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Proteomic Screening for Rho-kinase Substrates by Combining Kinase and Phosphatase Inhibitors with 14-3-3ζ Affinity Chromatography

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ABSTRACT. **The small GTPase RhoA is a molecular switch in various extracellular signals. Rho-kinase/ROCK/ROK, a major effector of RhoA, regulates diverse cellular functions by phosphorylating cytoskeletal proteins, endocytic proteins, and polarity proteins. More than twenty Rho-kinase substrates have been reported, but the known substrates do not fully explain the Rho-kinase functions. Herein, we describe the comprehensive screening for Rho-kinase substrates by treating HeLa cells with Rho-kinase and phosphatase inhibitors. The cell lysates containing the phosphorylated substrates were then subjected to affinity chromatography using beads coated with 14-3-3 protein, which interacts with proteins containing phosphorylated serine or threonine residues, to enrich the phosphorylated proteins. The identities of the molecules and phosphorylation sites were determined by liquid chromatography tandem mass spectrometry (LC/MS/MS) after tryptic digestion and phosphopeptide enrichment. The phosphorylated proteins whose phosphopeptide ion peaks were suppressed by treatment with the Rho-kinase inhibitor were regarded as candidate substrates. We identified 121 proteins as candidate substrates. We also identified phosphorylation sites in Partitioning defective 3 homolog (Par-3) at Ser143 and Ser144. We found that Rho-kinase phosphorylated Par-3 at Ser144 both *in vitro* and *in vivo*. The method used in this study would be applicable and useful to identify novel substrates of other kinases.**

Key words: phosphorylation/protein kinase/signal transduction/proteomics/mass spectrometry

Introduction

The small GTPase RhoA is a molecular switch in various extracellular signals. RhoA cycles between an inactive GDP-bound form and an active GTP-bound form through GDP/GTP exchange and a GTPase reaction (Jaffe and Hall, 2005) and is implicated in a variety of biological functions, including cell migration, contraction, adhesion, and polarization (Jaffe and Hall, 2005; Kaibuchi *et al.*, 1999). RhoA regulates these functions through specific effectors, such as Rho-kinase/ROCK/ROK, PKN, mDia, and phosphatidylinositol 5-kinase (Kaibuchi *et al.*, 1999; Narumiya *et al.*, 2009). Among these effectors, Rho-kinase appears to have a key role in mediating the critical functions of RhoA (Amano *et al.*, 2010a; Riento and Ridley, 2003). We previously found that Rho-kinase phosphorylates myosin phosphatase target protein 1 (MYPT1), a subunit of myosin phosphatase, and thereby inactivates the phosphatase activity of myosin phosphatase, resulting in a cell contraction with an increase in the level of phosphorylation of the myosin light chain (Kimura *et al.*, 1996). Since then, several groups, including our group, have identified specific substrates for Rho-kinase, including ezrin/radixin/moesin, adducin, Lim-kinase, endophilin, MARCKS, CRMP-2, MAP-2, intermediate filaments, Par-3, Tiam1/2, and p190 RhoGAP (Amano *et al.*, 2010a; Riento and Ridley, 2003). These substrates include cytoskeletal proteins, endocytic proteins, polarity regulators, and Rho family regulators and partly account for the modes of action of Rho-kinase (Amano *et al.*, 2010a). Rho-kinase phosphorylates Par-3 and FilGAP to regulate Rac activity for cell polarization (Nakayama *et al.*, 2008; Ohta *et al.*, 2006), but the known

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Abbreviations: LC/MS/MS, liquid chromatography tandem mass spectrometry; Par-3, Partitioning defective 3 homolog; MYPT1, myosin phosphatase target protein 1; CLA, calyculin A; FBS, fetal bovine serum; FDR, False discovery rate; GO, Gene Ontology.

substrates do not fully explain the functions of Rho-kinase in the establishment and maintenance of cell polarity.

The phosphorylation levels of most proteins are regulated *in vivo* by a balance between protein phosphatases and protein kinases (Salazar and Höfer, 2006). We previously found that the phosphorylation of the Rho-kinase substrates, including MYPT1, vimentin, and doublecortin, is increased by the treatment of the cells with calyculin A (CLA), a specific inhibitor of type 1 and 2A phosphatases, and that these phosphorylations are specifically inhibited by treatment with Y-27632, a Rho-kinase-specific inhibitor (Amano *et al.*, 2010b; Goto *et al.*, 1998). These results indicate that the substrates undergo a rapid turnover between their phosphorylated and dephosphorylated states; this combination of the inhibitors allowed us to examine the phosphorylation of the Rho-kinase substrates *in vivo*.

The 14-3-3 proteins are homologs of *C. elegans* polarity protein Par-5, and they regulate many cellular processes, such as cell division, signal transduction and cell polarization. 14-3-3 proteins regulate diverse target proteins by binding to phosphorylated Ser/Thr motifs (Bridges and Moorhead, 2005; Morrison, 2009). Moreover, 14-3-3 proteins appear to regulate cellular functions by inducing conformational changes in their target proteins, masking the phosphorylated region, or facilitating the binding of other proteins (Bridges and Moorhead, 2005; Morrison, 2009). To isolate the *in vivo* substrates of Rho-kinase associated with cell polarity, we took advantage of the 14-3-3 proteins, which specifically bind with proteins containing phospho-Ser/Thr (Bridges and Moorhead, 2005; Morrison, 2009).

In this study, we performed the comprehensive screening for Rho-kinase substrates using Y-27632 and CLA. The lysates of HeLa cells treated with Y-27632 and/or CLA were subjected to 14-3-3 pull-down assays to enrich the phosphorylated proteins. The molecular identity and phosphorylation sites of the enriched phosphorylated proteins were then determined by LC/MS/MS. The phosphorylated proteins with phosphopeptide ion peaks that were suppressed by the Y-27632 treatment were regarded as candidate substrates. We identified more than 100 proteins as candidate substrates for Rho-kinase, including novel Rho-kinase phosphorylation sites in Par-3.

Materials and Methods

Materials and chemicals

The cDNA encoding rat Par-3 was provided by Dr. Ohno (Yokohama City University School of Medicine). The cDNAs encoding 14-3-3ζ and 14-3-3ε were cloned from a human fetal brain cDNA library (Clontech Laboratories, Inc., California, USA). The polyclonal anti-MYPT1 antibodies were produced as described previously (Kawano *et al.*, 1999). The pCAGGS vector was provided by Dr. Nakafuku (Cincinnati Children's Hospital Medical Center, USA). Polyclonal anti-Adducin pT445, anti-p190A RhoGAP pS1150, and anti-CRMP2 pT514 were produced as described previously (Fukata *et al.*, 1999; Mori *et al.*, 2009; Yoshimura *et al.*, 2005). The polyclonal anti-Par-3 pS144 antibody was produced using the phosphopeptide Gly-Cys-His-Val-Arg-Arg-Ser-phospho Ser144-Asp-Pro-Ala-Leu-Thr by Biologica Co. (Aichi, Japan). The antiserum obtained was then affinity purified using that phosphopeptide. The polyclonal anti-phospho-MYPT1 (Thr850) antibody and the polyclonal anti-Par-3 antibody were supplied by Millipore (Massachusetts, USA). The polyclonal anti-phospho-Akt (Thr308) antibody was obtained from CST (Massachusetts, USA). Monoclonal anti-p190 antibody and the polyclonal anti-14-3-3ε antibody were obtained from BD Transduction Laboratories (New Jersey, USA). The polyclonal anti-14-3-3ζ antibody was obtained from Santa Cruz (California, USA). The monoclonal anti-tubulin antibody was obtained from Sigma-Aldrich (Missouri, USA). The monoclonal anti-His antibody was supplied by Qiagen (Hilden, Germany). Other materials and chemicals were obtained from commercial sources.

Plasmid construction

The mutants Par-3-1N-143A and Par-3-1N-144A were generated using a site-directed mutagenesis kit (Stratagene, California, USA) by changing Ser143 and Ser144 into alanines. The cDNAs encoding the Par-3 full-length and mutant fragments and full-length 14-3-3ε and ζ were subcloned into pGEX (GE healthcare, Buckinghamshire, England) or pCAGGS-myc-kk1, as described previously (Nishimura *et al.*, 2005). Protein production and purification were performed as described previously (Nishimura *et al.*, 2005). Briefly, GST- and histidine (His)-tagged proteins were produced in BL21(DE3) *Escherichia coli* cells and purified using glutathione Sepharose 4B beads (GE Healthcare) and Ni-NTA agarose (Qiagen), respectively.

Cell culture and transfection

HeLa and N1E-115 cells were cultured in DMEM with 10% fetal bovine serum (FBS), and the transfection was performed with Lipofectamine or Lipofectamine 2000 (Invitrogen, California, USA), according to the manufacturer's instructions.

CLA/Y-27632 treatment and 14-3-3ζ pull-down followed by LC/MS/MS

HeLa cells (5×10^5 cells/10-cm dish) were seeded and cultured for 24 hours and then cultured in DMEM without FBS. After 24 hours of serum starvation, the cells were treated with 20 μM Y-27632 or 0.1% DMSO for 30 minutes and then treated with 50 nM CLA or 0.05% DMSO for 12 minutes. The cells were gently washed with ice-cold PBS and scraped off the plate with ice-cold lysis buffer (20 mM Tris/HCl, 1% NP-40, 1 mM EDTA, 1 mM dithiothreitol, 150 mM NaCl, 50 nM CLA, 0.1 μM APMSF [*p*-amidinophenyl methansulfonyl fluoride], 1 μg/ml aprotinin, and 4 μg/ml leupep-

tin, pH 7.5). The lysate was incubated for 30 minutes at 4°C with rotation and centrifuged at 17,000×g for 20 minutes at 4°C; the supernatant was used as the cell lysate. Glutathione Sepharose 4B beads immobilized with 1 nmol of GST-14-3-3ζ were incubated with the cell lysate for 1 hour at 4°C with rotation. The beads were then washed with lysis buffer three times, and then with wash buffer (20 mM Tris/HCl, 1 mM EDTA, 1 mM dithiothreitol, 150 mM NaCl, 50 nM CLA, 0.1 μM APMSF, 1 μg/ml aprotinin, and 4 μg/ml leupeptin, pH 7.5) three times to remove the detergent from the beads. The bound proteins were extracted from the beads with guanidine solution (7 M guanidine and 50 mM Tris) and then reduced by incubating with 5 mM dithiothreitol for 30 minutes and alkylated with 10 mM iodoacetamide for 1 hour in the dark. The proteins were demineralized, concentrated by methanol/chloroform precipitation and digested with trypsin (50 mM NH_4HCO_3, 1.2 M urea, and 0.5 μg trypsin). The phosphopeptide enrichment was performed with a Titansphere® Phos-TiO Kit (GL Sciences, Tokyo, Japan) according to the manufacturer's instructions. LC/MS/MS was performed using an LTQ Orbitrap XL mass spectrometer (Thermo Scientific, Massachusetts, USA) connected to an HTC-PAL autosampler and a Paradigm MS4 HPLC (Michrom Bioresources, California, USA) with a C_{18} reversed-phase column and Michrom's ADVANCE Plug and Play Nano Source. The mass spectrometer was operated in the data-dependent MS^2 mode with multistage activation triggered by 97.97, 48.99, or 32.66 Da of neutral loss.

A peak list was generated and calibrated using MaxQuant software (version 1.1.1.25) (Cox and Mann, 2008). Database searches were performed against the complete proteome set of *Homo sapiens* (20,252) in UniProtKB 2010_11 concatenated with reversed copies of all sequences (Peng *et al.*, 2003) and supplemented with a contaminant list in MaxQuant (porcine trypsin, *Achromobacter lyticus* lysyl endopeptidase, and human keratins) using Andromeda, a search engine integrated into MaxQuant. The carbamidomethylation of cysteine was set as fixed, and the oxidation of methionine, the phosphorylation of serine/threonine/tyrosine, and N-terminal acetylation were set as variable modifications. A total of three missed cleavages by trypsin were allowed. The mass tolerance for fragment ions was set to 0.5 Da, and the maximum PEP was set to 1. False discovery rates (FDRs) for the peptide, protein, and site levels were set to 0.01. The minimum required peptide length was six amino acids. Three independent experiments were performed. The CLA-treated samples and untreated samples were analyzed independently because the CLA treatment substantially increased the number of phosphorylated peptides. Peptide ion intensities (log2 scale) were normalized by subtracting the median of all of the identified peptide intensities in each experiment. The ion intensities of the identified peptides in the controls and the CLA-treated samples were compared with those of the Y-27632-treated samples and the Y-27632+CLA-treated samples, respectively, and those phosphorylation sites with more than twofold higher ion intensity in at least two independent experiments were used as the candidate substrates for Rho-kinase.

Pull-down assays

The proteins in rat brain (P6 and P7) were extracted by the addition of lysis buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 150 mM NaCl, 1 mM dithiothreitol, 1 μM APMSF, 10 μg/ml leupeptin, 0.5 μM CLA, 0.5 μM okadaic acid, and 1.0% NP-40), followed by centrifugation at 100,000×g for 30 min at 4°C. The supernatant was incubated with the indicated GST fusion proteins for one hour at 4°C. The GST fusion proteins were then precipitated with glutathione Sepharose beads. The beads were washed three times with lysis buffer, eluted by boiling in SDS-PAGE sample buffer, and then subjected to immunoblot analysis with the indicated antibodies.

Phosphorylation assay

The phosphorylation assay was performed as previously described (Amano *et al.*, 1996). In brief, the kinase reaction of Rho-kinase for Par-3-1N was carried out in 50 μl reaction mixtures (50 mM Tris/HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, and 5 mM $MgCl_2$) containing 100 μM [γ-^{32}P]ATP (1–20 GBq/mmol), 0.1 μM of purified GST-Rho-k cat, and 1 μM of purified GST-Par-3 fragments. After incubation for 1, 4 or 10 min at 30°C, the reaction mixtures were boiled in SDS sample buffer and subjected to SDS-PAGE. The radiolabeled bands were visualized, and the density of each band was estimated using a BAS2000 image analyzer (Fujifilm, Tokyo, Japan).

Results

Combination of phosphatase inhibitor and kinase inhibitor followed by 14-3-3ζ pull-down

The treatment of HeLa cells with CLA induced the phosphorylation of Rho-kinase substrates, including adducin, MYPT1, and p190A RhoGAP, whereas the treatment with Y-27632 partially inhibited the CLA-induced phosphorylation (Fig. 1A), as previously described (Amano *et al.*, 2010b; Goto *et al.*, 1998). To enrich the phosphorylated proteins, the HeLa cell lysates treated with CLA and/or Y-27632 were subjected to affinity chromatography with beads coated with GST-14-3-3ζ or GST alone. The co-precipitated proteins were eluted with SDS sample buffer and were then subjected to SDS-PAGE, followed by silver staining. The CLA treatment increased the number and amount of proteins bound to GST-14-3-3ζ but not to GST, whereas Y-27632 had no apparent effect (Fig. 1B), suggesting that CLA induced the phosphorylation of a lot of proteins which associated with 14-3-3ζ, but Y-27632 did not affect the phosphorylation state of the most of the proteins. The immunoblot analysis with antibodies against MYPT1 and phosphorylated MYPT1 revealed that the associations between both the total MYPT1 and phosphorylated MYPT1 with GST-14-3-3ζ (but not with GST) were increased by

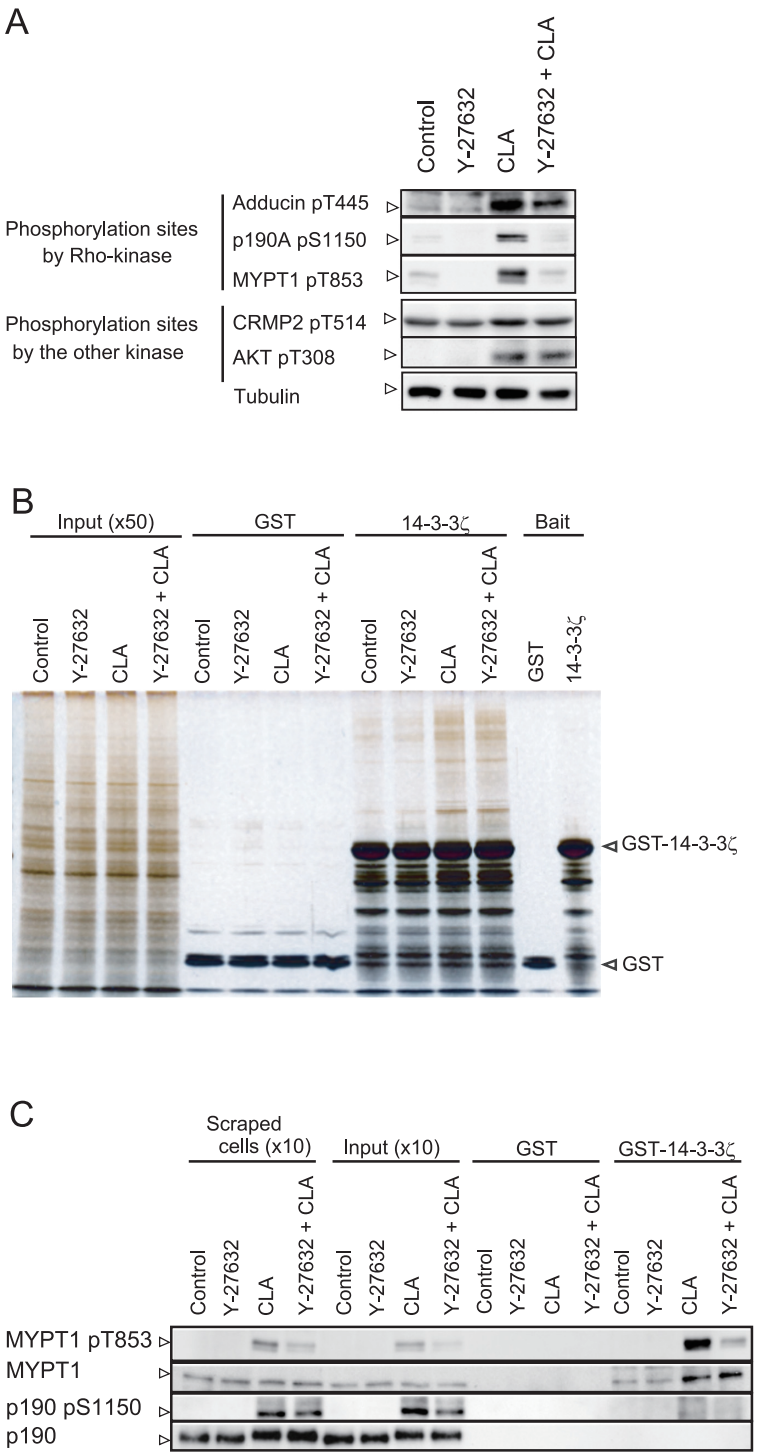
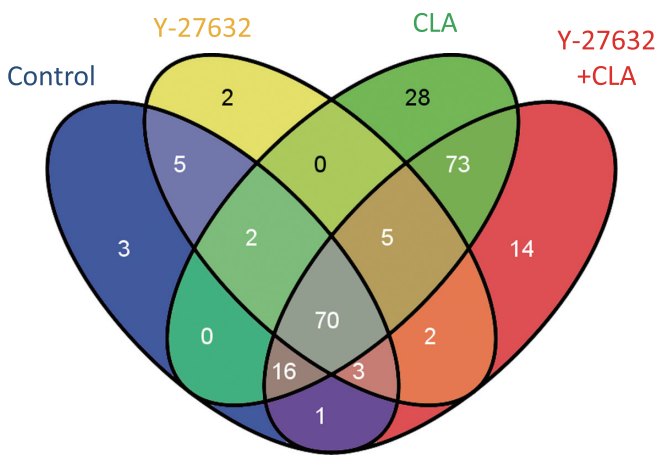


Fig. 1. Treatment with a phosphatase inhibitor and a kinase inhibitor followed by 14-3-3ζ pull-down. (A) Amplification of the effect of the kinase inhibitors by phosphatase inhibitor treatment. HeLa cells with 24-hour serum starvation were treated with 20 μM Y-27632 for 30 minutes and then with 50 nM calyculin A for 12 minutes. The cell lysates were analyzed by immunoblot analysis with the indicated antibodies. (B), (C) GST-14-3-3ζ pull-down assay enriched phosphoproteins. Phosphoproteins were examined immediately after cell lysis (Scraped cells), before the pull-down assay (Input) and in the eluate (GST and GST-14-3-3ζ). Proteins were detected by silver staining (B) or immunoblotting with the indicated antibodies (C).

A



B

Number of candidate substrates for Rho-kinase

Samples	Sites	Proteins
Candidates *		
CLA treated	227	100
CLA untreated	95	46
Total	297	121
Total phosphorylation sites	946	224
CLA-increased phosphorylation sites**	592	175

C

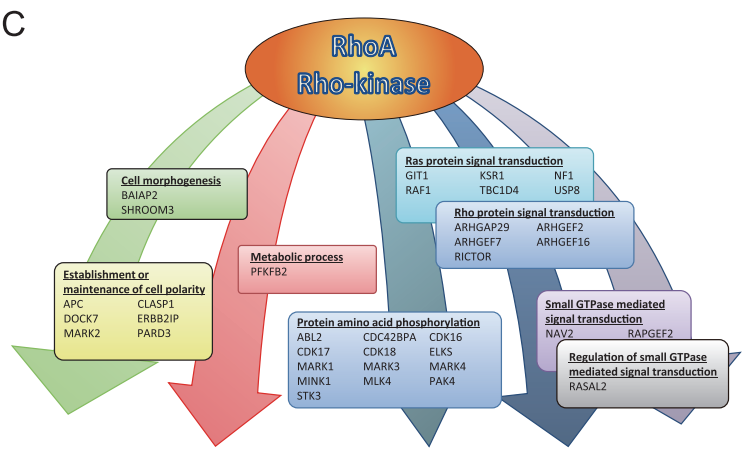


Fig. 2. Identification of the phosphoproteins induced by CLA/Y-27632 treatment. (A) The number of identified phosphoproteins in each sample is shown in Venn diagram (created by VENNY. Oliveros, J.C. <http://bioinfogp.cnb.csic.es/tools/venny/index.html>, 2007). (B) The number of candidate substrates for Rho-kinase. Phosphorylation sites with twofold higher ion intensity relative to the Y-27632-treated (*) or control (**) samples in at least two independent experiments were counted. C) Accumulation of candidate proteins in the biological processes in which Rho-kinase plays important roles. The distribution of GO biological processes of the 121 proteins potentially regulated by Rho-kinase was compared to that of the total human genome (analyzed by FatiGO (Al-Shahrour *et al.*, 2004), <http://www.babelomics.org.>, $p<0.01$, GO levels from 6 to 9), and proteins with significantly enriched ontologies are shown.

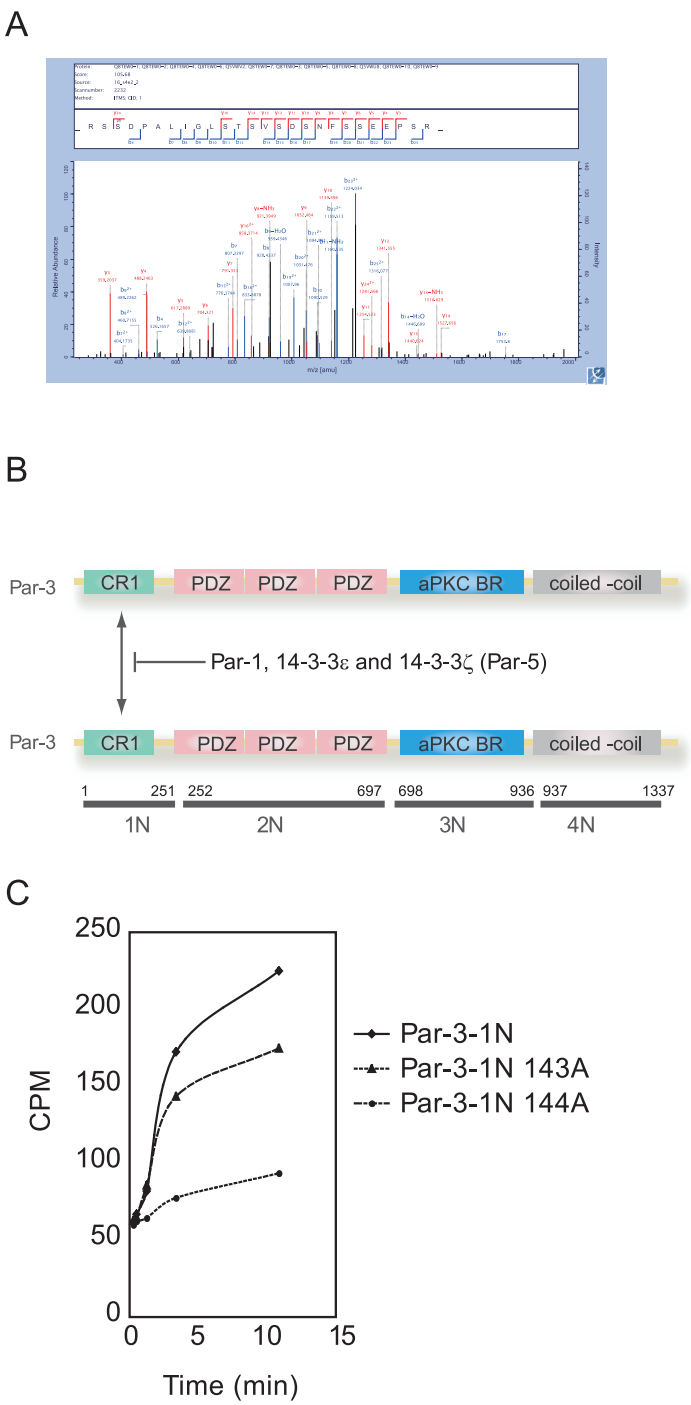
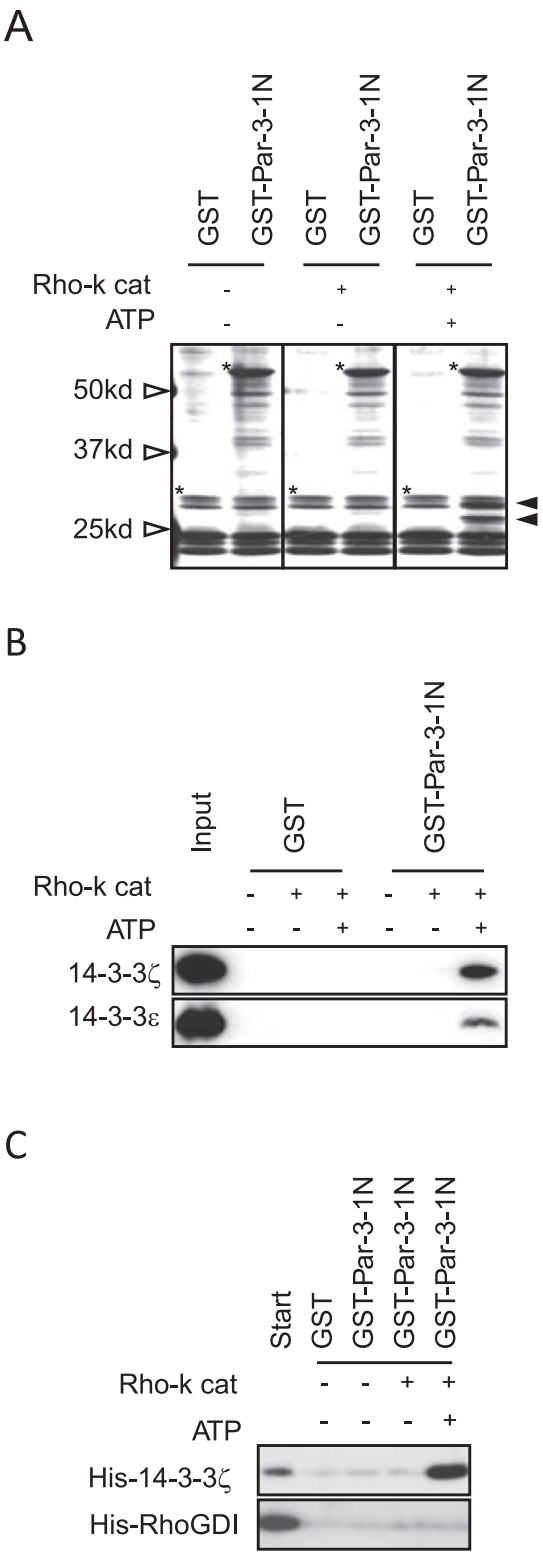


Fig. 3. Phosphorylation of Par-3 at Ser143 and Ser144 by Rho-kinase *in vitro*. (A) A representative MS/MS spectrum of the Par-3 peptides with pS144. (B) The domain structure of Par-3. Par-3 forms oligomers via CR1. Par-1 phosphorylates Par-3 and promotes the interaction of Par-3 with 14-3-3ε and ζ, thereby disrupting Par-3 oligomer formation. CR1, conserved region 1; PDZ, PSD95/Dlg/ZO-1; aPKC BR, aPKC binding region. C) Phosphorylation of Par-3-1N mutants by Rho-kinase. The GST fusion Par-3-1N mutants were incubated with Rho-k cat for 1, 4, or 10 min in the presence of [γ-^{32}P]ATP. The reaction mixtures were subjected to SDS-PAGE, and the radioactive proteins were detected by autoradiography. The results shown are representative of three independent experiments. CPM: count per minute.



the treatment with CLA (Fig. 1C). Interestingly, the association of phosphorylated MYPT1, but not of total MYPT1, with GST-14-3-3ζ was decreased by the treatment with Y-27632, suggesting that MYPT1 is phosphorylated by other kinases, such as PKA, in addition to Rho-kinase and that the association of MYPT1 with 14-3-3ζ is mediated by these other kinases, as previously described (Koga and Ikebe, 2008). p190A RhoGAP, one of the substrates of Rho-kinase, was not associated with GST-14-3-3ζ (Fig. 1C), indicating that this screening excluded unbound 14-3-3 Rho-kinase substrates.

LC/MS/MS analysis of enriched phosphopeptides

To identify the proteins bound to GST-14-3-3ζ, the bound proteins were extracted and digested by trypsin, and the phosphopeptides were enriched using a titania column, followed by LC/MS/MS using an LTQ Orbitrap XL mass spectrometer. The phosphopeptides that were identified at least twice in three independent experiments were used in further analyses, and 946 phosphorylation sites in 224 proteins were identified (Supplementary Table 1, Fig. 2A, B). Of the identified proteins, 25% (56 proteins) overlapped with previously reported 14-3-3ζ binding proteins (Supplementary Table 1), and 61.2% of the identified proteins had 14-3-3 binding motifs with high stringency (Supplementary Table 2, predicted by Scansite (Obenauer, 2003)).

We obtained 297 sites in 121 proteins as potential substrate sites of Rho-kinase (Supplementary Table 1, Fig. 2B). Among the candidates, three proteins (MYPT1, vimentin, and Par-3) were among the 28 known Rho-kinase substrates (Amano *et al.*, 2010a), and 10 proteins overlapped with the previously identified substrates of Rho-kinase that were identified by affinity column chromatography using the catalytic domain of Rho-kinase (Amano *et al.*, 2010b) (Supplementary Table 3). The substrate candidates we identified were significantly enriched in the Gene Ontology (GO) biological process categories in which Rho-kinase is known to play an essential role (Fig. 2C), indicating that our screen-

Fig. 4. Promotion of the interaction of Par-3 with 14-3-3ε and 14-3-3ζ by Rho-kinase phosphorylation. (A), (B) Effect of the phosphorylation of Par-3-1N by Rho-kinase on the interaction of Par-3 with 14-3-3ε and 14-3-3ζ. GST-Par-3-1N was phosphorylated by Rho-kinase *in vitro*. Phosphorylated or non-phosphorylated GST protein-immobilized beads were incubated with rat brain lysate. (A) The bound proteins were subjected to SDS-PAGE and were analyzed by silver staining. Asterisks indicate each GST fusion protein. Arrowheads indicate phospho-Par-3-specific-interacting proteins. (B) The samples were analyzed by immunoblotting with the indicated antibodies. (C) Promotion of a direct interaction of Par-3 with 14-3-3ζ by Rho-kinase. GST fusion Par-3-1N was phosphorylated by Rho-kinase *in vitro*. Phosphorylated or non-phosphorylated GST protein-immobilized beads were incubated with His-14-3-3ζ or His-RhoGDI. The binding proteins were analyzed by immunoblotting with anti-His-tag antibodies.

ing of Rho-kinase substrates effectively identified the candidate substrates for Rho-kinase.

Rho-kinase phosphorylation of Par-3 Ser144

To determine whether this method is useful for novel Rho-kinase substrate screening, we focused on Par-3 (Fig. 3A). Par-3 plays an important role in various cell polarization events by forming a polarity complex with its binding partners (Suzuki and Ohno, 2006). Par-3, MARK2, and 14-3-3 are homologs of *C. elegans* polarity regulating Par proteins (Par-3, Par-1, and Par-5, respectively), and Par-1 is known to phosphorylate Par-3 at Ser144 and to recruit 14-3-3, resulting in the prevention of the dimerization and activity of Par-3 (Fig. 3B) (Kemphues *et al.*, 1988; Hurd *et al.*, 2003; Benton and St Johnston, 2003). We previously found that Rho-kinase phosphorylates Par-3 at Thr833 and thereby inhibits its binding to Tiam1 (a Rac1 GEF), leading to the inactivation of Rac1. However, this phosphorylation does not fully explain how Rho-kinase regulates the functions of Par-3. Thus, we further explored the physiological relevance of the phosphorylation of Par-3 at Ser144 by Rho-kinase. To confirm the phosphorylation of Par-3 at Ser144 by Rho-kinase, we performed a Rho-kinase phosphorylation assay using the Ser144-containing Par-3-1N fragment (amino acids 1-251) and found that GST-Rho-k cat (a constitutively active form of Rho-kinase) efficiently phosphorylated Par-3-1N (Fig. 3C). The efficiency of the phosphorylation of GST-Par-3-1N-144A was substantially reduced relative to that of the wild-type protein but that of GST-Par-3-1N-143A was not substantially reduced (Fig. 3C), suggesting that Rho-kinase phosphorylates Par-3 and that its major phosphorylation site is Ser144.

Promotion of the interaction of Par-3 with 14-3-3ε and 14-3-3ζ by Rho-kinase phosphorylation

To confirm the interaction of Par-3 with 14-3-3, GST-Par-3-1N was stoichiometrically phosphorylated by GST-Rho-k cat and then incubated with rat brain lysate. A 30 kDa protein was specifically precipitated with the GST-Par-3-1N that had been phosphorylated by Rho-kinase (Fig. 4A), and this 30 kDa protein was recognized by antibodies against 14-3-3ε and 14-3-3ζ (Fig. 4B). To examine the direct interaction of phosphorylated Par-3-1N with 14-3-3 proteins, phosphorylated or non-phosphorylated GST-Par-3-1N was incubated with His-14-3-3ζ. His-14-3-3ζ interacted with phosphorylated GST-Par-3-1N but not with GST or unphosphorylated GST-Par-3-1N (Fig. 4C). These results indicate that Rho-kinase phosphorylates Par-3 at Ser144 and promotes the interaction of Par-3 with 14-3-3.

Phosphorylation of Par-3 at Ser144 by Rho-kinase in vivo

To examine the Par-3 phosphorylation by Rho-kinase *in vivo*, we prepared an antibody that specifically recognized Par-3 phosphorylated at Ser144. The specificity of the anti-Par-3 pS144 antibody was examined by immunoblot analysis. The anti-Par-3 pS144 antibody specifically recognized GST-Par-3-1N (but not GST-Par-3-1N-144A) that was phosphorylated by Rho-kinase in a dose-dependent manner (Fig. 5A). Last, we investigated the *in vivo* phosphorylation of Par-3 at Ser144 in HeLa cells. The immunoblot analysis with the anti-Par-3 pS144 antibody revealed that the treatment of HeLa cells with CLA increased the level of phosphorylation of Par-3 at Ser144, whereas Y-27632 inhibited the CLA-induced phosphorylation of Par-3 at Ser144 (Fig. 5B). The treatment of the cells with CLA also increased the association of Par-3 phosphorylated at Ser144 with GST-14-3-3ζ, and Y-27632 inhibited this association (Fig. 5B), suggesting that Rho-kinase phosphorylates Par-3 at Ser144 *in vivo* and that this phosphorylation promotes the association of Par-3 with 14-3-3.

To monitor the phosphorylation of Par-3 at Ser144 that is induced by RhoA activation, we stimulated HeLa cells and N1E-115 cells with LPA because LPA is known to stimulate the Rho/Rho-kinase pathway in certain types of cells. However, the phosphorylation of this site in these cells was not affected by LPA-mediated stimulation or Y-27632-mediated inhibition (data not shown), suggesting that LPA did not induce the Par-3 phosphorylation under the condition.

Discussion

In this study, we identified more than 100 substrate candidates for Rho-kinase (Supplementary Table 1, Fig. 2A, B). Among the candidate sites, we found that Rho-kinase directly phosphorylated Par-3 at Ser144 (Fig. 3C), inducing the association of 14-3-3 with Par-3 (Fig. 4C). This association inhibits the dimerization of Par-3, which is required for the function of Par-3 in cell polarization (Benton and St Johnston, 2003; Hurd *et al.*, 2003). We previously found that Rho-kinase phosphorylates Par-3 at Thr833 and thereby inhibits the interaction of Par-3 with Tiam1 (a Rac1 GEF) to prevent Par-3-mediated Rac1 activation (Nakayama *et al.*, 2008). Thus, Rho-kinase appears to inhibit the functions of Par-3 through the phosphorylation of at least two distinct sites in Par-3.

We also identified many peptides and phosphorylation sites without using the pull-down technique with the 14-3-3-coated beads. These phosphorylation sites were diverse, and the results were not reproducible; thus, it is difficult to determine the physiological relevance of these phosphorylation sites (data not shown). It should be noted that most of

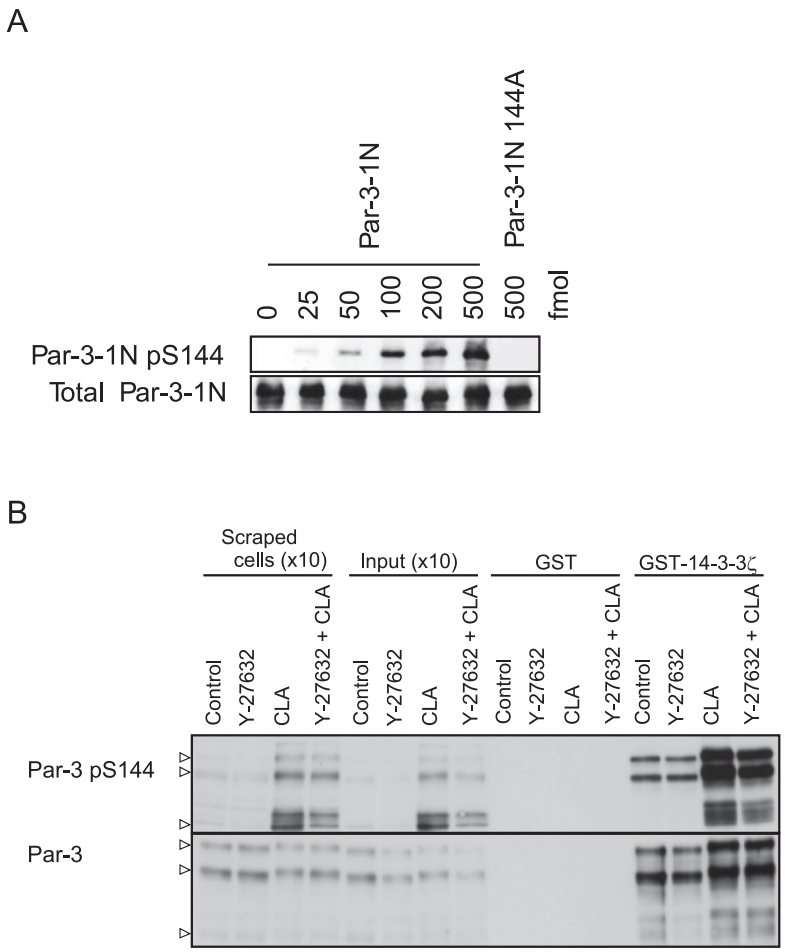


Fig. 5. Phosphorylation of Par-3 at Ser144 by Rho-kinase *in vivo*. (A) Specificity of the phospho-specific anti-Par-3-pS144 antibody. GST-Par-3-1N (2 nmol) containing the indicated amount of Par-3-1N; Par-3-1N-144A phosphorylated by Rho-k cat was subjected to SDS-PAGE. Immunoblot analysis with anti-Par-3-pS144 antibody (upper panel) or anti-GST antibody (lower panel) was carried out. (B) Phosphorylation of Par-3 at Ser144 by Rho-kinase *in vivo*. Control, Y-27632-treated, CLA-treated, and Y-27632+CLA-treated HeLa cells were subjected to a GST-14-3-3ζ pull-down assay. Phospho-Ser144 of Par-3 was examined with anti-Par-3 pS144 antibodies immediately after cell lysis (Scraped cells), before the pull-down assay (Input) and in the eluate (GST and GST-14-3-3ζ).

these phosphorylated proteins are abundant and involved in housekeeping processes and do not overlap with the proteins obtained after the pull-down, suggesting that the pull-down with the beads coated with 14-3-3 effectively enriched a specific population of the phosphorylated proteins (data not shown). Because 14-3-3 specifically interacts with proteins through a consensus motif containing phosphate and regulates protein functions through inhibiting dimerization, intramolecular association or association with other partners (Bridges and Moorhead, 2005; Morrison, 2009), our proteomic approach with the 14-3-3 beads appears to enrich the functionally important phosphoproteins.

In addition to Par-3, we obtained more than 100 candidate substrates for Rho-kinase; these proteins are involved in cell polarity, cell morphogenesis, metabolic processes or Rho signaling. Moreover, several of these candidates appear to be substrates of Rho-kinase, but we need to determine whether they are in fact phosphorylated by Rho-kinase to conclude that they are real substrates. A certain population of the candidates may be phosphorylated by Rho-kinase and associated with 14-3-3 through its phosphorylation sites, whereas the remaining candidates may be phosphorylated by other kinases prior to association with 14-3-3. It is also possible that the remaining candidates may be phosphorylated by Rho-kinase at sites that are different from the 14-3-3 binding site, as the phosphorylation was inhibited by Y-27632. Further detailed analyses of the substrate candidates should provide important insight into the RhoA/Rho-kinase-mediated signal network.

We have recently reported more than three hundreds candidate substrates for Rho-kinase by combining affinity

chromatography using Rho-kinase and LC/MS/MS (Amano *et al.*, 2010b). We found that several candidates, including APC, PFKFB2, and NF1, were obtained in both methods. These candidates appear to be good substrates for Rho-kinase both *in vitro* and *in vivo*. The number of overlapping candidates was less than expected. This smaller-than-expected overlap may be explained by the notion that 14-3-3 enriches the specific population of Rho-kinase substrates because 14-3-3 preferentially associates with a consensus motif that contains phosphate. Another reason may be the difference between the protein profiles in HeLa cells and rat brain.

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