The asterisk indicates the phosphorylated His-EF-1α that broadly mobility-shifted on SDS–PAGE. Bovine serum albumin used as a blocking protein was detected mainly in the S fraction, irrespective of F-actin (just above His-EF-1α on SDS–PAGE). (B) Low-speed sedimentation assay. Phosphorylated (with ATP, +) or nonphosphorylated (without ATP, −) His-EF-1α prepared as above was incubated with F-actin (3.3 μM), and separated into S and P fractions by low-speed centrifugation (at 10,000*g*). As a control experiment, buffer prepared in the absence of His-EF-1α with (+) or without (−) ATP was separately incubated with F-actin. Each fraction was subjected to SDS–PAGE and Coomassie brilliant blue staining. Arrow and arrowhead denote the position of His-EF-1α and F-actin, respectively. The asterisk indicates the phosphorylated His-EF-1α. The results shown are representative of three independent experiments.

column was phosphorylated by GST-Rho-kinase (data not shown). By an immunoblot analysis with anti-EF-1α antibody, the binding of EF-1α to GST-MBS-ANK was confirmed (Fig. 5B). These results suggest that the phosphorylation state of EF-1α might be dually regulated by Rho-kinase and myosin phosphatase downstream of Rho as in the case of other substrates.

DISCUSSION

We have previously shown that Rho-kinase and myosin phosphatase dually regulate the phosphorylation level of various substrates, such as MLC (8, 9), the ERM family (18, 19), and adducin (20). These signaling pathways play crucial roles in a variety of cellular functions downstream of Rho (reviewed in 4). Here, EF-1α was identified as a novel substrate of Rho-kinase, and the phosphorylation of EF-1α by Rho-kinase was shown to decrease the F-actin-binding and -bundling activities of EF-1α. This is the first evidence indicating that the modification of EF-1α by phosphorylation directly affects its activities toward F-actin. Furthermore, MBS of myosin phosphatase bound to EF-1α, suggesting that the state of phosphorylation of EF-1α is regulated by Rho-kinase and myosin phosphatase, as described for other substrates of Rho-kinase.

The next step we are facing is to clarify the physiological role of the phosphorylation of EF-1α by Rho-kinase *in vivo*. It has been recently reported that EF-1α from highly metastatic cells has reduced binding affinity to F-actin (28), suggesting that the difference in the EF-1α activities toward F-actin is important for cytoskeletal organization in motile processes. We hypothesize that modulation of the activities of EF-1α by Rho-kinase might influence the cytoskeletal organization and/or regulate the protein synthesis associated with the actin cytoskeleton as described below.

The intracellular concentration of EF-1α is very high, and more than half of the total EF-1α (~60%) appears to bind to the actin cytoskeleton (28). EF-1α binds to the same site on actin as several other actin-binding proteins such as myosin (37, 40, 41), so the weakened F-actin binding activity of EF-1α by Rho-

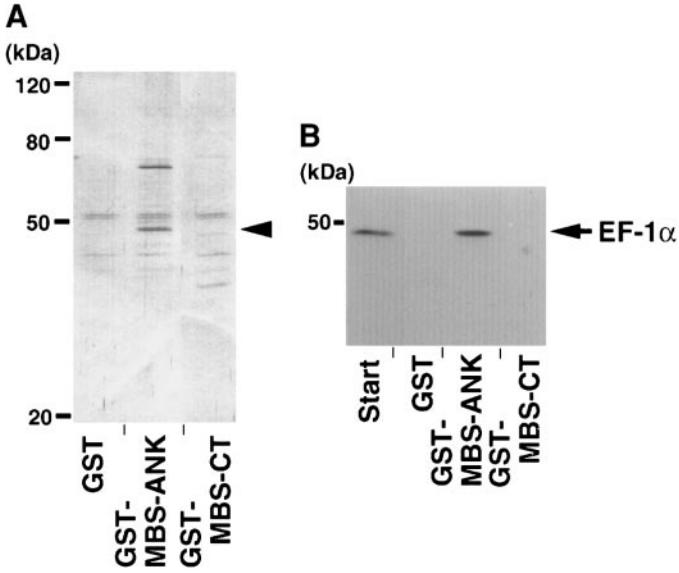

FIG. 5. Interaction of EF-1α with MBS of myosin phosphatase. Proteins from a bovine brain membrane extract that bound to GST, GST-MBS-ANK, or GST-MBS-CT immobilized on a column were evaluated by silver staining (A) or by immunoblot analysis with anti-EF-1α antibody (B). Arrowhead denotes the position of p48. The results shown are representative of three independent experiments.

kinase would help other actin binding proteins, e.g., myosin, bind to the actin cytoskeleton. Thus, when Rho is activated, Rho-kinase may phosphorylate EF-1α and MLC, sequester EF-1α away from F-actin, and in turn facilitate the interaction of myosin with F-actin. As a result, cells could alter the actin cytoskeleton to exert various cellular functions, such as stress fiber formation (see below) and membrane ruffling.

It is now recognized that protein synthesis machinery containing mRNAs, ribosomes, initiation factors, and elongation factors, is associated with the actin cytoskeleton (42, 43), and that the localized translation of specific proteins is essential for cell motility. For example, β-actin mRNA is localized at the leading edge of motile cells, where it is actively translated to actin protein. Mislocalization of β-actin mRNA disrupts cell polarity (44, 45). Because F-actin and aminoacyl-tRNA compete with each other for binding to EF-1α (41), Rho-kinase phosphorylates EF-1α to weaken its F-actin-binding, thereby inducing the binding of aminoacyl-tRNA to EF-1α. This might promote the localized protein synthesis associated with the actin cytoskeleton.

It has been reported that in the yeast *Saccharomyces cerevisiae*, Bni1p, which is one of the Rho1p effectors (46), interacts with EF-1α, and that the binding of Bni1p to EF-1α inhibits F-actin-binding and -bundling activities of EF-1α (47). In a mammalian system, cooperation between Rho-kinase and mDia, an effector of Rho and a mouse homologue of Bni1p, is necessary for the formation of fully refined stress fibers (48). Although MLC is apparently the most important substrate of Rho-kinase in the formation of stress fiber, EF-1α may also participate in the formation of stress fibers as a common downstream molecule of not only Rho-kinase but also mDia. Further experiments are necessary to understand in better detail the relationship between Rho-kinase/mDia and EF-1α.

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REFERENCES

1. Nobes, C., and Hall, A. (1994) Regulation and function of the Rho subfamily of small GTPases. *Curr. Opin. Genet. Dev.* **4,** 77–81.
2. Van Aelst, L., and D'Souza-Schorey, C. (1997) Rho GTPases and signaling networks. *Genes Dev.* **11,** 2295–2322.
3. Hall, A. (1998) Rho GTPases and the actin cytoskeleton. *Science* **279,** 509–514.
4. Kaibuchi, K., Kuroda, S., and Amano, M. (1999) Regulation of the cytoskeleton and cell adhesion by the Rho family GTPases in mammalian cells. *Annu. Rev. Biochem.* **68,** 459–486.
5. Leung, T., Manser, E., Tan, L., and Lim, L. (1995) A novel serine/threonine kinase binding the Ras-related RhoA GTPase which translocates the kinase to peripheral membranes. *J. Biol. Chem.* **270,** 29051–29054.
6. Matsui, T., Amano, M., Yamamoto, T., Chihara, K., Nakafuku, M., Ito, M., Nakano, T., Okawa, K., Iwamatsu, A., and Kaibuchi, K. (1996) Rho-associated kinase, a novel serine/threonine kinase, as a putative target for small GTP binding protein Rho. *EMBO J.* **15,** 2208–2216.
7. Ishizaki, T., Maekawa, M., Fujisawa, K., Okawa, K., Iwamatsu, A., Fujita, A., Watanabe, N., Saito, Y., Kakizuka, A., Morii, N., and Narumiya, S. (1996) The small GTP-binding protein Rho binds to and activates a 160 kDa Ser/Thr protein kinase homologous to myotonic dystrophy kinase. *EMBO J.* **15,** 1885–1893.
8. Amano, M., Ito, M., Kimura, K., Fukata, Y., Chihara, K., Nakano, T., Matsuura, Y., and Kaibuchi, K. (1996) Phosphorylation and activation of myosin by Rho-associated kinase (Rho-kinase). *J. Biol. Chem.* **271,** 20246–20249.
9. Kimura, K., Ito, M., Amano, M., Chihara, K., Fukata, Y., Nakafuku, M., Yamamori, B., Feng, J., Nakano, T., Okawa, K., Iwamatsu, A., and Kaibuchi, K. (1996) Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase). *Science* **273,** 245–248.
10. Leung, T., Chen, X. Q., Manser, E., and Lim, L. (1996) The p160 RhoA-binding kinase ROKa is a member of a kinase family and is involved in the reorganization of the cytoskeleton. *Mol. Cell. Biol.* **16,** 5313–5327.
11. Amano, M., Chihara, K., Kimura, K., Fukata, Y., Nakamura, N., Matsuura, Y., and Kaibuchi, K. (1997) Formation of actin stress fibers and focal adhesions enhanced by Rho-kinase. *Science* **275,** 1308–1311.
12. Ishizaki, T., Naito, M., Fujisawa, K., Maekawa, M., Watanabe, N., Saito, Y., and Narumiya, S. (1997) p160ROCK, a Rho-associated coiled-coil forming protein kinase, works downstream of Rho and induces focal adhesions. *FEBS. Lett.* **404,** 118–124.
13. Kureishi, Y., Kobayashi, S., Amano, M., Kimura, K., Kanaide, H., Nakano, T., Kaibuchi, K., and Ito, M. (1997) Rho-associated kinase directly induces smooth muscle contraction through myosin light chain phosphorylation. *J. Biol. Chem.* **272,** 12257–12260.
14. Uehata, M., Ishizaki, T., Satoh, H., Ono, T., Kawahara, T., Morishita, T., Tamakawa, H., Yamagami, K., Inui, J., Maekawa, M., and Narumiya, S. (1997) Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. *Nature* **389,** 990–994.
15. Katoh, H., Aoki, J., Ichikawa, A., and Negishi, M. (1998) p160 RhoA-binding kinase ROKa induces neurite retraction. *J. Biol. Chem.* **273,** 2489–2492.
16. Amano, M., Chihara, K., Nakamura, N., Fukata, Y., Yano, T., Shibata, M., Ikebe, M., and Kaibuchi, K. (1998) Myosin II activation promotes neurite retraction during the action of Rho and Rho-kinase. *Genes Cells* **3,** 177–188.
17. Hirose, M., Ishizaki, T., Watanabe, N., Uehata, M., Kranenburg, O., Moolenaar, W. H., Matsumura, F., Maekawa, M., Bito, H., and Narumiya, S. (1998) Molecular dissection of the Rho-associated protein kinase (p160ROCK)-regulated neurite remodeling in neuroblastoma N1E-115 cells. *J. Cell Biol.* **141,** 1625–1636.
18. Matsui, T., Maeda, M., Doi, Y., Yonemura, S., Amano, M., Kaibuchi, K., Tsukita, S., and Tsukita, S. (1998) Rho-kinase phosphorylates COOH-terminal threonines of ezrin/radixin/moesin (ERM) proteins and regulates their head-to-tail association. *J. Cell Biol.* **140,** 647–657.
19. Fukata, Y., Kimura, K., Oshiro, N., Saya, H., Matsuura, Y., and Kaibuchi, K. (1998) Association of the myosin-binding subunit of myosin phosphatase and moesin: Dual regulation of moesin

phosphorylation by Rho-associated kinase and myosin phosphatase. *J. Cell Biol.* **141,** 409–418.

20. Kimura, K., Fukata, Y., Matsuoka, Y., Bennett, V., Matsuura, Y., Okawa, K., Iwamatsu, A., and Kaibuchi, K. (1998) Regulation of the association of adducin with actin filaments by Rho-associated kinase (Rho-kinase) and myosin phosphatase. *J. Biol. Chem.* **273,** 5542–5548.
21. Oshiro, N., Fukata, Y., and Kaibuchi, K. (1998) Phosphorylation of moesin by Rho-associated kinase (Rho-kinase) plays a crucial role in the formation of microvilli-like structures. *J. Biol. Chem.* **273,** 34663–34666.
22. Fukata, Y., Oshiro, N., Kinoshita, N., Kawano, Y., Matsuoka, Y., Bennett, V., Matsuura, Y., and Kaibuchi, K. (1999) Phosphorylation of adducin by Rho-kinase plays a crucial role in cell motility. *J. Cell Biol.* **145,** 347–361.
23. Riis, B., Rattan, S. I., Clark, B. F., and Merrick, W. C. (1990) Eukaryotic protein elongation factors. *Trends Biochem. Sci.* **15,** 420–424.
24. Demma, M., Warren, V., Hock, R., Dharmawardhane, S., and Condeelis, J. (1990) Isolation of an abundant 50,000-dalton actin filament bundling protein from *Dictyostelium* amoebae. *J. Biol. Chem.* **265,** 2286–2291.
25. Yang, F., Demma, M., Warren, V., Dharmawardhane, S., and Condeelis, J. (1990) Identification of an actin-binding protein from *Dictyostelium* as elongation factor 1a. *Nature* **347,** 494–496.
26. Owen, C. H., DeRosier, D. J., and Condeelis, J. (1992) Actin crosslinking protein EF-1 a of *Dictyostelium discoideum* has a unique bonding rule that allows square-packed bundles. *J. Struct. Biol.* **109,** 248–254.
27. Shiina, N., Gotoh, Y., Kubomura, N., Iwamatsu, A., and Nishida, E. (1994) Microtubule severing by elongation factor 1 alpha. *Science* **266,** 282–285.
28. Edmonds, B. T., Wyckoff, J., Yeung, Y. G., Wang, Y., Stanley, E. R., Jones, J., Segall, J., and Condeelis, J. (1996) Elongation factor-1 alpha is an overexpressed actin binding protein in metastatic rat mammary adenocarcinoma. *J. Cell Sci.* **109,** 2705–2714.
29. Amano, M., Mukai, H., Ono, Y., Chihara, K., Matsui, T., Hamajima, Y., Okawa, K., Iwamatsu, A., and Kaibuchi, K. (1996) Identification of a putative target for Rho as a serine-threonine kinase, PKN. *Science* **271,** 648–650.
30. Kawano, Y., Fukata, Y., Oshiro, N., Amano, M., Nakamura, T., Matsumura, F., Inagaki, M., and Kaibuchi, K. (1999) Phosphorylation of myosin-binding subunit (MBS) of myosin phosphatase by Rho-kinase during cell migration and cytokinesis. *J. Cell Biol.* **147,** 1023–1037.
31. Pardee, J. D., and Spudich, J. A. (1982) Purification of muscle actin. *Methods Enzymol.* **85,** 164–181.
32. Arimura, N., Inagaki, N., Chihara, K., Menager, C., Nakamura, N., Amano, M., Iwamatsu, A., Goshima, Y., and Kaibuchi, K. (2000) Phosphorylation of collapsin response mediator protein-2 by Rho-kinase. *J. Biol. Chem.* **275,** 23973–23980.
33. Iwamatsu, A. (1992) S-carboxymethylation of proteins transferred onto polyvinylidene difluoride membranes followed by *in situ* protease digestion and amino acid microsequencing. *Electrophoresis* **13,** 142–147.
34. Jensen, O. N., Vorm, O., and Mann, M. (1996) Sequence patterns produced by incomplete enzymatic digestion or one-step Edman degradation of peptide mixtures as probes for protein database searches. *Electrophoresis* **17,** 938–944.
35. Kawajiri, A., Itoh, N., Fukata, M., Nakagawa, M., Yamaga, M., Iwamatsu, A., and Kaibuchi, K. (2000) Identification of a novel β-catenin-interacting protein. *Biochem. Biophys. Res. Commun.* **273,** 712–717, doi:10.1006/bbrc.2000.3002.
36. Kuriyama, M., Harada, N., Kuroda, S., Yamamoto, T., Nakafuku, M., Iwamatsu, A., Yamamoto, D., Prasad, R., Croce, C., Canaani, E., and Kaibuchi, K. (1996) Identification of AF-6 and canoe as putative targets for Ras. *J. Biol. Chem.* **271,** 607–610.
37. Edmonds, B. T., Murray, J., and Condeelis, J. (1995) pH regulation of the F-actin binding properties of *Dictyostelium* elongation factor 1 alpha. *J. Biol. Chem.* **270,** 15222–15230.
38. Landon, F., Gache, Y., Touitou, H., and Olomucki, A. (1985) Properties of two isoforms of human blood platelet alpha-actinin. *Eur. J. Biochem.* **153,** 231–237.
39. Shimizu, H., Ito, M., Miyahara, M., Ichikawa, K., Okubo, S., Konishi, T., Naka, M., Tanaka, T., Hirano, K., Hartshorne, D. J., and Nakano, T. (1994) Characterization of the myosin-binding subunit of smooth muscle myosin phosphatase. *J. Biol. Chem.* **269,** 30407–30411.
40. Rayment, I., Holden, H. M., Whittaker, M., Yohn, C. B., Lorenz, M., Holmes, K. C., and Milligan, R. A. (1993) Structure of the actin-myosin complex and its implications for muscle contraction. *Science* **261,** 58–65.
41. Liu, G., Tang, J., Edmonds, B. T., Murray, J., Levin, S., and Condeelis, J. (1996) F-actin sequesters elongation factor 1alpha from interaction with aminoacyl-tRNA in a pH-dependent reaction. *J. Cell Biol.* **135,** 953–963.
42. Singer, R. H. (1992) The cytoskeleton and mRNA localization. *Curr. Opin. Cell Biol.* **4,** 15–19.
43. Bassell, G., and Singer, R. H. (1997) mRNA and cytoskeletal filaments. *Curr. Opin. Cell Biol.* **9,** 109–115.
44. Kislauskis, E. H., Zhu, X., and Singer, R. H. (1994) Sequences responsible for intracellular localization of beta-actin messenger RNA also affect cell phenotype. *J. Cell Biol.* **127,** 441–451.
45. Latham, V. M., Jr., Kislauskis, E. H., Singer, R. H., and Ross, A. F. (1994) Beta-actin mRNA localization is regulated by signal transduction mechanisms. *J. Cell Biol.* **126,** 1211–1219.
46. Kohno, H., Tanaka, K., Mino, A., Umikawa, M., Imamura, H., Fujiwara, T., Fujita, Y., Hotta, K., Qadota, H., Watanabe, T., Ohya, Y., and Takai, Y. (1996) Bni1p implicated in cytoskeletal control is a putative target of Rho1p small GTP binding protein in *Saccharomyces cerevisiae. EMBO J.* **15,** 6060–6068.
47. Umikawa, M., Tanaka, K., Kamei, T., Shimizu, K., Imamura, H., Sasaki, T., and Takai, Y. (1998) Interaction of Rho1p target Bni1p with F-actin-binding elongation factor 1alpha: Implication in Rho1p-regulated reorganization of the actin cytoskeleton in *Saccharomyces cerevisiae. Oncogene* **16,** 2011–2016.
48. Watanabe, N., Kato, T., Fujita, A., Ishizaki, T., and Narumiya, S. (1999) Cooperation between mDia1 and ROCK in Rho-induced actin reorganization. *Nat. Cell Biol.* **1,** 136–143.