Identification of the novel substrates of Rho-kinase

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Identification of calponin as a novel substrate of Rho-kinase

ABSTRACT

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, I found that Rho-kinase phosphorylated basic calponin stoichiometrically *in vitro*. I 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. I 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)

INTRODUCTION

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*.

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, I 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, I found that Rho-kinase phosphorylated basic calponin stoichiometrically *in vitro*. I determined the major sites of phosphorylation of calponin by Rho-kinase, and prepared antibodies that specifically recognized calponin phosphorylated by Rho-kinase. Furthermore, I 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). [g-^{32}P]ATP was purchased from Amersham Corp. Guanosine 5′-(3-*O*-thio)-triphosphate (GTPgS) was purchased from Beringer Mannheim Biochem. Other materials and chemicals were obtained from commercial sources.

Phoshorylation assay. The kinase reaction of GST-Rho-kinase CAT was carried out in 50 ml 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 mM [g-^{32}P]ATP (1-20 GBq/mmol), recombinant kinase (0.1 mM), and calponin (0.5 mM). After incubation for 1 hr 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 ml 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 mM [g-^{32}P]ATP (1-20 GBq/mmol), purified enzyme (6.0 nM), and calponin (1.0 mM) with GTPgS·GST-RhoA or GDP·GST-RhoA (1.7 mM). 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 ml of Buffer A containing 100 mM [g-^{32}P]ATP for 1 hr at 30˚C, and the phosphorylated calponin was digested with *Achromobacter* protease I at 37˚C for 20 hr. The obtained peptides were applied onto a C18 reverse phase column (SG120; 4.6 X 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-CMTAYGpT^{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 mM) was phosphorylated or not with GST-Rho-kinase CAT (2.5 mM) in 125 ml Buffer C (50 mM Tris/HCl at pH 7.5, 5 mM $MgCl_2$, 1 mM DTT, 100 mM ATP, 18 mM KCl, 0.7 mM calyculin A) for 1 hr at 30˚C. F-actin (16 mM) 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 mM calyculin A, 8.6%(w/v) sucrose). After the incubation, 50 ml of each reaction mixture was layered onto a 100-ml sucrose barrier (20%(w/v) sucrose in Buffer D) and centrifuged at 100,000×g for 1 hr 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, I explored the substrate of Rho-kinase and myosin phosphatase. I hypothesized that the phosphorylation level of calponin, which is one of actin-binding proteins and a substrate of myosin phosphatase, might be regulated by Rho-kinase and myosin phosphatase in smooth muscle contraction. So, I first examined whether basic calponin could be phosphorylated by Rho-kinase. Rho-kinase purified from bovine brain phosphorylated basic calponin in a GTPgS·GST-RhoA-dependent manner (**Fig. 1A**). GTPgS is a nonhydrolyzable GTP analog. Because I could not obtain a sufficient amount of purified Rho-kinase, I 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 I 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**). I 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, I 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 calponin by Rho-kinase modulates F-actin binding activity of calponin, I 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*.

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 I found that Rho-kinase phosphorylated 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 I 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). I 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 I 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. I 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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LEGENDS TO FIGURES

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 GTPgS·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.

FIG. 2. Determination of the phosphorylation sites of calponin recognized by Rho-kinase. Basic calponin was incubated with GST-Rho-kinase CAT as described in 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. I determined the amino acid sequences and phosphorylated amino acids of peaks 2, 3, 5, and 6.

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, I 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.

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.

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 mM) and incubated at 25˚C for 20 min. After the incubation, 50 ml of each reaction mixture was layered onto 100 ml of 20% (w/v) sucrose barrier and centrifuged at 100,000×g for 1 hr at 25˚C. Pellets were subjected to immunoblot analysis using anti-calponin antibody (CP-93). Arrow denotes the basic calponin.

FOOTNOTES

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; GTPgS, guanosine 5'-(3-*O*-thio)-triphosphate; DTT, dithiothreitol

Fig. 1

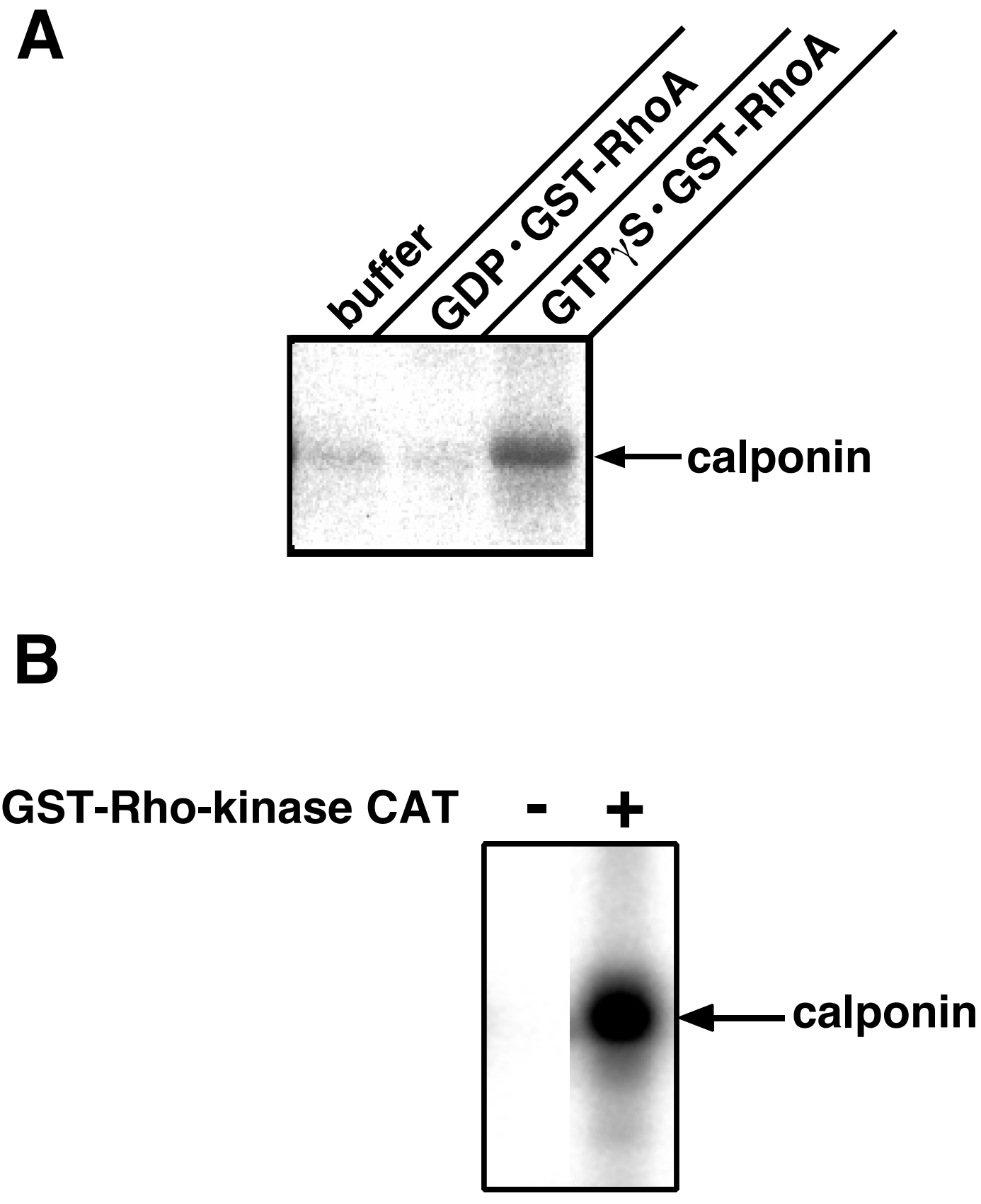
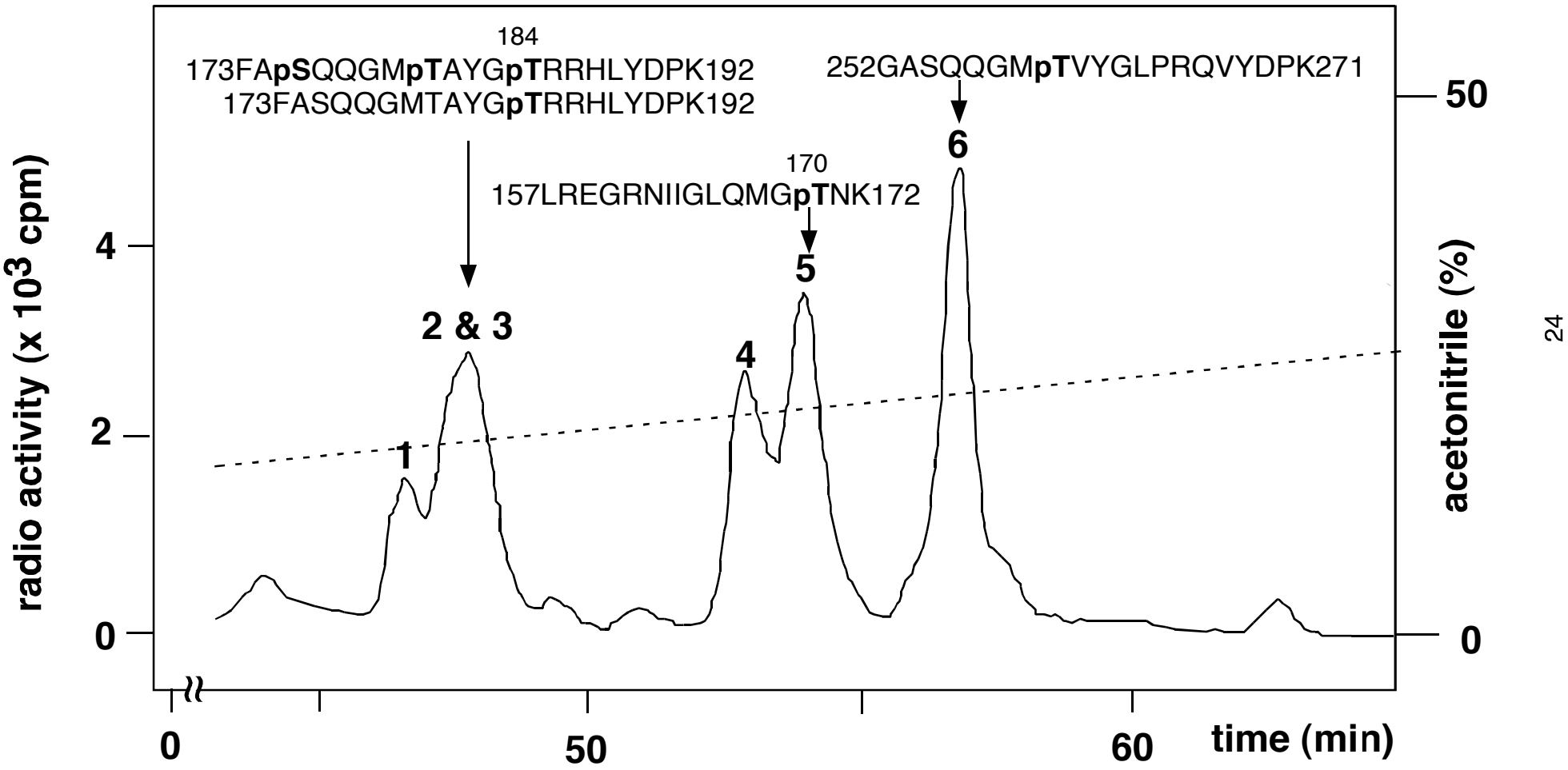

A
buffer
GDP・GST-RhoA
GTPγS・GST-RhoA
calponin
B
GST-Rho-kinase CAT
-
+
calponin

Fig. 2


184
173FA**pS**QQGM**pT**AYG**pT**RRHLYDPK192
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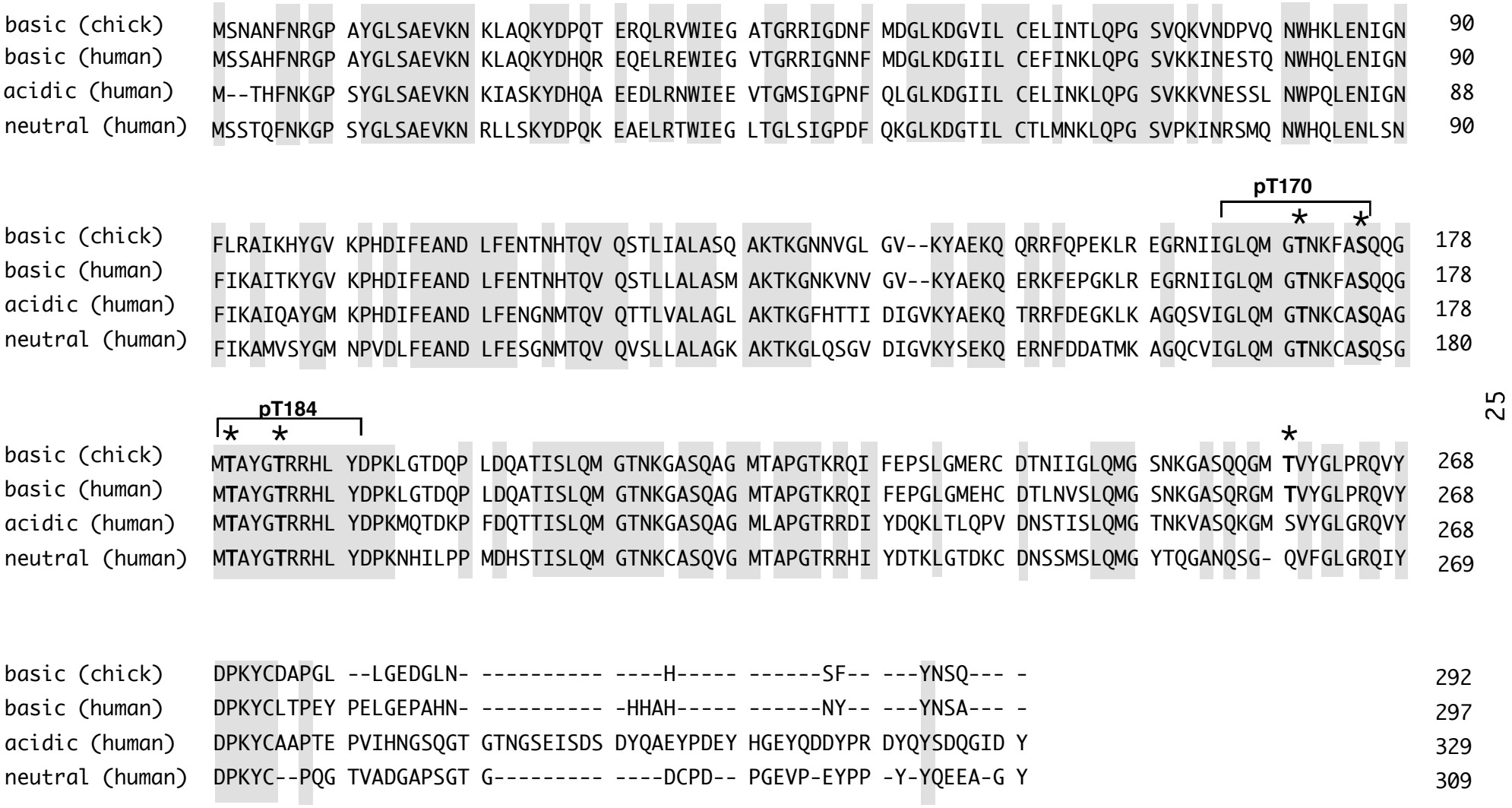
252GASQQGM**pT**VYGLPRQVYDPK271
170
157LREGRNIIGLQMG**pT**NK172
1
2 & 3
4
5
6
radio activity (x 10^3 cpm)
acetonitrile (%)
time (min)
0
2
4
0
50
60
50
0

Fig. 3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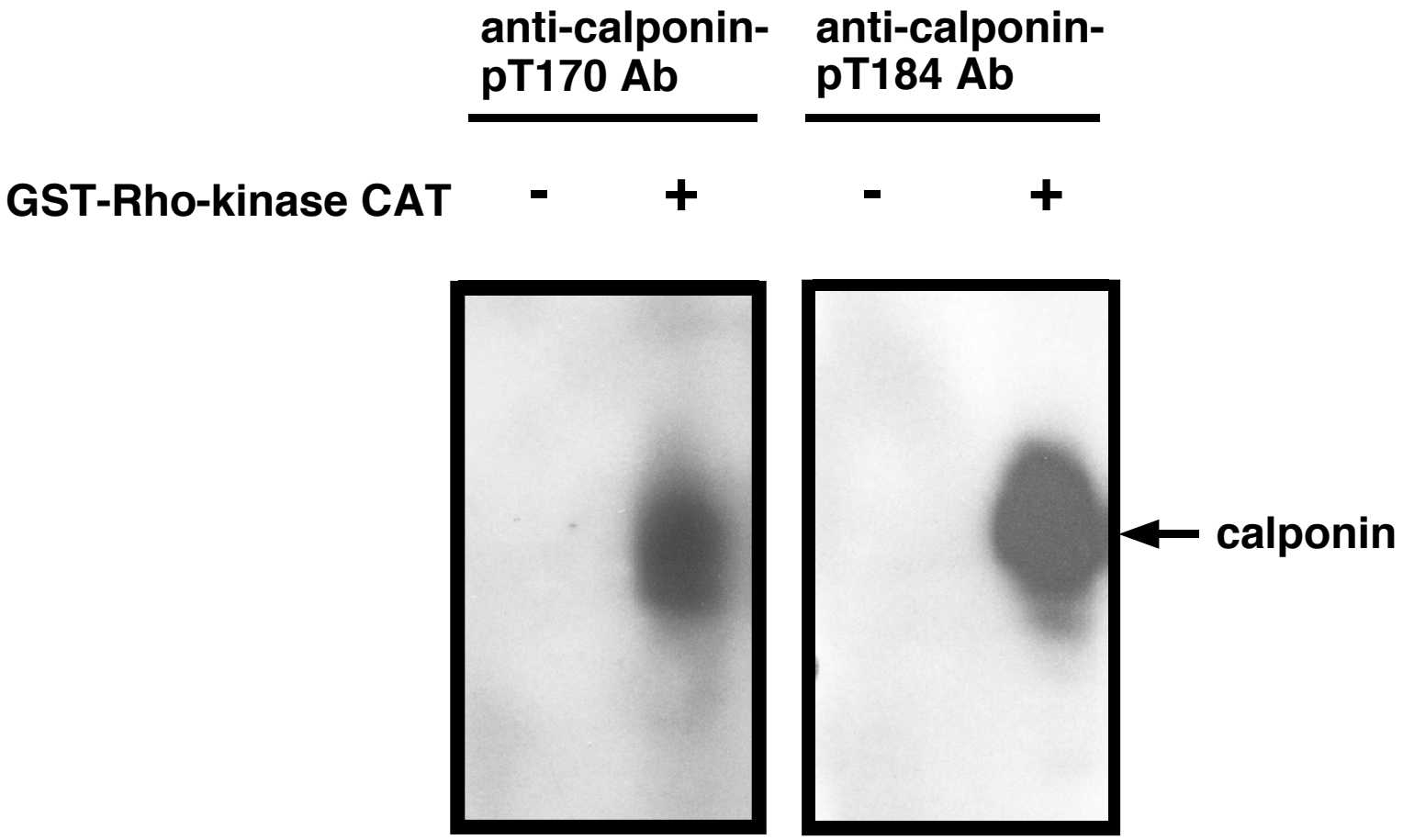
basic (human) MSSAHFNRGP AYGLSAEVKN KLAQKYDHQR EQELREWIEG VTGRRIGNNF MDGLKDGIIL CEFINKLQPG SVKKINESTQ NWHQLENIGN 90
acidic (human) M--THFNKGP SYGLSAEVKN KIASKYDHQA EEDLRNWIEE VTGMSIGPNF QLGLKDGIIL CELINKLQPG SVKKVNESSL NWPQLENIGN 88
neutral (human) MSSTQFNKGP SYGLSAEVKN RLLSKYDPQK EAELRTWIEG LTGLSIGPDF 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

Fig. 4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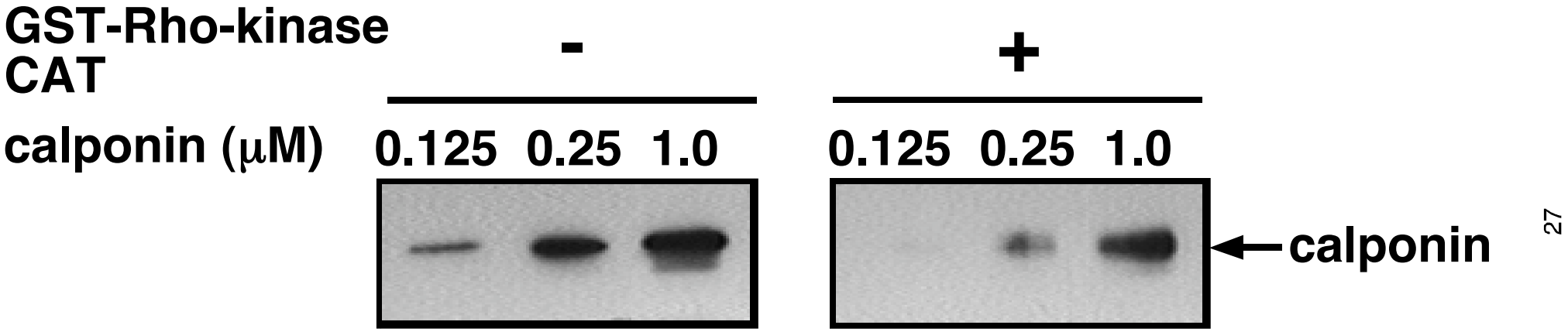
anti-calponin-pT184 Ab
GST-Rho-kinase CAT
-
+
-
+
calponin

Fig. 5


GST-Rho-kinase CAT
-
+
calponin (μM)
0.125 0.25 1.0
0.125 0.25 1.0
calponin

Identification of endophilin A1 as a novel substrate of Rho-kinase

Abstract

Rho-associated protein kinase (Rho-kinase) as a specific effector of Rho. Here, I identified endophilin A1 as a novel substrate of Rho-kinase. Endophilin A1 interacts with synaptojanin and dynamin. Endophilin is involved in the clathrin-mediated endocytotic complex. I found that Rho-kinase phosphorylated endophilin A1 at Thr-14. Furthermore, overexpression of constitutively active Rho or endophilin^{T14D} (substitution of Thr-14 by Asp) inhibited the clathrin-mediated endocytosis in cells, whereas endophilin^{T14A} (substitution of Thr-14 by Ala) counteracted the inhibitory effect of constitutively active Rho. Taken together, these results suggest that endophilin is a substrate of Rho-kinase and that Rho/Rho-kinase regulates the clathrin-mediated endocytosis through the phosphorylation of endophilin.

Introduction

Clathrin-mediated endocytosis is one of the primary mechanisms by which eukaryotic cells internalize nutrients, antigens, and growth factors and recycle receptors and vesicles (1,2). Many compartments of the clathrin-mediated endocytosis machinery have been identified, including dynamin, endophilin, and synaptojanin (3,4).

The small GTPase Rho regulates the formation of actin stress fibers and focal adhesions (5-7) and membrane ruffring (8,9), and cell motility (10,11) in certain types of cells, cell morphology (12), cell aggregation (13), smooth muscle contraction (14,15), neurite retraction in neuronal cells (16,17), the formation of microvilli (18), and cytokinesis (19,20). Recently, the study has shown that Rho regulates the clathrin-mediated endocytosis (21). Rho exerts its biological functions through interaction with specific effectors (22).

Previously, a novel serine/threonine kinase, Rho-associated kinase (Rho-kinase) /ROKa/ROCK II, was identified as an effector of Rho (23-25). Rho-kinase is activated by the GTP-bound active form of Rho (24-26). Rho-kinase appears to regulate various cellular responses downstream of Rho (27). Rho-kinase regulates the phosphorylation of myosin light chain (MLC), resulting in actomyosin contractility, by the direct phosphorylation of MLC (28) and by the inactivation of myosin phosphatase through the phosphorylation of myosin binding subunit (MBS) (29). However, the function of Rho-kinase has not yet been clarified.

In this study, I searched for novel substrates of Rho-kinase in brain, and identified endophilin A1. Endophilin has been implicated in several stages of clathrin-mediated endocytosis, form

generating membrane curvature, an early step, to later events such as vesicle fission and uncoating (3,4,30,31). Endophilin displays lysophosphatidic acid acyltransferase activity at its N terminus (32). Its C-terminal Src-homology-3 (SH3) domain selectively interacts with other endocytotic proteins, such as dynamin and synaptojanin, and may recruit these proteins to the nerve terminal (31,32). I also identified Thr-14 as the site of phosphorylation in endophilin A1 by Rho-kinase. The phosphorylation of endophilin A1 by Rho-kinase appears to plays a crucial role in clathrin-mediated endocytosis.

Materials and Methods

Materials and chemicals

Anti-endophilin antibody was kindly provided by Dr. P. De Camili (Yale Univ., New Haven, USA). cDNAs-encoding mouse endophilin A1 and $RhoA^{V14}$ were provided by Dr. K. Takei (Okayama univ., Okayama, Japan) and Dr. S. Narumiya (Kyoto Univ., Kyoto, Japan), respectively. pCAGGS vector was provided by Dr. M. Nakafuku (Tokyo University, Tokyo, Japan). Other materials and chemicals were obtained from commercial sources.

Plasmid constructs

The cDNA fragments encoding endophilin A1 1-352 aa (full length), 35-352 aa, 1-290 aa, 126-290 aa, and 291-352 aa were amplified by PCR using the obtained clone as a template, containing the entire open reading frame of mouse endophilin A1. The mutants of $endophilin^{T14D}$ and ^{T14A} were generated with a site-detected mutagenesis kit (Stratagene, La Jolla, CA) by changing Thr-14 into Asn-14 or Ala-14. The cDNA fragments were subcloned into pGEX-4T-1 (Amersham Pharmacia Biotech, Buckinghamshire, UK), pCAGGS-myc, and pEGFP-C1 (Clontech) vector.

Purification of a Rho-kinase substrate, p40

Bovine brain gray matter was homogenized and fractionated with centrifugation at 20,000 g. The proteins contained in the precipitate (Ppt 1) were extracted with 2 M NaCl and centrifuged at 10,000 g. The proteins contained in the precipitate (Ppt 2) were subsequently solubilized with 1% 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonic acid (CHAPS) and the solubilized proteins were recovered by the centrifugation at 100,000 g as the supernatant (Sup 3). Sup 3 was diluted to 0.5%

CHAPS and subjected to Mono Q column chromatography. Proteins were eluted with a linear gradient of NaCl (0-0.5 M). The eluates from Mono Q column were phosphorylated with GST-Rho-kinase CAT (catalytic domain). The fractions containing phosphorylated proteins were collected and further fractionated by S-sepharose column chromatography with a stepwise gradient of NaCl (0-0.5 M). The eluates from S-sepharose were phosphorylated with GST-Rho-kinase CAT.

Phosphorylation assay

The phosphorylation assay of the samples was carried out as described (28). In brief, the kinase reaction for Rho-kinase was carried out in 50 μl of a reaction mixture (50 mM Tris-HCl at pH 7.5, 2 mM EDTA, 1 mM DTT, 7 mM $MgCl_2$, 10 μM [γ-^{32}P]ATP [1-20 GBq/mmol] and purified GST [glutathione-*S*-transferase]-constitutively active form of Rho-kinase [GST-Rho-kinase CAT]) for 10 to 60 min at 30˚C. GST-Rho-kinase CAT was produced in Sf9 cells with a baculovirus system (33), and purified on glutathione-Sepharose 4B beads (Pharmacia) (25). Then the reaction mixtures were boiled in SDS sample buffer and subjected to SDS-PAGE. The radiolabeled bands were visualized by an image analyzer (BAS 2000; Fuji, Tokyo, Japan).

Mass spectral analysis

The fractions containing p40 were separated by SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (Problot, Applied Biosystems, Foster, CA). The immobilized protein was reduced, S-carboxymethylated, and digested *in situ* with *Achromobacter* protease I (a Lys-C) (34). Molecular mass analyses of Lys-C fragments were performed by matrix-assisted laser

desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry using a PerSeptive Biosystem Voyager-DE/RP (35). I identified protein by comparing the molecular weight determined by v/MS and theoretical peptide masses from the proteins registered in NCBInr.

Culture preparation

HeLa cells were seeded at a density of 0.5 X 10^5 cells onto 13-mm glass coverslips coated with poly-*d*-lysine (Sigma) in DMEM with 10% FBS. Transfection of plasmids was carried out using Lipofectamine plus reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol.

Internalization assay

Internalization assay with transferrin in HeLa cells was performed 24 h after transfection. The cells were washed three times in warmed PBS and incubated with 20 mg/ml tetrametylrhodamine-conjugated transferrin (Molecular Probes) in PBS containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose, and 0.2% BSA for 15 min at 37˚C (36). The cells were washed three times in cold PBS and fixed with 3.7% formaldehyde in PBS buffer for 10 min at room temperature, rinsed three times with PBS, and mounted. The cells were observed using a Zeiss Axiophoto microscope (Carl Zeiss), and the percentage of the transfected cells displaying significantly reduced levels of endocytosis were counted as described (37).

Results

Identification of endophilin A1 as a novel Rho-kinase substrate in bovine brain

To search for Rho-kinase substrates, I separated bovine brain membrane and peripheral membrane proteins by Mono Q column chromatography and subjected the fractions to phosphorylation assay using a constitutively active form of Rho-kinase (GST-Rho-kinase CAT). Among the several proteins detected in this assay, one with a mass of about 40 kDa (p40) was recognized as a major band phosphorylated in a GST-Rho-kinase CAT dependent manner (**Fig. 2**, fractions No. 14-16). P40 was further purified by S-sepharose column chromatography **(Fig. 3).** To clarify the molecular identity of p40, the purified protein was subjected to mass spectrometry as described in Materials and Methods. Nine peptide sequences derived from p40 were determined;KDLREIQHHLK, KLEGRRLDFDYK, KVDVTSRAVMEIMTK, KQNFIDPLQNLHDK, KTIEYLQPNPASRAK, KGPGYPQAEALLAEAMLK, KGGPGYPQAEALLAEAMLK, KFGRELGDDCNFGPALGEVGEAMRELSEVK, and KFGRELGDDCNFGPALGEVGEAMRELSEVK. These sequences were almost identical to that of human endophilin A1. Furthermore, the antibody raised against endophilin A1 cross-reacted with p40 **(Fig. 4).** The endophilins are a family of proteins identified in search for SH3 domain-containing proteins (31,38-41). Via its C-terminal SH3 domain, endophilin A1 binds to proline-rich domains of amphiphysin (42), dynamin (31), and synaptojanin (31,39), three proteins also involved in synaptic vesicle endocytosis (4).

Phosphorylation of recombinant endophilin A1 with Rho-kinase

To examine whether Rho-kinase phosphorylates endophilin A1,

endophilin A1 was produced as GST-fusion protein and subjected to the phosphorylation assay. GST-endophilin A1 was phosphorylated in the presence of GST-Rho-kinase CAT **(Fig. 5).** These results indicate that Rho-kinase directly phosphorylates endophilin A1 *in vitro.*

Identification of phosphorylation site of endophilin A1 by Rho-kinase

To determine the phosphorylated region of endophilin A1 by Rho-kinase, indicated fragments of endophilin A1 were produced as GST-fusion proteins **(Fig.6A).** The purified GST-endophilin A1 1-352 aa, and 1-290 aa were phosphorylated by GST-Rho-kinase CAT in the presence of [γ-^{32}P]ATP *in vitro.* However, GST-endophilin A1 35-352 aa, 126-290 aa and 291-352 aa were not phosphoryated by Rho-kinase CAT **(Fig. 6B).** These results suggest that a region containing 1-34 aa of endophilin A1 is phosphorylated by Rho-kinase.

R/KXXS/T or R/KXS/T are known as the phosphorylation consensus sequence of Rho-kinase. Thr-14 and Ser-18 are putative phosphorylation sites in 34 amino acid of endophilin A1 **(Fig. 6C).** To determine phosphorylation site of endophilin A1 by Rho-kinase, I produced endophilin A1 mutants (endophilin^{T14D}, ^{S18D}, and $^{T14D, S18D}$) in which Thr-14 and/or Ser-18 were replaced by Asp and subjected to the phosphorylation assay. GST-endophilin^{S18D} was phosphorylated by Rho-kinase CAT, whereas GST-endophilin^{T14D} and $^{T14D, S18D}$ were not **(Fig. 6D).** Taken together, these results indicate that Thr-14 is the major site of endophilin A1 phosphorylated by Rho-kinase *in vitro.*

Inhibition of the receptor-mediated endocytosis by endophilin^{T14D}

I assayed the endocytic accumulation of rhodamine-conjugated transferrin in order to examine whether endophilin mutants inhibit the clathrin-dependent endocytosis. In untransfected cells, the rhodamine-transferrin signal was detected in the perinuclear endosomal compartment and in early endosomal structures dispersed throughout the cytoplasm. Control EGFP and endophilin WT did not affect the internalization **(Fig. 7)**. In contrast, the expression of endophilin^{T14D}, which was expected to mimic phosphorylated endophilin, inhibited the internalization. However, endophilin^{T14A} (substitution of Thr-14 by Ala) also inhibited the internalization. The transition between the phosphorylation and dephosphorylation states of endophilin by Rho-kinase may regulate the receptor internalization. The expression of constitutively active form of Rho (RhoDA) inhibited the receptor internalization. However, endophilin^{T14A} counteracted the inhibitory effect of constitutively active Rho **(Fig. 7)**. Taken together, these results suggest that Rho/Rho-kinase regulates the clathrin-mediated endocytosis through the phosphorylation of endophilin.

Discussion

In the present study, I identified endophilin A1 as a novel Rho-kinase substrate **(Fig. 1-5).** Rho-kinase phosphorylated endophilin A1 at Thr-14 *in vitro* **(Fig. 6).** Furthermore, I demonstrated that endophilin^{T14D} inhibited the receptor internalization, and that the overexpression of endophilin^{T14A} attenuated the inhibition of internalization by RhoDA **(Fig. 7).** Taken together, these results suggest that the Rho/Rho-kinase regulates the clathrin-mediated endocytosis through the phosphorylation of endophilin.

What is the function of the endophilin phosphorylation in the clathrin-mediated endocytosis? It has been known that several proteins associated with clathrin-coated vesicles are substrates for protein kinases (43). These proteins include dynamin 1, synaptojanin 1, and amphiphysins (43). Dynamin 1 is phosphorylated by protein kinase C (PKC), casein kinase (CK2) (44), mitogen-activated protein kinase (MAPK) (45), and cdc2 (46). Amphiphysin is phosphorylated by CK2 (47), cyclin-dependent protein kinase 5 (cdk5) and cdc2 (48). PKC and cdk5 phosphorylate synaptojanin (49,50). These phosphoproteins called dephosphins are coordinately dephosphorylated by calcineurin, the Ca^{2+}-dependent protein phophatase (51). It was shown that phosphorylation of dynamin 1 and synaptojanin 1 inhibits their binding to amphiphysin, while phosphorylated amphiphyisin has an impaired affinity for AP-2 and clathrin (52). Recently, Lee and colleague reported that synaptojanin phosphorylated by cdk5 inhibited the binding to endophilin A1 (50). The phosphorylation of endophilin by Rho-kinase may inhibit its binding to several binding partner and the clathrin-mediated endocytosis.

The present study has also indicated that Rho regulates the clathrin-mediated receptor internalization (21). However, the molecular mechanisms have not been known. Identification of endophilin as a substrate of Rho-kinase has shed new light on the mechanisms involved in the regulation of receptor endocytosis through Rho/Rho-kinase pathway. Further work will be required to elucidate the physiological meaning of endophilin as the substrate of Rho-kinase.

Acknowledgment

I thank Dr. De Camili and K. Takei for kindly providing anti-endophilin Ab and the mouse cDNA of endophilin A1, and Dr. S. Narumiya for providing cDNA of $RhoA^{V14}$. pCAGGS vector was provided by Dr. M. Nakafuku. I thank Dr. S. Shiosaka, Dr. K. Kaibuchi, Dr. N. Inagaki and Dr. M. Amano for valuable discussion and technical advices, T. Ishii for secretarial assistance.

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Figure Legends

Figure 1 Purification of novel substrates of Rho-kinase from bovine brain. Bovine brain gray matter was homogenized and fractionated with centrifugation. The proteins contained in the precipitate (Ppt 1) were extracted with NaCl and centrifuged. The proteins contained in the precipitate (Ppt 2) were subsequently solubilized with CHAPS and the solubilized proteins were recovered by the centrifugation as the supernatant (Sup 3). Sup 3 was diluted to CHAPS and subjected to Mono Q column chromatography. Proteins were eluted with a linear gradient of NaCl. The eluates from Mono Q column were phosphorylated with GST-Rho-kinase CAT. The fractions containing phosphorylated proteins were collected and further fractionated by S-sepharose column chromatography with a stepwise gradient of NaCl. The eluates from S-sepharose were phosphorylated with GST-Rho-kinase CAT.

Figure 2 Phosphorylation of p40 of MonoQ eluates with Rho-kianse. Bovine brain membrane and peripheral membrane fraction was loaded onto the Mono Q column. Proteins were eluted with a linear gradient of NaCl (0-0.5 M). Each fraction was subjected to the phosphorylation assay with or without GST-Rho-kinase CAT in the presence of [γ-^{32}P]ATP. The phosphorylated proteins were imaged by the autoradiography. Fractions 15 contained a Rho-kinase substrate, p40. The arrow denotes p40.

Figure 3 Phosphorylation of p40 with Rho-kinase. Fraction 15 of Mono Q column was subjected to S-sepharose column chromatography and eluted with a stepwise gradient of NaCl. The eluates from S-sepharose were phosphorylated with or without GST-Rho-kinase CAT

in the presence of [γ -^{32}P]ATP. The phosphorylated proteins were imaged by the autoradiography. The arrow indicates p40.

Figure 4 Immunoblot analyses with anti-endophilin antibody. Samples of each step of purification were subjected to immunoblot analysis. The bands corresponding to p40 was recognized with anti-endophilin.

Figure 5 Phosphorylation of recombinant endophilin A1 with Rho-kinase. GST-endophilin A1 was subjected to phosphorylation assay with or without GST-Rho-kinase CAT in the presence of [γ -^{32}P]ATP. The image was analyzed by the autoradiography. GST-endophilin A1 was phosphorylated stoichiometrically with Rho-kinase. The arrow denotes GST-endophilin A1.

Figure 6 Identification of phosphorylation site of endophilin A1 by Rho-kinase. (A) Schematic representation of constructs used in this study. Numbers refer to amino acid positions. (B) Mapping of the region in endophilin phosphorylated by Rho-kinase. GST-endophilin 1-352 aa, 35-352 aa, 1-290 aa, 126-290 aa, and 291-352 aa were phosphorylated by Rho-kinase CAT in the presence of [γ -^{32}P]ATP. The phosphorylated proteins were imaged by the autoradiography. (C) R/KXXS/T or R/KXS/T is known as the phosphorylation consensus sequence of Rho-kinase. Two putative consensus sequences are involved in 34 amino acid of endophilin A1. Red color shows the putative phosphorylation site of endophilin by Rho—kinase. (D) Identification of phosphorylation site of endophilin by Rho—kinase. GST-endophilin WT, 14D, 18D and 14D18D were phosphorylated by Rho-kinase CAT in the presence of [γ -^{32}P]ATP. The phosphorylated proteins were imaged by the

autoradiography.

Figure 7 Rho/Rho-kinase inhibit the receptor endocytosis through the phosphorylation of endophilin. HeLa cells expressing the indicated proteins were incubated with rhodamine-conjugated transferrin for 15 min to allow for endocytosis and fixed. The transferrin internalization was quantified. The percentages of the transfected cells lacking transferrin endocytosis are shown.

Fig.1 Purification of novel substrates of Rho-kinase from bovine brain

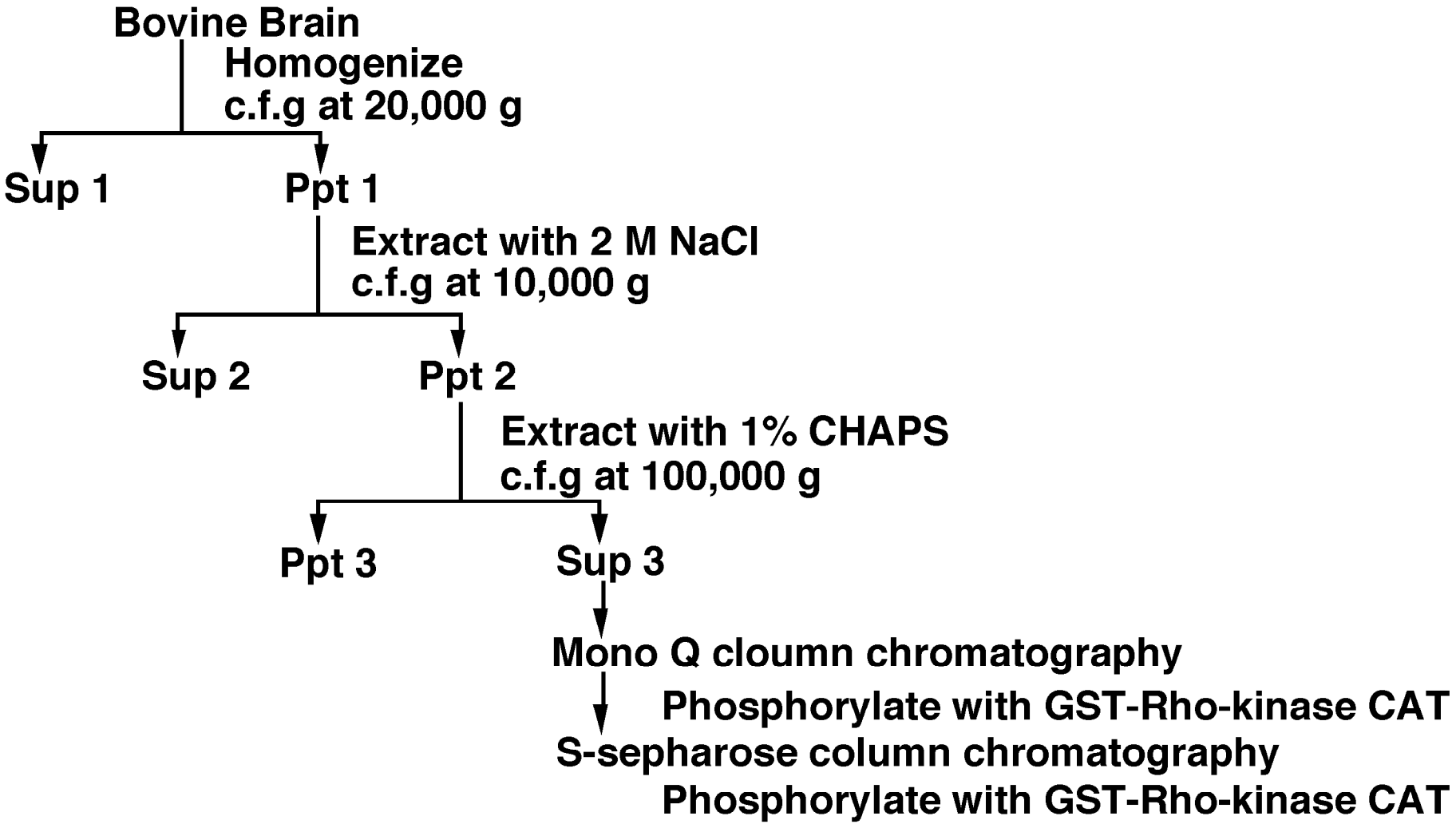


Fig.2 Phosphorylation of p40 of Mono Q elautes with GST-Rho-kinase CAT

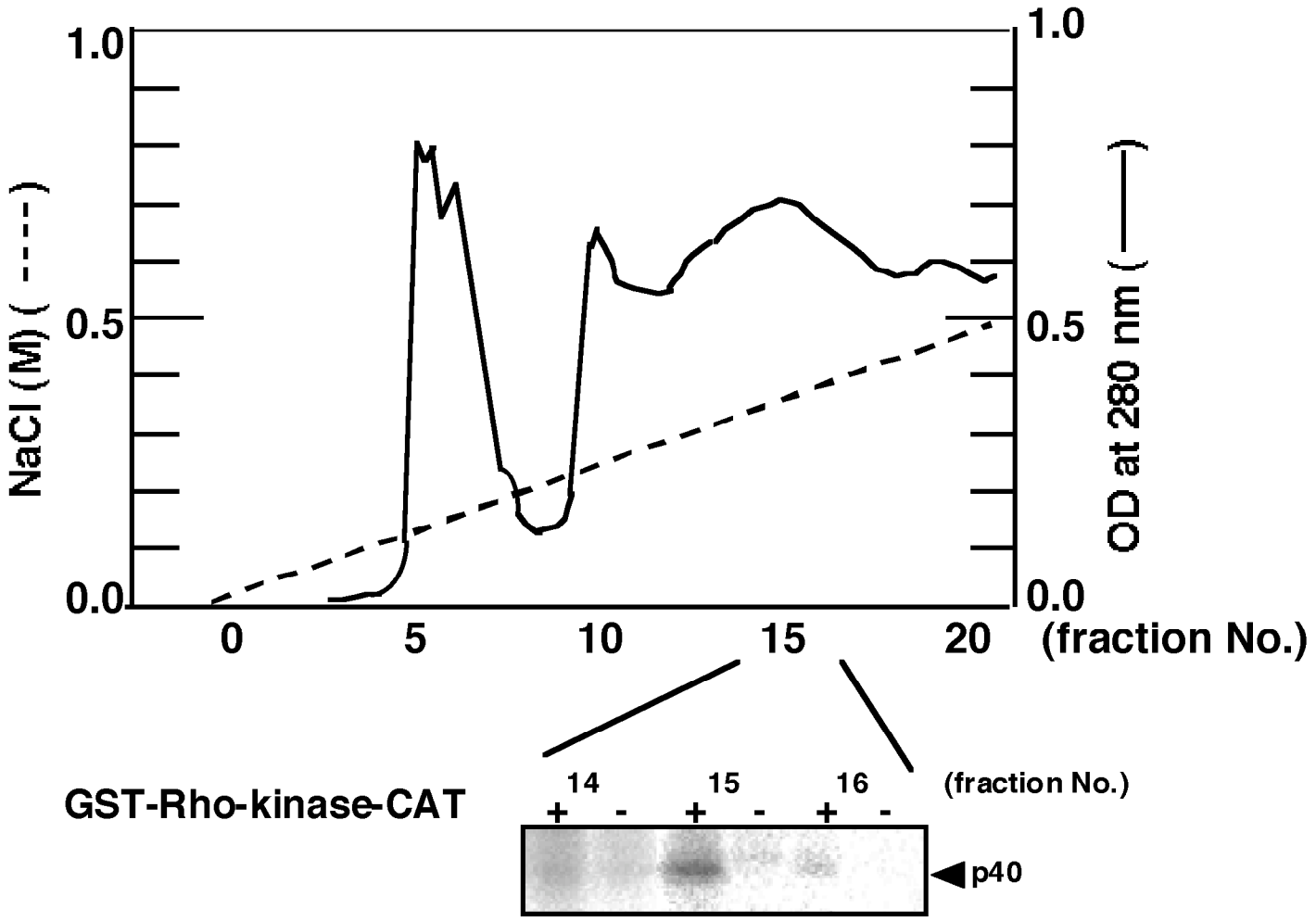


Fig.3 Phosphorylation of p40 with GST-Rho-kinase CAT

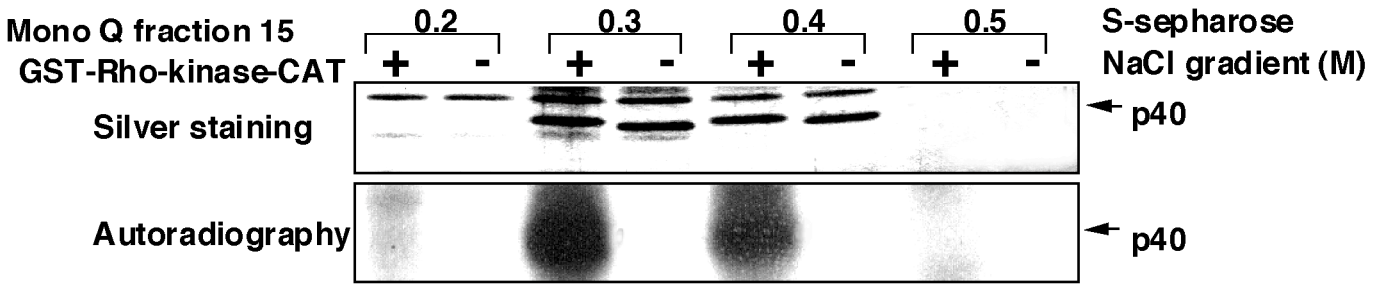


Fig.4 Immunoblot analyses with anti-endophilin A1 antibody

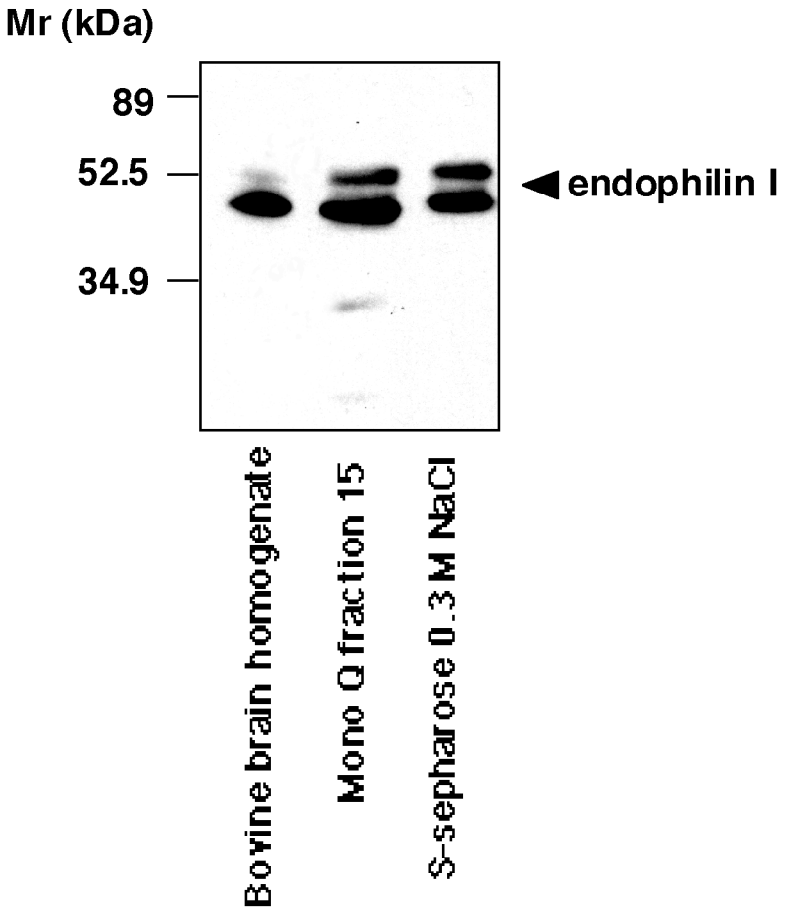


Fig.5 Phosphorylation of recombinant endophilin A1 with Rho-kinase

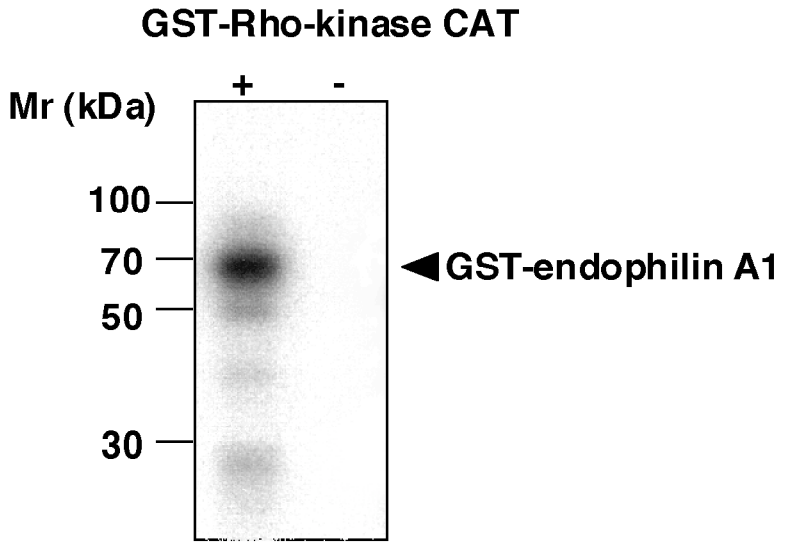
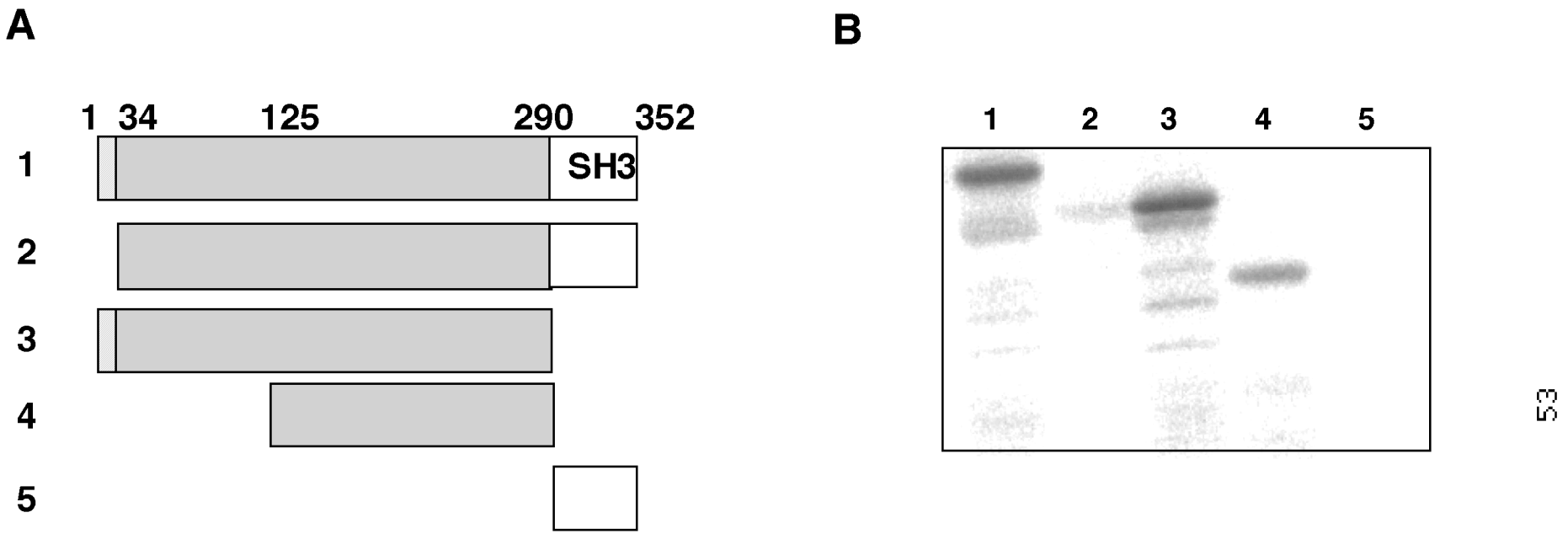


Fig. 6 Identification of phosphorylation site of endophilin A1 by Rho-kinase



C

1 14 18 34 a.a.

endophilin A1 MSVAGLKKQFHKATQKVSEKVGGAEGTKLDDDFK

Fig. 6 Identification of phosphorylation site of endophilin A1 by Rho-kinase

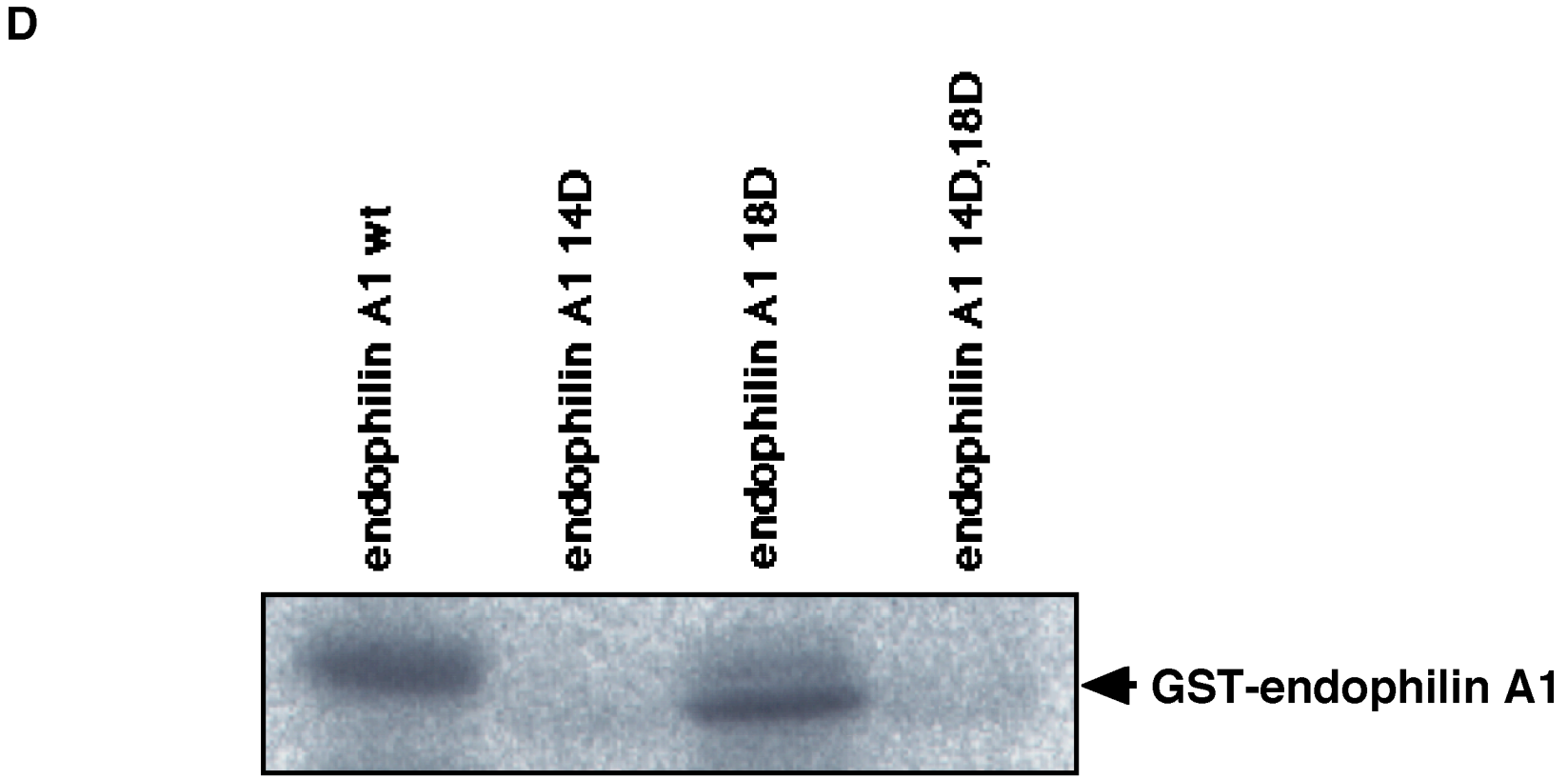


Fig. 7 Rho/Rho-kinase inhibit the receptor endocytosis through the phosphorylation of endophilin