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Identification of Calponin as a Novel Substrate of Rho-Kinase

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Calponin, an F-actin-associated protein implicated in the regulation of smooth muscle contraction, is known to be phosphorylated *in vitro* by protein kinase C (PKC) and Ca^{2+}/calmodulin dependent protein kinase II (CaM kinase II). Unphosphorylated calponin binds to F-actin and inhibits the actin-activated myosin ATPase activity; these properties are lost on phosphorylation. In the present study, we found that Rho-kinase phosphorylated basic calponin stoichiometrically *in vitro*. We identified the sites of phosphorylation of calponin by Rho-kinase as Thr-170, Ser-175, Thr-180, Thr-184, and Thr-259, and prepared antibodies that specifically recognized calponin phosphorylated at Thr-170 and Thr-184. We showed that the phosphorylation of calponin by Rho-kinase inhibited the binding of calponin to F-actin. Taken together, these results suggest that calponin is a substrate of Rho-kinase and that Rho-kinase regulates the interaction of calponin with F-actin.

Key Words: **small guanosine triphosphatase (GTPase); Rho; Rho-kinase; calponin; F-actin; smooth muscle; myosin light chain (MLC).**

Rho is a small guanosine triphosphatase (GTPase) that exhibits both GDP/GTP binding and GTPase activities. Rho has GDP-bound inactive (GDP · Rho) and GTP-bound active (GTP · Rho) forms, which are interconvertible by GDP/GTP exchange and GTPase reactions (for reviews, see 1–3). When cells are stimulated with certain extracellular signals such as lysophosphatidic acid, GDP · Rho is converted to GTP · Rho, which binds to specific effectors and then exerts its biological functions. Rho participates in signaling pathways that regulate stress fiber and focal adhesion formation in fibroblasts (4, 5). Rho is also involved in the regulation of cell morphology (6), cell motility (7), cytokinesis (8, 9), membrane ruffling (10), smooth muscle contraction (11, 12), and the synthesis of phosphatidylinositol 4,5-diphosphate via phosphatidylinositol 5-kinase (13). Recently, several effector proteins of Rho have been identified: *e.g.*, protein kinase N, Rho-kinase/ROK/ROCK, myosin binding subunit (MBS) of myosin phosphatase, Rhophilin, Rhotekin, Citron, Citron-kinase and m-Dia (for a review, see 14).

Among these effectors, Rho-kinase has been implicated in many processes downstream of Rho: *e.g.*, stress fiber and focal adhesion formation (15–17), smooth muscle contraction (18), intermediate filament disassembly (19, 20), neurite retraction (21–23), microvillus formation (24), cytokinesis (25), and cell migration (26). Rho-kinase regulates the phosphorylation of myosin light chain (MLC) by direct phosphorylation and by inactivation of myosin phosphatase through the phosphorylation of MBS (27, 28). In addition to MLC, the ezrin/radixin/moesin (ERM) family of proteins and adducin were found to be substrates of both Rho-kinase and myosin phosphatase (29–31). Rho-kinase and myosin phosphatase are thought to control the phosphorylation level of a subset of substrates and to regulate cytoskeletal organization cooperatively *in vivo*.

Abbreviations used: PKC, protein kinase C; CaM kinase II, Ca^{2+}/calmodulin-dependent kinase II; MBS, myosin-binding subunit; MLC, myosin light chain; ERM, ezrin/radixin/moesin; GST, glutathione-*S*-transferase; GTPγS, guanosine 5′-(3-*O*-thio)-triphosphate; DTT, dithiothreitol.

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Calponin was originally discovered in smooth muscle as an F-actin-, calmodulin-, and tropomyosin-binding protein (32). Recently, three types of calponin isoforms, acidic, neutral, and basic calponin, have been classified on the basis of their isoelectric point (33–35). All calponin isoforms are composed of Vav/CDC24 homology domain, actin-binding consensus sequence, and COOH-terminal UNC-87 repeats domain (32). Basic calponin is distributed relatively specifically in smooth muscle tissues (33) and has been well characterized *in vitro*. The binding of basic calponin to F-actin has an inhibitory effect on the actin-activated myosin ATPase activity (32), and this inhibitory effect is reduced through the phosphorylation of calponin by certain kinases such as protein kinase C (PKC) and Ca^{2+}/calmodulin dependent protein kinase II (CaM kinase II) (36). Basic calponin was shown to be dephosphorylated by smooth muscle myosin phosphatase (37). The addition of smooth muscle basic calponin reduced Ca^{2+}-activated tension of permeabilized smooth muscle (38). Taken together, the data implicate basic calponin in the regulation of smooth muscle contraction, which regulation depends on the phosphorylation state of calponin, although neither has direct evidence been obtained nor has the underlying mechanism been demonstrated. Acidic and neutral calponins are expressed in both muscle and non-muscle tissues (35, 39), but their functions are still not clear. Because basic calponin is a probable substrate of myosin phosphatase, we hypothesize that calponin is phosphorylated by Rho-kinase and that its activity is regulated by Rho-kinase/myosin phosphatase pathway in smooth muscle contraction.

In the present study, we found that Rho-kinase phosphorylated basic calponin stoichiometrically *in vitro*. We determined the major sites of phosphorylation of calponin by Rho-kinase, and prepared antibodies that specifically recognized calponin phosphorylated by Rho-kinase. Furthermore, we demonstrated that phosphorylation of calponin by Rho-kinase inhibited the binding of calponin to F-actin.

MATERIALS AND METHODS

Materials and chemicals. Glutathione-S-transferase (GST)-Rho-kinase CAT (6–553 amino acids) was produced by baculovirus-infected Sf9 cells (40) and purified on a glutathione-Sepharose column (41). Rho-kinase was purified from bovine brain (41). GST-RhoA was purified from *Escherichia coli* and loaded with guanine nucleotides as described (42). Chicken gizzard basic calponin was purified by previously described (36). F-actin was purified from an acetone powder prepared from rabbit skeletal muscle as described (43). [γ-^{32}P] ATP was purchased from Amersham Corp. Guanosine 5′-(3-*O*-thio)-triphosphate (GTPγS) was purchased from Beringer Mannheim Biochem. Other materials and chemicals were obtained from commercial sources.

Phosphorylation assay. The kinase reaction of GST-Rho-kinase CAT was carried out in 50 μl of Buffer A (50 mM Tris–HCl at pH 7.5, 2 mM EGTA, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 6 mM KCl) containing 100 μM [γ-^{32}P]ATP (1–20 GBq/mmol), recombinant kinase (0.1 μM), and calponin (0.5 μM). After incubation for 1 h at 30°C, the reaction mixture was boiled in SDS sample buffer and subjected to SDS–PAGE. The kinase reaction of native Rho-kinase was carried out in 50 μl of Buffer B (50 mM Tris–HCl at pH 7.5, 2.2 mM EDTA, 7 mM $MgCl_2$, 1 mM DTT, 6 mM KCl, 0.2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) containing 50 μM [γ-^{32}P]ATP (1–20 GBq/mmol), purified enzyme (6.0 nM), and calponin (1.0 μM) with GTPγS · GST-RhoA or GDP · GST-RhoA (1.7 μM). After incubation for 10 min at 30°C, the reaction mixtures were boiled in SDS sample buffer and subjected to SDS–PAGE. An image analyzer (FujiX Bioimage analyzer Bas2000 System; Fuji Film Co. Ltd. Tokyo, Japan) was used to visualize the radiolabeled bands.

Determination of phosphorylation sites of calponin by Rho-kinase. Calponin (340 pmol of protein) was phosphorylated with GST-Rho-kinase CAT (58 pmol of protein) in 500 μl of Buffer A containing 100 μM [γ-^{32}P]ATP for 1 h at 30°C, and the phosphorylated calponin was digested with *Achromobacter* protease I at 37°C for 20 h. The obtained peptides were applied onto a c18 reverse phase column (SG120; 4.6 × 250 mm, Shiseido, Japan) and eluted with a linear gradient of 0–48% acetonitrile for 100 min at a flow rate of 1.0 ml/min by HPLC (System Gold, Beckman), and the radioactive peaks were separated. Phosphoamino acid sequence of each peptide was carried out with a peptide sequencer (PPSQ-10, Shimadzu, Japan). The fractions obtained from each Edman degradation cycle were counted for ^{32}P in a Beckman liquid scintillation counter.

Production of site- and phosphorylation state-specific antibodies for calponin. Rabbit polyclonal antibodies against calponin phosphorylated at Thr-170 (anti-calponin-pT170 antibody) and at Thr-184 (anti-calponin-pT184 antibody) were raised as described (44). The phosphopeptides (CGLQMGpT^{170}NKFAS and acetyl-CMT-AYGpT^{184}RRHLY) for calponin were chemically synthesized for use as immunogens and bound to a carrier protein, keyhole limpet hemocyanin, via the NH_2-terminal cysteine residue of the peptides by Peptide Institute Inc. (Osaka, Japan). The antisera obtained were then affinity-purified against the respective phosphopeptides.

Co-sedimentation assay. Calponin (3.5 μM) was phosphorylated or not with GST-Rho-kinase CAT (2.5 μM) in 125 μl Buffer C (50 mM Tris–HCl at pH 7.5, 5 mM $MgCl_2$, 1 mM DTT, 100 μM ATP, 18 mM KCl, 0.7 μM calyculin A) for 1 h at 30°C. F-actin (16 μM) was mixed with indicated amount of calponin. Phosphorylated or non-phosphorylated calponin was incubated for 30 min at 25°C in Buffer D (20 mM Tris–HCl at pH 7.5, 30 mM KCl, 2 mM $MgCl_2$, 1 mM ATP, 1 mM DTT), 0.2 mM $CaCl_2$, 0.2 μM calyculin A, 8.6% (w/v) sucrose). After the incubation, 50 μl of each reaction mixture was layered onto a 100-μl sucrose barrier (20% (w/v) sucrose in Buffer D) and centrifuged at 100,000*g* for 1 h at 25°C. The supernatants and pellets were separated and subjected to immunoblot analysis using monoclonal anti-calponin antibody (CP-93; SIGMA).

Other procedures. SDS–PAGE was performed as described previously (45).

RESULTS

Rho-kinase and MBS of myosin phosphatase dually regulate the phosphorylation levels of MLC, adducin, and ERM, and are thought to modulate cytoskeletal organization (27–31).

To further understand the molecular mechanisms of the regulation of cytoskeletal organization, we explored the substrate of Rho-kinase and myosin phosphatase. We hypothesized that the phosphorylation level of calponin, which is one of actin-binding proteins

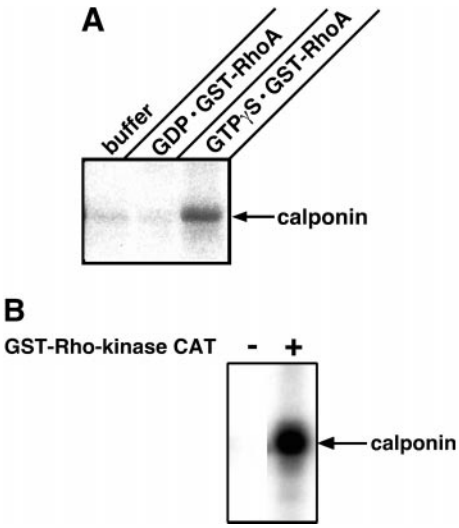

FIG. 1. Phosphorylation of calponin by Rho-kinase. (A) Basic calponin was phosphorylated by Rho-kinase purified from bovine brain. The phosphorylated protein was resolved by SDS–PAGE and visualized by an image analyzer. Phosphorylation of basic calponin was markedly enhanced by the addition of GTPγS · GST-RhoA, but not by that of GDP · GST-RhoA. Arrow denotes the phosphorylated basic calponin. (B) Basic calponin was phosphorylated by constitutively activated Rho-kinase (GST-Rho-kinase CAT). The kinase reaction was carried out in the presence or absence of GST-Rho-kinase CAT. About 1.3 pmol of phosphate was maximally incorporated into 1.0 pmol of basic calponin by GST-Rho-kinase CAT.

and a substrate of myosin phosphatase, might be regulated by Rho-kinase and myosin phosphatase in smooth muscle contraction. So, we first examined whether basic calponin could be phosphorylated by Rho-kinase. Rho-kinase purified from bovine brain phosphorylated basic calponin in a GTPγS · GST-RhoA-dependent manner (Fig. 1A). GTPγS is a nonhydrolyzable GTP analog. Because we could not obtain a sufficient amount of purified Rho-kinase, we employed GST-Rho-kinase CAT in subsequent experiments, which was previously shown to be constitutively active *in vitro* and *in vivo* (16, 28). GST-Rho-kinase CAT phosphorylated basic calponin (Fig. 1B), and the amount of phosphate incorporated into calponin was approximately 1.3 mol per 1 mol of protein under the condition. Basic calponin phosphorylated by Rho-kinase showed slower mobility than non-phosphorylated calponin on SDS–PAGE gels (data not shown).

Then we determined the major sites of basic calponin phosphorylated by Rho-kinase. Phosphorylated basic calponin was digested with *Achromobacter* protease I, separated by HPLC, and subjected to peptide sequencing. Six radioactive peaks (named peaks 1 to 6) were obtained (Fig. 2). We did not determine the phosphorylation sites in peaks 1 and 4, because these peaks were minor ones. The amino acid sequences determined were FASQQGMTAYGTRRHLYDPK (peaks 2, 3), LREGRNIIGLQMGTNK (peak 5), and GASQQGMTVYGLPRQVYDPK (peak 6), corresponding to residues 173–192, 157–172, and 252–271 of basic calponin, respectively (Fig. 2), and the phosphorylation sites were identified as Thr-170, Ser-175, Thr-180, Thr-184, and Thr-259 (Fig. 2). Rho-kinase phosphorylated Thr-184 more efficiently than Ser-175 and Thr-180, judging from the radioactivity of each amino acid from the Edman degradation cycle (data not shown). Thr-170 also seemed to be one of the major phosphorylation sites. The identified sites except Thr-259 are conserved among the calponin isoforms (Fig. 3).

All these isoforms of calponin are composed of Vav/CDC24 homology domain, actin-binding consensus sequence, and UNC-87 repeats (32). Phosphorylation sites of Thr-170, Ser-175, Thr-180 and Thr-184 are located in the first UNC-87 repeat, and Thr-259 is located in the third one.

To investigate the phosphorylation of calponin by Rho-kinase, we prepared rabbit polyclonal antibodies raised against the synthetic phosphopeptides pT170 (CGLQMGpT^{170}NKFAS) for anti-calponin-pT170 antibody and pT184 (acetyl-CMTAYGpT^{184}RRHLY) for anti-calponin-pT184 antibody (Fig. 3). Equal amounts of phosphorylated and non-phosphorylated forms of calponin (1.0 pmol) were loaded onto a gel. Anti-calponin-pT170 antibody and anti-calponin-pT184 antibody specifically recognized basic calponin phosphorylated by Rho-kinase, but not the non-phosphorylated basic calponin (Fig. 4). These results confirm that Thr-170 and Thr-184 are phosphorylated by Rho-kinase.

It has been shown that the activity of calponin is affected by phosphorylation. Phosphorylation of calponin by some kinases decreases its F-actin binding activity (36). To examine whether the phosphorylation of

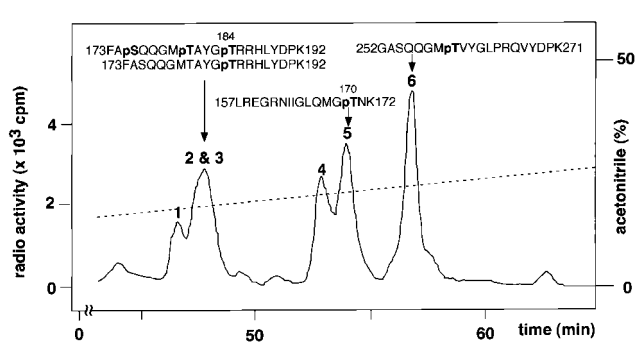

FIG. 2. Determination of the phosphorylation sites of calponin recognized by Rho-kinase. Basic calponin was incubated with GST-Rho-kinase CAT as described under Materials and Methods, and the phosphorylated protein was digested with *Achromobacter* protease I. The obtained peptides were applied onto a C18 reverse phase column and eluted by HPLC with a linear gradient of 0–48% acetonitrile (dotted line) for 100 min. The radioactive peptides were separated, and phosphoamino acid sequencing was carried out with a peptide sequencer. Six major radioactive peptides (peaks 1–6) were obtained as demonstrated. We determined the amino acid sequences and phosphorylated amino acids of peaks 2, 3, 5, and 6.

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basic (chick)     MSNANFNRGP AYGLSAEVKN KLAQKYDPQT ERQLRVWIEG ATGRRIGDNF MDGLKDGVIL CELINTLQPG SVQKVNDPVQ NWHKLENIGN  90
basic (human)     MSSAHFNRGP AYGLSAEVKN KLAQKYDHQR EQELREWIEG VTGRRIGNNF MDGLKDGIIL CEFINKLQPG SVKKINESTQ NWHQLENIGN  90
acidic (human)    M--THFNKGP SYGLSAEVKN KIASKYDHQA EEDLRNWIEE VTGMSIGPNF QLGLKDGIIL CELINKLQPG SVKKVNESSL NWPQLENIGN  88
neutral (human)   MSSTQFNKGP SYGLSAEVKN RLLSKYDPQK EAELRTWIEG LTGLSIGPDF QKGLKDGTIL CTLMNKLQPG SVPKINRSMQ NWHQLENLSN  90

                                                                                                    pT170
                                                                                                  *   *
basic (chick)     FLRAIKHYGV KPHDIFEAND LFENTNHTQV QSTLIALASQ AKTKGNNVGL GV--KYAEKQ QRRFQPEKLR EGRNIIGLQM GTNKFASQQG 178
basic (human)     FIKAITKYGV KPHDIFEAND LFENTNHTQV QSTLLALASM AKTKGNKVNV GV--KYAEKQ ERKFEPGKLR EGRNIIGLQM GTNKFASQQG 178
acidic (human)    FIKAIQAYGM KPHDIFEAND LFENGNMTQV QTTLVALAGL AKTKGFHTTI DIGVKYAEKQ TRRFDEGKLK AGQSVIGLQM GTNKCASQAG 178
neutral (human)   FIKAMVSYGM NPVDLFEAND LFESGNMTQV QVSLLALAGK AKTKGLQSGV DIGVKYSEKQ ERNFDDATMK AGQCVIGLQM GTNKCASQSG 180

                  pT184
                *   *                                                                                 *
basic (chick)     MTAYGTRRHL YDPKLGTDQP LDQATISLQM GTNKGASQAG MTAPGTKRQI FEPSLGMERC DTNIIGLQMG SNKGASQQGM TVYGLPRQVY 268
basic (human)     MTAYGTRRHL YDPKLGTDQP LDQATISLQM GTNKGASQAG MTAPGTKRQI FEPGLGMEHC DTLNVSLQMG SNKGASQRGM TVYGLPRQVY 268
acidic (human)    MTAYGTRRHL YDPKMQTDKP FDQTTISLQM GTNKGASQAG MLAPGTRRDI YDQKLTLQPV DNSTISLQMG TNKVASQKGM SVYGLGRQVY 268
neutral (human)   MTAYGTRRHL YDPKNHILPP MDHSTISLQM GTNKCASQVG MTAPGTRRHI YDTKLGTDKC DNSSMSLQMG YTQGANQSG- QVFGLGRQIY 269

basic (chick)     DPKYCDAPGL --LGEDGLN- ---------- ----H----- ------SF-- ---YNSQ--- -                            292
basic (human)     DPKYCLTPEY PELGEPAHN- ---------- -HHAH----- ------NY-- ---YNSA--- -                            297
acidic (human)    DPKYCAAPTE PVIHNGSQGT GTNGSEISDS DYQAEYPDEY HGEYQDDYPR DYQYSDQGID Y                               329
neutral (human)   DPKYC--PQG TVADGAPSGT G--------- ----DCPD-- PGEVP-EYPP -Y-YQEEA-G Y                               309
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FIG. 3. Phosphorylation sites of calponin by Rho-kinase. The phosphorylation sites of basic calponin (Thr-170, Ser-175, Thr-180 Thr-184, and Thr-259) recognized by Rho-kinase are indicated (*). All of the identified sites except Thr-259 are conserved among the calponin isoforms. To examine the phosphorylation of calponin by Rho-kinase, we prepared the phosphorylation state-specific antibodies against pT170 (anti-calponin-pT170 antibody) and pT184 (anti-calponin-pT184 antibody). Parts of sequence used for chemical synthesis of immunogen are indicated by brackets.

calponin by Rho-kinase modulates F-actin binding activity of calponin, we performed a cosedimentation assay using basic calponin and F-actin. Basic calponin bound to F-actin in a dose-dependent manner. Basic calponin stoichiometrically phosphorylated by GST-Rho-kinase CAT cosedimented with F-actin less efficiently than non-phosphorylated calponin (Fig. 5). These results indicate that the phosphorylation of basic calponin by Rho-kinase decreased the F-actin binding activity of calponin *in vitro.*

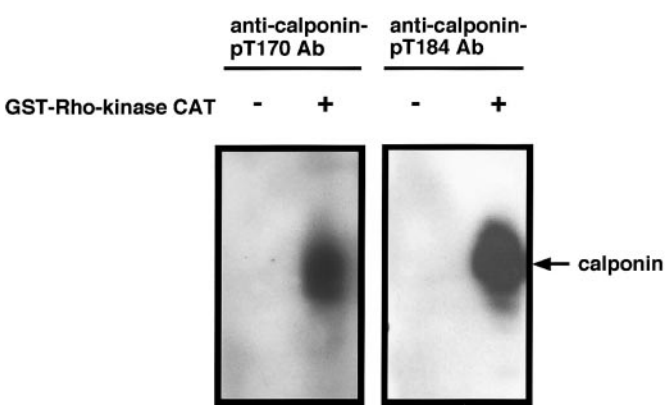

FIG. 4. Immunoblot analysis with phosphorylated calponin antibodies. One pmol of basic calponin was incubated without (−) or with (+) GST-Rho-kinase CAT and then subjected to SDS–PAGE. Immunoblot analysis with anti-calponin-pT170 antibody (left) or anti-calponin-pT184 antibody (right) was carried out. Both antibodies specifically recognized the calponin phosphorylated by Rho-kinase. Arrow denotes the phosphorylated basic calponin.

DISCUSSION

It was previously shown that Rho-kinase and myosin phosphatase regulate the phosphorylation level of a subset of substrates, including MLC (27, 28), ERM (29, 30), and adducin (31), downstream of Rho. MLC, ERM, and adducin are phosphorylated by Rho-kinase, and dephosphorylated by myosin phosphatase. Here we found that Rho-kinase phosphorylated basic calponin

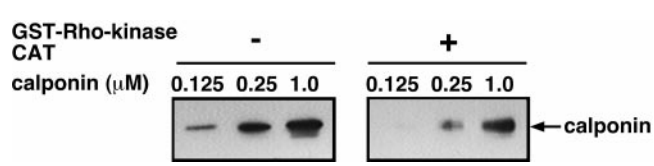

FIG. 5. Effect of phosphorylation of calponin by Rho-kinase on the F-actin binding activity of calponin. Purified basic calponin was incubated without (−) or with (+) GST-Rho-kinase CAT. Indicated doses of calponin were mixed with F-actin (16 μM) and incubated at 25°C for 20 min. After the incubation, 50 μl of each reaction mixture was layered onto 100 μl of 20% (w/v) sucrose barrier and centrifuged at 100,000*g* for 1 h at 25°C. Pellets were subjected to immunoblot analysis using anti-calponin antibody (CP-93). Arrow denotes the basic calponin.

in vitro (Fig. 1). Basic calponin was shown earlier to be dephosphorylated by smooth muscle myosin phosphatase (37). These results raise the possibility that the phosphorylation level of basic calponin is regulated by Rho-kinase and myosin phosphatase, as in the case of MLC, ERM, and adducin.

Basic calponin was shown previously to be phosphorylated by PKC and CaM kinase II, at both Ser-175 and Thr-184 (36, 46, 47). Phosphorylation of basic calponin by either kinase resulted in the loss of the ability of calponin to inhibit the actin-activated myosin ATPase (36), and Thr-184 is thought to be functionally important site of phosphorylation (48). Here we found that basic calponin is phosphorylated at Thr-170, Ser-175, Thr-180, Thr-184, and Thr-259 by Rho-kinase (Figs. 2 and 3). Phosphorylation of basic calponin by Rho-kinase appeared to inhibit the binding of calponin to F-actin (Fig. 5), which would be expected to result in an increase in actin-activated myosin ATPase activity. In fact, Rho-kinase was shown to induce the contraction in smooth muscle (18). We prepared anti-calponin-pT170 antibody and anti-calponin-pT184 antibody, because Thr-170 and Thr-184 are thought to be the major phosphorylation sites. Thr-170, Thr-180, and Thr-259 have not been reported as sites of phosphorylation by any other kinase. The effects of phosphorylation at those sites on the activity of calponin are not clear. Thr-170 is a major site of phosphorylation of calponin by Rho-kinase and is conserved among the three isoforms. Thus we propose that Thr-170 would be suitable to monitor the Rho-kinase specific phosphorylation and that Thr-184 would be suitable to monitor the function of calponin. These antibodies will be useful tools to explore *in vivo* the effects of calponin phosphorylated by Rho-kinase.

There are three isoforms of calponin: basic, acidic, and neutral calponin (32). Basic calponin is expressed specifically in smooth muscle tissues (32), and has been implicated in smooth muscle contraction. Acidic calponin is expressed in brain, lung, aorta, kidney, intestine, and stomach (35), whereas neutral calponin is expressed in fibroblasts, vascular endothelial cells and keratinocytes (39). UNC-87 repeats, Vav/CDC24 homology domain and actin-binding sequence, which were originally identified in the basic calponin isoform, are conserved in both acidic and neutral calponins (Fig. 3). Although the functions of acidic and neutral calponin are not known, they are predicted to be similar to those of basic calponin.

Basic calponin is known to regulate smooth muscle contraction. We showed here that Rho-kinase phosphorylated basic calponin and that the phosphorylation of calponin by Rho-kinase inhibited the binding of calponin to F-actin. Basic calponin has been reported to be dephosphorylated by smooth muscle myosin phosphatase (37). It is likely that the phosphorylation levels of basic calponin are regulated by Rho-kinase and myosin phosphatase, which are Rho effectors. These findings suggest that Rho-kinase and myosin phosphatase can regulate the interaction of basic calponin with F-actin downstream of Rho, resulting in the regulation of the contraction of the actomyosin system. As the phosphorylation sites of basic calponin recognized by Rho-kinase are conserved among the three isoforms except Thr-259 (Fig. 3), Rho-kinase and myosin phosphatase may regulate not only basic calponin but also acidic and neutral calponins in various tissues.

Most importantly, future studies need to be conducted to determine if calponin is phosphorylated by Rho-kinase and if Rho-kinase regulates the interaction of calponin with F-actin *in vivo*. Also, further experiments are necessary to understand in better detail relationship between Rho-kinase/myosin phosphatase and calponin.

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