バイオサイエンス研究科　博士論文要旨

所　属 (主指導教官)	バイオサイエンス研究科・細胞生物学専攻・細胞内情報学講座 貝淵　弘三		
氏　名	千原　一泰	提　出	平成１１年　１月２７日
題　目	Mechanisms underlying the Rho family GTPase-mediated neuronal network formation. (低分子量G蛋白質Rho familyによる神経回路網形成機構)		

要旨

The Rho family small GTPases are thought to regulate cytoskeletal organization of various types of cells. We previously identified Rho-associated protein kinase (Rho-kinase) as a specific target of Rho, and found that Rho-kinase elevates the phosphorylation level of myosin light chain (MLC) of myosin II, thereby regulating the formation of stress fibers and focal adhesions. Here, I examined whether Rho-kinase regulates growth cone morphology and axon guidance via the regulation of cytoskeletal rearrangements in neuronal cells. I showed that the expression of constitutively active forms of Rho-kinase induced neurite retraction of N1E-115 cells, whereas the expression of dominant negative forms of Rho-kinase inhibited the lysophosphatidic acid (LPA)-induced neurite retraction of N1E-115 cells. The expression of mutant MLC^{T18D,S19D} (substitution of Thr and Ser to Asp), which is known to lead to the activation of myosin II when reconstituted with myosin heavy chain *in vitro*, also promoted neurite retraction. These result indicated that Rho-kinase is involved in the LPA-induced neurite retraction downstream of Rho and Rho-kinase. To further understand the functions of Rho-kinase

in the nervous system, I searched for the substrates of Rho-kinase from bovine brain. I identified collapsin response mediator protein-2 (CRMP-2) which is thought to be essential for collapsin-induced growth cone collapse as a novel Rho-kinase substrate. Rho-kinase phosphorylated CRMP-2 at Thr-555 *in vitro*. I produced here an antibody that recognizes CRMP-2 phosphorylated at Thr-555. By use of this antibody, I found that CRMP-2 was phosphorylated by Rho-kinase downstream of Rho *in vivo*. These results raised the possibility that Rho-kinase regulates the morphology of growth cone and axon guidance by phosphorylating CRMP-2 in addition to MLC.

Mechanisms of the Rho family GTPase-induced neuronal network formation

低分子量G蛋白質Rho familyによる神経回路網の形成

千原　一泰
奈良先端科学技術大学院大学
バイオサイエンス研究科　細胞内情報学講座
(貝淵　弘三　教授)

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Summary

The Rho family small GTPases are thought to regulate cytoskeletal organization of various types of cells. We previously identified Rho-associated kinase (Rho-kinase) as a specific target of Rho, and found that Rho-kinase elevates the phosphorylation level of myosin light chain (MLC) of myosin II, thereby regulating the formation of stress fibers and focal adhesions. Here, I examined whether Rho-kinase regulates growth cone morphology and axon guidance via the regulation of cytoskeletal rearrangements in neuronal cells. I showed that the expression of constitutively active forms of Rho-kinase induced neurite retraction of N1E-115 cells, whereas the expression of dominant negative forms of Rho-kinase inhibited the lysophosphatidic acid (LPA)-induced neurite retraction of N1E-115 cells. The expression of mutant $MLC^{T18D,S19D}$ (substitution of Thr and Ser to Asp), which is known to lead to the activation of myosin II when reconstituted with myosin heavy chain *in vitro*, also promoted neurite retraction. These results indicated that Rho-kinase is involved in the LPA-induced neurite retraction downstream of Rho, and that myosin II activation promotes neurite retraction downstream of Rho and Rho-kinase. To understand the functions of Rho-kinase in the nervous system, I searched for the substrates of Rho-kinase from bovine brain. I identified collapsin response mediator protein-2 (CRMP-2) which is thought to be essential for collapsin-induced growth cone collapse as a novel Rho-kinase substrate. Rho-kinase phosphorylates CRMP-2 at Thr-555 *in*

vitro. I produced here an antibody that recognized CRMP-2 phosphorylated at Thr-555. By use of this antibody, I found that CRMP-2 was phosphorylated by Rho-kinase downstream of Rho *in vivo*. These results raised the possibility that Rho-kinase regulates the morphology of growth cone and axon guidance by phosphorylating CRMP-2 in addition to MLC.

Introduction

In the developing brain, neuronal axons are guided toward the appropriate targets by a variety of extracellular guidance cues (1, 2). The axonal guidance cues regulate the morphology and growing directions of the growth cone, the expanded tip of growing axons (1, 2). Such regulations are considered to be achieved by the reorganization of the cytoskeleton in the growth cone such as actin filaments and microtubles (3). Although recent studies have identified several guidance cue molecules and their receptors, the mechanisms of their intracellular signaling are poorly understood.

The Rho family small GTPases including Rho, Rac and Cdc42 are intracellular signaling molecules that are thought to regulate cytoskeletal organizations (4, 5). They function as molecular switches that cycle between GDP-bound inactive and GTP-bound active forms in response to extracellular stimuli (6, 7). In Swiss 3T3 fibroblast, Rho is required for the formation of stress fiber and focal adhesion induced by lysophosphatidic acid (LPA) (8). On the other hand, Cdc42 is required for by the bradykinin-induced filopodia formation (9), while Rac1 is required for membrane ruffling induced by platelet-derived growth factor or insulin (10). The Rho family GTPases are also reported to regulate the neurite outgrowth and cell morphology of neuronal cell lines (11, 12). In N1E-115 cells, Rho is required for LPA- and thrombin-induced neurite retraction and growth cone collapse (11, 12). Cdc42 and Rac1 are

involved in acetylcholine-induced neurite outgrowth of N1E-115 cells (12, 13) and play critical roles in neurite formation and axonal guidance in the developing nervous systems of *Drosophila* and mouse (14, 15). Rac1 also reported to be involved in collapsin-induced growth cone collapse in chick dorsal root ganglion (DRG) neuron (16).

Recently, a novel serine/threonine kinase, Rho-associated kinase (Rho-kinase) was identified as a target molecule of Rho (17). ROCK I/ROKβ is an isoform of Rho-kinase (18, 19). Rho-kinase is activated by GTP-bound active form of Rho (17, 20). A lot of putative Rho targets have been identified; protein kinase N (21, 22), the myosin-binding subunit (MBS) of myosin phosphatase (23), p140mDia (24), citron (25), citron-kinase (26), rhophilin, rhotekin (25), Kv1.2 (27), and phospholipase D (28). Rho-kinase is implicated in many processes downstream of Rho; stress fiber and focal adhesion formation (29-31), smooth muscle contraction (32), intermediate filament disassembly (33, 34), microvilli formation (35), cytokinesis (36) and cell migration (37). Rho-kinase regulates the phosphorylation of myosin light chain (MLC) by the direct phosphorylation of MLC and by the inactivation of myosin phosphatase through the phosphorylation of MBS (20, 23). In addition to MLC and MBS, Rho-kinase phosphorylates the ERM family proteins and adducin *in vitro* (38, 39). But the functions of Rho-kinase in neuronal cells have not yet been determined.

In order to determine possible roles of Rho-kinase in the regulation of neuronal cell morphology and axon guidance, I here examined whether Rho-kinase regulates the

morphology of growth cones and axon guidance via the reorganization of the cytoskeleton in neuronal cells. I found that Rho-kinase is involved in the LPA-induced neurite retraction of N1E-115 cells downstream of Rho, and identified MLC of myosin II as a major effector molecule of Rho-kinase-mediated neurite retraction in these cells. Furthermore, I also identified CRMP-2, which was previously identified as a protein required for collapsin-induced growth cone collapse as a novel substrate of Rho-kinase.

Materials and Methods

Materials and chemicals ——— Glutathione *S*-transferase (GST)-CAT was produced and purified as described (20, 40). Mouse monoclonal antibody against CRMP-2 (486-528 amino acids) was kindly provided from Dr. Y. Ihara (University of Tokyo, Tokyo, Japan). Rabbit polyclonal antibody against CRMP-2 phosphorylated at Thr555 (anti-pT555) was generated with chemically synthesized phosphopeptide Cys-Ile^{550}-Pro-Arg-Arg-Thr-phosphoThr-Gln-Arg-Ile-Val-Ala^{560}. Fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG, Texas red-conjugated anti-mouse antibody and [γ-^{32}P]ATP were purchased from Amersham (Arlington Heights, IL). LPA was purchased from Sigma Chemical CO. (St. Louis, MO, USA). Human fetal brain cDNA library was kindly provided by Dr. H. Saya (Kumamoto University, Kumamoto, Japan). N1E-115 cells were kindly provided by Dr. T. Kato (Nagoya City University, Aichi, Japan). Other materials and chemicals were obtained from commercial sources.

Plasmid constructs ——— The cDNA fragments of RhoA^{V14}, RhoA^{N19}, Rac1^{V12}, Cdc42^{V12}, Rho-kinase, CAT, COIL, RB/PH, ΔRB/PH and RB were subcloned into pEF-BOS-myc vector (21, 30, 41). The cDNA fragments of RB/PH (TT) and RB (TT) in which Asn-1036 and Lys-1037 were changed to Thr were generated by polymerase chain reaction (PCR) primer mutagenesis of Asn-1036 and Lys-1037 to Thr and subcloned into pEF-BOS-myc vector. The cDNA of human CRMP-2 was amplified by PCR from human fetal brain cDNA library with

primers 5′-AGATCTATGTCTTATCAGGGGAAGAAAAA-3′ and 5′-AGATCTCTAGCCCAGGCTGGTGATGT-3′, then subcloned into pBluescript SK (-). The mutant CRMP-2 T555A in which Thr555 was altered to Ala was generated by PCR primer mutagenesis of Thr-555 to Ala-555. The cDNA fragments of CRMP-2 and CRMP-2 T555A were subcloned into pEF-BOS-HA vector.

Transfection of plasmids into N1E-115 cells

——— N1E-115 cells maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal calf serum (FCS) were seeded at a density of 2 x 10^4 cells on 13-mm-round glass coverslips coated with poly-D-lysine (Sigma). Transfection of plasmids into N1E-115 was carried out using a Lipofectamine-mediated DNA transfection procedure (GIBCO-BRL, Gaithersburg, MD). To identify the transfected cells, N1E-115 cells were cotransfected with cDNAs and pME18S-lacZ (42) mixed in 2-4:ratios and stained for β-galactosidase activity.

Purification of a Rho-kinase substrate p70

——— Bovine brain cytosol fraction (20 mg of protein) prepared as described (43, 44) was loaded onto Mono Q HR5/5 column (Pharmacia Biotech Inc., Grand Island, NY) preequilibrated with 10 ml of buffer A (20 mM Tris-HCl at pH 7.5, 1 mM EDTA, 1 mM dithiothreitol (DTT), 5 mM MgCl2). After washing with buffer A, proteins were eluted with a linear gradient of NaCl (0-0.5 M) at a flow rate of 0.5 ml/min. Fractions (1 ml each) were collected, and the aliquot of each fraction (20 μl) was analyzed by the

phosphorylation assay as described below. The peak fractions containing the major phosphorylated protein were diluted with two volume of buffer A, and further loaded onto Mono S HR5/5 column (Pharmacia) using the same flow rate and gradient of NaCl. Each fraction (20 µl) was used for the phosphorylation assay.

Phosphorylation assay ——— The phosphorylation assay of the samples was carried out as described (20). In brief, the kinase reaction for Rho-kinase was carried out in 50 µl of the reaction mixture (50 mM Tris/HCl at pH 7.5, 2 mM EDTA, 1 mM DTT, 7 mM MgCl2, 10 µM [γ-^{32}P]ATP [1-20 GBq/mmol] and purified GST-CAT [1 µg of protein]) for 10 to 60 min at 30°C. Then the reaction mixtures were boiled in SDS-sample buffer and subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and the radiolabeled bands were visualized by an image analyzer (Fuji, Tokyo, Japan). To confirm the phosphorylation site of CRMP-2 by Rho-kinase, HA-CRMP-2 and HA-CRMP-2 T555A were used as substrates. HA-CRMP-2 or HA-CRMP-2 T555A were prepared by immunoprecipitation from COS7 cells transiently transfected with each expression vector as described (44).

Peptide sequencing ——— Peptide sequencing of p70 was carried out as described (45). In brief, partially purified p70 was subjected to SDS-PAGE and blotted onto a polyvinyllidene difluoride membrane, and stained with 0.1% Ponceau S in 1% acetic acid. The band corresponding to p70 was cut out, digested with lysyl endopeptidase,

Achromobacter protease I. The obtained peptides were fractionated by C18 column chromatography and subjected to amino acid sequencing for identification.

Identification of CRMP-2 phosphorylation sites by Rho-kinase ——— The phosphorylation sites of CRMP-2 by Rho-kinase were identified as according to the method described (46). In brief, CRMP-2 (77 μg of protein) was phosphorylated by GST-CAT (70 μg of protein), and digested with endopeptidase, Asp-N, then the obtained peptides were fractionated by C18 column chromatography and subjected to amino acid sequencing. The fractions obtained from each Edoman degradation cycle were counted for ^{32}P in a liquid scintillation counter.

Transfection of plasmids into COS7 cells ——— COS7 cells were cultured in DMEM containing 10% FCS. Transfection of plasmids into COS7 cells was carried out using a Lipofectamine-mediated DNA transfection procedure (GIBCO-BRL). Cells were seeded on 100 mm culture dish at a density of 1.8×10^6 cells. 24 h after the transfection, the culture medium was replaced by DMEM without FCS and the cells were cultured for another 24 h. For immunoblot studies, the cells were treated with 10% (w/v) trichloroacetic acid, and the resulting precipitates were analyzed as described previously (42).

Immunofluorescene analysis ——— COS7 cells seeded on 13-mm-round glass coverslips were fixed with 3.7%

formaldehyde in phosphate buffer saline (PBS) for 10 min at room temperature and washed with PBS, and permeabilized with ice-cold methanol for 10 min at 4°C. The fixed cells were incubated with 10% normal goat serum in PBS for 1 h at room temperature. The fixed cells were incubated with rabbit anti-pT555 (1:1) and anti-HA antibody (12CA5; 1:100) in PBS for 6 h at 4°C, and washed three times for 10 min with PBS. After the first labeling, the cells were incubated with FITC-labeled and Texas Red-labeled secondary antibodies for 1 h at room temperature. Cells were viewed by using confocal laser scanning microscope (Carl Zeiss, Inc., Thornwood, NY).

Results

Production of dominant active and negative mutants of Rho-kinase.

Rho-kinase is composed of catalytic (CAT), coiled coil (COIL), Rho-binding (RB), and pleckstrin-homology (PH) domains (17). We previously constructed CAT (6-553 amino acids), COIL (421-701 amino acids) and RB (941-1075 amino acids) (30). CAT served as a dominant active form, and RB served as a dominant negative form of Rho-kinase, respectively (20, 30). For further investigation of Rho-kinase functions, here I produced cDNAs encoding Rho-kinase mutants in addition to the above constructs (Fig. 1). ΔRB/PH (6-980 amino acids), which lacks the COOH-terminal regulatory region including RB and PH domains, is thought to be a constitutively active form (29). RB/PH (941-1388 amino acids), which is composed of RB and PH domains, is thought to be a dominant negative form. Since RB/PH or RB was thought to titrate out GTP·Rho in cells, I also produced RB/PH (TT) and RB (TT) in which Asn-1036 and Lys-1037 were replaced by Thr in the RB domain of Rho-kinase. Rho-kinase has been shown to abolish its binding activity to GTP·Rho by these substitutions (29). I also confirmed that RB (TT) did not bind to GTPγS·Rho *in vitro* under the conditions in which RB bound to GTPγS·Rho (data not shown). These cDNA fragments of Rho-kinase were cloned into pEF-BOS-myc vector to express them in mammalian cells.

Rho-kinase regulates the cell morphology and neurite retraction of N1E-115 neuroblastoma

Next, I utilized these dominant active and negative mutants of Rho-kinase for determining the roles of Rho-kinase in the regulation of neurites of a neuronal cell line, N1E-115 cells. N1E-115 cells showed a flattened morphology and extended neurites when cultured in serum-free medium (Fig. 2Aa), and the addition of LPA induced rapid neurite retraction and cell rounding within a few minuets (Fig. 2Ab) as described previously (11). Rho^{V14}, Rac^{V12} are structurally equivalent to $H\text{-}Ras^{V12}$ (substitution of Gly to Val) and thought to be the constitutively active forms (6, 47, 48). N1E-115 cells were transfected with the cDNA encoding either $HA\text{-}Rho^{V14}$, $HA\text{-}Rac^{V12}$, myc-Rho-kinase, myc-ΔRB/PH, or myc-CAT. The cDNA encoding β-galactosidase was cotransfected with these cDNAs for the identification of the transfected cells. 24 h after the transfection, the cells were serum-starved for another 12 or 24 h and then fixed for morphological examination. The cells expressing $HA\text{-}Rho^{V14}$ became rounded and failed to extend neurites (Fig. 2Ac), whereas the cells expressing $HA\text{-}Rac^{V12}$ spread out and lost both neurites and filopodia (Fig. 2Ad) as described previously (13). The cells expressing myc-ΔRB/PH or myc-CAT showed a morphology similar to that of the cells expressing $HA\text{-}Rho^{V14}$ (Fig. 2Ae, f), indicating that active Rho-kinase led to neurite retraction and cell rounding. Myc-Rho-kinase showed the weak activity in this capacity (Fig. 2B). The effects of each molecule became stronger as time passed after the transfection (Fig. 2B). It is possible that Rho-kinase prevents neurite extension rather than induces neurite retraction. However, this possibility is less

likely, because the microinjection of Rho^{V14} or GST-CAT into serum-starved N1E-115 cells induced a rapid withdrawal of neurites (data not shown).

Rho^{N19} is structurally equivalent to H-Ras^{N17} (substitution of Thr to Asn) and thought to be a dominant negative form of Rho (6, 10, 49). The expression of Rho^{N19}, RB, or RB/PH, which are thought to interfere with Rho functions, inhibited the LPA-induced neurite retraction (Fig. 3A, B). RB/PH (TT) and RB (TT) enabled us to evaluate the effects of Rho-kinase without titrating out GTP·Rho. RB/PH (TT) showed an inhibitory effect on the LPA-induced neurite retraction and cell-rounding to an extent similar to that of RB/PH, while RB (TT) had a small effect (Fig. 3A, B). RB/PH (TT) may inhibit the endogenous Rho-kinase more effectively than does RB (TT). COIL (421-701 amino acids) had no effect. The coexpression of myc-RB or myc-RB/PH (TT) inhibited the HA-Rho^{V14}-induced morphological changes, but not the myc-CAT-induced morphological changes (data not shown), suggesting that RB and RB/PH (TT) inhibited the endogenous Rho-kinase activity but not completely blocked the high level of the exogenously added CAT.

Myosin II activation induced by the MLC mutant promotes neurite retraction in N1E-115 neuroblastoma.

Since Rho-kinase is known to regulate the level of MLC phosphorylation (20, 23), I expected MLC to be one of the major effectors of Rho-kinase in neurite retraction of N1E-115 cells. The phosphorylation of MLC of myosin II at Ser-19 is essential for the actin-activated ATPase of myosin II

in smooth muscle contraction (50-53), and the phosphorylation of MLC at Thr-18 enhances the ATPase activity (51, 54). Protein kinases, such as MLC kinase and Rho-kinase, phosphorylate MLC at these sites (20, 51). Recently, I demonstrated that Rho^{V14} and CAT elevated the level of MLC phosphorylation by the use of a specific antibody against MLC phosphorylated at Ser-19 in COS7 cells (42). Kureishi *et al.* (32) also demonstrated that CAT provoked a contraction and proportional increase in the level of MLC phosphorylation in permeabilized smooth muscle.

To examine whether the activation of myosin II is sufficient for neurite retraction, I examined the effect of MLC mutants in N1E-115 cells. The replacement of MLC by recombinant $MLC^{T18D,S19D}$ (MLC-DD; substitution of Thr-18 and Ser-19 to Asp) in myosin II partially activates the ATPase activity and forms myosin filaments when reconstituted with myosin heavy chain *in vitro* (55). I produced cDNAs encoding MLC, MLC-DD and $MLC^{T18A,S19A}$ (MLC-AA; substitution of Thr-18 and Ser-19 to Ala, unphosphorylatable form) and cloned them into pEF-BOS-myc vector (Fig. 4A).

I transfected the MLC mutant cDNAs into N1E-115 cells. The cells expressing myc-MLC or myc-MLC-AA extended neurites when deprived of serum, whereas the cells expressing myc-MLC-DD failed to extend neurites (Fig. 4B, C). The expression of myc-MLC or myc-MLC-AA had no or minimal effect on the LPA-induced neurite retraction in N1E-115 cells (Fig. 4B). To confirm that the morphological changes induced by MLC-DD is independent of Rho and Rho-kinase, I examined the effects of RB and RB/PH (TT). The coexpression of myc-RB or

myc-RB/PH (TT) with myc-MLC-DD showed a minimal effect on the MLC-DD-induced morphological changes (data not shown). Thus, it is likely that the morphological changes induced by MLC-DD are independent of Rho and Rho-kinase. These results indicate that Rho-kinase phosphorylates and sequentially activates myosin II, and the myosin II activation promotes neurite retraction downstream of Rho and Rho-kinase in N1E-115 cells. However, the morphological changes induced by MLC-DD were mild in comparison with those induced by active forms of Rho-kinase. The cells expressing myc-MLC-DD became either flattened cells or modestly rounded cells without neurites, while most cells were rounded when treated with active forms of Rho-kinase. This may be because MLC-DD cannot exert the full activity of myosin II. Alternatively, other substrates of Rho-kinase may be necessary for the complete alteration of morphology by Rho-kinase.

Purification of a novel Rho-kinase substrate p70 from bovine brain cytosol.

To examine the latter possibility, I searched for Rho-kinase substrates other than MLC in bovine brain. Proteins in bovine brain cytosol fraction were separated by Mono Q column and subjected to phosphorylation assay using GST-CAT. Among the several proteins phosphorylated in this assay, a protein with mass of about 70 kDa (p70) was phosphorylated strongly in a GST-CAT-dependent manner (Fig. 5). p70 was eluted in peaks between 200 and 300 mM NaCl. p70 was further purified by Mono S column (Fig. 6).

Identification of p70 as CRMP-2.

To clarify the molecular identity of p70, purified p70

was subjected to amino acid sequencing as described in "Materials and Methods". Six peptide sequences derived from p70 were determined. The obtained peptide sequences from p70 were KQIGENLIVP, KSSAEVIAQARK, KMDENQFVAV, KVFNLYPR, KIVLED and KAIEALAEIRXVP, where X indicates an unidentified residue. These peptide sequences were almost identical to that of bovine collapsin response mediator protein-2 (CRMP-2, Fig. 7A). Furthermore, the antibody raised against CRMP-2 (1-572 amino acids) cross-reacted with p70 (data not shown). Recently, CRMP-2 homologues have been identified from various species (Fig. 7B). Chick CRMP-62, a chick homologue, is required for the growth cone collapse of DRG neurons induced by collapsin (56) (98% identity). Unc-33, a *C. elegans* homologue, is identified by a mutation resulting in severely uncoordinated movement, abnormalities in axon guidance, and superabundance of microtubles in neuron (57) (30% homology). I also found that CRMP-2 has a structure homologous with *Bacillus stearothermophylus* D-hydantoinase enzyme (36% homology).

Identification of the CRMP-2 phosphorylation site by Rho-kinase *in vitro*.

Next, I determined the phosphorylation site of bovine CRMP-2 by GST-CAT. CRMP-2 was phosphorylated in the presence of [γ-^{32}P]ATP *in vitro*, and digested with endopeptidase, Asp-N. The digested peptides were separated by C18 column chromatography. One radioactive peak was obtained (Fig. 8A). The peak was fractionated, and subjected to amino acid sequencing. The sequence obtained from this fraction was DNIQPRRTTQRIVAPPGGR, corresponding to

548-565 amino acids of CRMP-2. The fractions obtained from each Edoman degradation cycle were counted for ^{32}P in a liquid scintillation counter. As a result, Thr-555 was turned out to be phosphorylated by GST-CAT (Fig. 8B). But I could not rule out the possibility that GST-CAT phosphorylates not only Thr-555, but also Thr-554. To confirm the phosphorylation site of CRMP-2 by Rho-kinase, I produced a CRMP-2 mutant (HA-CRMP-2 T555A) in which Thr-555 was replaced by Ala. HA-CRMP-2 or HA-CRMP-2 T555A was expressed in COS7 cells and immunoprecipitated with anti-HA antibody from the lysate of the cells expressing the proteins. The resulting immunoprecipitates was subjected to the phosphorylation assay (Fig. 9). HA-CRMP-2 was phosphorylated by GST-CAT, whereas HA-CRMP-2 T555A was not phosphorylated. These results confirm that Thr-555 is the major phosphorylation site of CRMP-2 by Rho-kinase *in vitro*.

Rho-kinase phosphorylates CRMP-2 at Thr-555 *in vivo*.

To examine *in vivo* CRMP-2 phosphorylation by Rho-kinase, I produced an antibody that specifically recognized CRMP-2 phosphorylated at Thr-555 (anti-pT555 antibody). I used a phosphopeptide corresponding to 550-560 amino acids of CRMP-2 in which Thr-555 is phosphorylated as an antigen. The specificity of the antibody was examined by immunoblot analysis. 2 pmol of CRMP-2 containing increasing amount of the phosphorylated CRMP-2 were loaded on the gel. Fig. 10 shows the specificity of anti-pT555 antibody. The antibody bound to the phosphorylated CRMP-2 in a dose-dependent manner but did not react with the unphosphorylated form. Thus, anti-pT555 antibody specifically recognizes the

phosphorylation of CRMP-2 at Thr-555 by Rho-kinase. This antibody was used for detection of CRMP-2 phosphorylation by Rho-kinase in COS7 cells. Because endogenous CRMP-2 was not detected in COS7 cells by immunoblot analysis using anti-CRMP-2, HA-CRMP-2 was expressed exogenously with myc-Rac1^{V12}, myc-Cdc42^{V12}, myc-RhoA^{V14}, myc-RhoA^{N19}, myc-Rho-kinase, myc-RhoA^{V14} and myc-Rho-kinase, or myc-CAT in COS7 cells. The HA-CRMP-2 phosphorylation was not detectable in serum-starved COS7 cells expressing HA-CRMP-2 alone (Fig. 11). On the other hand, the coexpression of myc-RhoA^{V14}, a dominant active form of RhoA increased the level of the HA-CRMP-2 phosphorylation, whereas myc-RhoA^{N19}, a dominant negative form of RhoA did not increase (Fig. 11). The coexpression of myc-Rho-kinase, myc-RhoA^{V14} and myc-Rho-kinase, or myc-CAT also increased the level of the HA-CRMP-2 phosphorylation. However, myc-CAT did not induce the HA-CRMP-2 T555A phosphorylation. In agreement with these immunoblot data, I also detected the HA-CRMP-2 phosphorylation by coexpression with myc-RhoA^{V14} or myc-CAT by Immunofluorescene analysis (Fig. 12). Both phosphorylated and unphosphorylated forms of CRMP-2 were located in the plasma membrane and cytoplasm, but absent from the nuclei. Interestingly, coexpression of myc-Rac1^{V12} or myc-Cdc42^{V12} also resulted in a small increase in the level of HA-CRMP-2 phosphorylation, raising the possibility that CRMP-2 may be also phosphorylated downstream of Rac and Cdc42 (Fig. 11). Taken together, these results indicate that Rho-kinase elevates the level of HA-CRMP-2 phosphorylation at Thr-555 downstream of Rho in COS7 cells.

Discussion

Involvement of Rho-kinase in Rho-induced neurite retraction.

The Rho family small GTPases are involved in the regulation of cell morphology and the organization of cytoskeleton such as actin filaments and microtubles in a various type of cells (58). We previously identified Rho-kinase as a target molecule of Rho. Rho-kinase is composed of catalytic-, coiled coil-, RB-, and PH domains (17). We previously produced CAT as the dominant active form of Rho-kinase, and RB as the dominant negative form (20, 30). We confirmed that CAT shows the constitutively active kinase activity, and that RB binds to GTPγS·Rho and inhibits the GTPγS·Rho-dependent Rho-kinase activity (30). In N1E-115 cells, serum, LPA or the active form of Rho ($RhoA^{V14}$) induce neurite retraction (11, 12). To examine whether Rho-kinase regulates neurite retraction downstream of Rho in N1E-115 cells, I here produced various Rho-kinase mutants besides the above constructs. In these mutants, ΔRB/PH, which lacks the COOH-terminal regulatory region including RB and PH domains induced the formation of stress fibers and focal adhesion in fibroblasts (data not shown). Thus, ΔRB/PH may serves as the constitutively active form. I showed here that ΔRB/PH induced neurite retraction in N1E-115 cells as well as CAT.

On the other hand, the expression of dominant negative form of Rho (Rho^{N19}) or the treatment of cells with *Botulinum* toxin C3 transferase, which ADP-ribosylates Rho

and inactivates it (59, 60), has been shown to induce neurite extension in N1E-115 cells when the cells are cultured in the presence of serum (11, 12). They also inhibit the LPA-induced neurite retraction in N1E-115 cells (11, 12). I also found that RB/PH (TT), which does not bind to active form of Rho, inhibited the LPA-induced neurite retraction in N1E-115 cells, suggesting that it serves as the powerful dominant negative form of Rho-kinase. Although RB is also dominant negative form, I consider that it is a less specific inhibitor for endogenous Rho-kinase than RB/PH (TT), because RB titrates out GTP·Rho to inhibit other Rho-targets. Thus, RB/PH (TT) is the most useful dominant negative form so far obtained. However, further studies are necessary to understand the molecular mechanism by which RB/PH (TT) inhibits the activity of endogenous Rho-kinase in intact cells. Taken together, these data indicate that Rho-kinase is involved in neurite retraction downstream of Rho in N1E-115 cells.

Involvement of myosin phosphorylation by Rho-kinase in Rho-induced neurite retraction.

We previously reported that Rho-kinase elevates the phosphorylation level of MLC leading to myosin II activation (20, 23, 42). I here found that the N1E-115 cells expressing the dominant active form of MLC, MLC-DD failed to extend neurites when deprived of serum. These results suggest that MLC is involved in neurite retraction of N1E-115 cells. MLC-AA is thought to serve as the dominant negative form of MLC *in vivo*, because the replacement of MLC

with MLC-AA prevents actin-activated myosin ATPase *in vitro* (55). However, I here found that the expression of MLC-AA did not affect the LPA-induced morphological changes in N1E-115 cells. I consider that MLC-AA may be inefficiently incorporated into actomyosin filaments in transfected cells or may not serve as the dominant negative form in intact cells. These data indicate that the phosphorylation of MLC by Rho-kinase is involved in neurite retraction via the Rho signaling pathway.

It is interesting that the morphological changes induced by MLC-DD were milder than those induced by LPA, Rho^{V14}, ΔRB/PH, or CAT. The N1E-115 cells expressing MLC-DD became either flattened or modestly rounded without neurites, while most cells were rounded when treated with LPA or when expressing $RhoA^{V14}$, ΔRB/PH, or CAT. This may be because MLC-DD cannot exert the full activity of myosin II or other substrates of Rho-kinase are involved in the Rho-kinase induced morphological change.

Rho-kinase phosphorylates CRMP-2 *in vitro* and *in vivo*.

The latter possibility prompted me to investigate Rho-kinase substrates other than MLC. Here I identified CRMP-2 as a novel substrate of Rho-kinase in the bovine brain. CRMP-2 constituted about 0.1% of total protein in the brain (data not shown). Recently, CRMP-2 homologues have been identified in various species. CRMP-62, a chick homologue of CRMP-2 was originally isolated as an intracellular mediator of collapsin which is thought to act as a repulsive

growth cone guidance cue (56). CRMP-2 is also homologous to Unc-33 protein of *C.elegans*. In *unc-33* mutants, several classes of neurons showed neurite outgrowth defects. Some axons followed abnormal pathways or terminated prematurely (57). These molecular similarities between CRMP-2 and its homologues have been suggesting that CRMP-2 is involved in the regulation of neurite outgrowth and axon guidance. My screening showed that CRMP-2 was the most prominent Rho-kinase phosphorylated protein in the brain. Thus, CRMP-2 is one of the major substrates of Rho-kinase in the brain. CRMP-2 is specifically expressed in the brain and the level of expression is elevated during the period of neuronal network formation (56, 61, 62). The Rho family GTPases are also highly expressed in developing nervous system (63, 64). In addition, Rho-kinase is highly expressed in brain (17, 18, 29) and chick E7 DRG (data not shown). Taken together, these observations support the idea that CRMP-2 is involved in the regulation of growth cone morphology by the Rho-Rho-kinase signaling.

In PC 12 cells, NGF regulates the phosphorylation level of CRMP-2 (61, 65). The amino acid sequence of CRMP-2 reveal consensus phosphorylation sites for several protein kinases. Here, I found that Rho-kinase phosphorylates Thr-555 of CRMP-2 *in vitro*. The level of CRMP-2 phosphorylation was not detectable in serum-starved COS7 cells expressing CRMP-2 alone. On the other hand, the coexpression of CRMP-2 with a dominant active form of Rho ($RhoA^{V14}$) or Rho-kinase remarkably increased the level of CRMP-2 phosphorylation. These results indicate that Rho-kinase phosphorylates Thr-

555 of CRMP-2 downstream of Rho *in vivo*. Interestingly, CRMP-2 was also phosphorylated to some extent by the coexpression of a dominant active form of Rac1 or Cdc42. Recently, myotonic dystrophy kinase-related Cdc42-binding kinase (MRCK) is identified as a target molecule of Cdc42 and Rac1. MRCK has a kinase domain similar to that of Rho-kinase (66). The constitutively active form of MRCK phosphorylated Thr-555 of CRMP-2 *in vitro* (data not shown). The expression of constitutively active form of MRCK in COS7 cells also led to phosphorylation of CRMP-2 at Thr-555 (data not shown). These results raise the possibility that MRCK phosphorylates Thr-555 of CRMP-2 downstream of Cdc42 or Rac1. It is reported that Cdc42 and Rac1 activate Rho in fibroblasts (4). Thus, there is another possibility that Cdc42 and Rac1 activate Rho, and then Rho-kinase phosphorylates Thr-555 of CRMP-2. Further studies are necessary to understand the precise mechanisms that account for the mode of phosphorylation of CRMP-2 downstream of Cdc42 or Rac1. Taken together, these results suggest that Rho-kinase regulates the morphology of growth cone and axon guidance by phosphorylating CRMP-2 downstream of Rho in addition to MLC.

Possible roles of CRMP-2 phosphorylated by Rho-kinase.

CRMP-2 is located in the plasma membrane and cytoplasm, but absent from the nuclei in DRG neuron (62). I also found that CRMP-2 is present in membrane fraction as well as cytosol fraction in the bovine brain (data not

shown). CRMP-2 is located in the filopodia and lamellipodia of growth cones in primary cultured neuron (62). Here, I produced an antibody that recognizes CRMP-2 phosphorylation at Thr-555. It is interesting to determine which of growth cone guidance molecules activates Rho and Rho-kinase signaling pathway that leads to CRMP-2 phosphorylation by the use of this antibody.

The regulations of morphology and growing directions of growth cones are considered to be achieved by the reorganization of the cytoskeleton such as actin filaments and microtubles (3, 67). Actin filaments extend as a meshwork throughout lamellipodia and are bundled in the filopodia of growth cone (3, 67). Microtubles are bundled in axons and the tip of microtubles often reach the leading edges of lamellipodia and occasionally enter the filopodia of growth cones (3, 67). CRMP-2 is also located in the lamellipodia and the filopodia of growth cone in DRG neuron (62). It is important to identify the proteins that associate with CRMP-2 and to examine whether the phosphorylated CRMP-2 is involved in the regulation of morphology of growth cone and axon guidance. The mutants CRMP-2 substituted of Thr-555 to Asp or Ala may be useful in such investigations.

Conclusion.

In conclusion, I found that the activation of Rho-kinase is necessary for neurite retraction induced by LPA and Rho in N1E-115 cells. In this neuronal response, MLC of myosin II was a major effector molecule, which is

phosphorylated by Rho-kinase downstream of Rho. I also found that Rho-kinase phosphorylates CRMP-2 both *in vitro* and *in vivo*. It is thought that CRMP-2 and Unc-33, which is *C. elegans* homologue of CRMP-2, are involved in axon guidance and the regulation of neuronal cell morphology. Taken together, these results raise the possibility that Rho-kinase regulates axon guidance and neuronal cell morphology by phosphorylation of MLC and CRMP-2 (Fig. 13).

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Figure legends

Fig. 1. Diagrams of a series of Rho-kinase mutants. Numbers indicate the amino acid residues. Functional and structural domains of Rho-kinase are schematically shown. DA; dominant active forms of Rho-kinase (induced neurite retraction), DN; dominant negative forms of Rho-kinase (inhibited the LPA-induced neurite retraction).

Fig. 2. Effects of dominant active forms of Rho, Rac, and Rho-kinase on neurite retraction of N1E-115 cells. (A) N1E-115 cells were transiently transfected with either pEF-BOS-myc vector (a and b), pEF-BOS-HA-Rho^{V14} (c), pEF-BOS-HA-Rac^{V12} (d), pEF-BOS-myc-ΔRB/PH (e), or pEF-BOS-myc-CAT (f). pME18S-lacZ was cotransfected with these plasmids to identify the transfected cells. 24 h after the transfection, the cells were cultured in serum-free medium for another 12 h, and subsequently fixed and stained for β-galactosidase activity (a, c-f). The cells transfected with pEF-BOS-myc were treated with 0.1 μM LPA for 10 min to induce neurite retraction (b). Bar represents 20 μm. (B) 12h (*open column*) or 24 h (*hatched column*) after the transfection of plasmids encoding indicated cDNAs, the cells were cultured in serum-free medium for 12 h. The percentage of lacZ-positive cells bearing neurites above 30 μm was scored. Data are means ± SEM of at least triplicate determinations.

Fig. 3. Effects of dominant negative forms of Rho and Rho-kinase on neurite retraction of N1E-115 cells. (A) N1E-115 cells were transiently transfected with either pEF-BOS-myc-RB (a), pEF-BOS-myc-RB/PH (TT) (b), pEF-BOS-HA-

Rho^{N19} (c), pEF-BOS-myc-vector (d), or pEF-BOS-myc-RB (TT) (e). 48 h after the transfection, the cells were cultured in serum-free medium for 24 h. The transfected cells were treated with 0.1 μM LPA for 10 min, and subsequently fixed and stained for β-galactosidase activity. Bar represents 20 μm. (B) 48 h after the transfection of plasmids encoding indicated cDNAs, the cells were cultured in serum-free medium for 24 h. The cells were treated with vehicle (*open column*) or 0.1 μM LPA (*hatched column*) for 10 min. The percentage of lacZ-positive cells bearing neurites longer than 30 μm was scored. Data are means ± SEM of at least triplicate determinations.

Fig. 4. Effects of MLC and MLC mutants on neurite retraction in N1E-115 cells. (A) Functional and substituted residues of MLC used in this assay are schematically shown. (B) N1E-115 cells were transiently transfected with either pEF-BOS-myc vector (a), pEF-BOS-myc-MLC (b), pEF-BOS-myc-MLC-AA (c), or pEF-BOS-myc-MLC-DD (d). pME18S-lacZ was cotransfected with these plasmids to identify the transfected cells. 48 h after the transfection, the cells were cultured in serum-free medium for 24 h, subsequently fixed and stained for β-galactosidase activity. Bar represents 20 μm. (C) 48 h after the transfection of plasmids encoding indicated cDNAs, the cells were cultured in serum-free medium for 24 h. The cells were treated with vehicle (*open column*) or 0.1 μM LPA (*hatched column*) for 10 min. The percentage of lacZ-positive cells bearing neurites above 30 μm was scored. Data are means ± SEM of at least triplicate determinations.

Fig. 5. Screening of a Rho-kinase substrate. Bovine brain cytosol fraction (20 mg of protein) was loaded onto Mono Q column. Proteins were fractionated by elution with the indicated linear gradient of NaCl. The protein content of eluted fractions was monitored by UV absorbance at 280 nm. The each fraction was subjected to the phosphorylation assay. The reaction mixture was subjected to SDS-PAGE and the radioactive proteins were detected by autoradiography. The fraction No. 10 and No. 11 contained a Rho-kinase substrate, p70.

Fig. 6. Purification of p70 by Mono S column chromatography. The fraction No. 10 and No. 11 obtained from the first Mono Q column chromatography were loaded onto Mono S column. The NaCl gradient and flow rate were the same as the first Mono Q column chromatography. 20 μl of each fraction eluted from Mono S column were subjected to the phosphorylation assay. The reaction mixture was subjected to SDS-PAGE and silver stained. The radioactive proteins were detected by autoradiography.

Fig. 7. Deduced amino acid sequence of p70. (A) The amino acid sequence of bovine CRMP-2 and the peptides obtained from p70 (*boxed sequence*) were shown. Amino acid sequence of p70 corresponding with bovine CRMP-2 were indicated by *underlines*. (B) Alignment of amino acid sequences of CRMP-2 and Unc-33 were shown. The percentages refer to identities within each region or at right within their whole length.

Fig. 8. Identification of the CRMP-2 phosphorylation site by Rho-kinase. (A) CRMP-2 phosphorylated by Rho-kinase

was digested by Asp-N, and resulting peptides were loaded onto C18 column, and phosphoamino acid sequence of radioactive fraction was carried out. (B) The fractions obtained from each Edoman degradation cycle were counted for ^{32}P in a liquid scintillation counter.

Fig. 9. *In vitro* phosphorylation of HA-CRMP-2 and HA-CRMP-2 T555A by Rho-kinase. HA-CRMP-2 and HA-CRMP-2 T555A expressed in COS7 cells were immunoprecipitated by anti-HA antibody. The immunoprecipitates were subjected to phosphorylation assay, separated by SDS-PAGE and detected by autoradiography (*left panel*). Each amount of proteins was normalized by immunoblot analysis with anti-HA antibody (*right panel*).

Fig. 10. Specificity of anti-pT555 antibody. CRMP-2 (2 pmol) containing the indicated amounts of CRMP-2 phosphorylated by CAT was subjected to SDS-PAGE. Immunoblot analysis with anti-pT555 (*upper panel*) or anti-CRMP-2 was carried out (*right panel*).

Fig. 11. Detection of phosphorylated CRMP-2 in COS7 cells expressing Rho family GTPases or Rho-kinase. pEF-BOS-HA-CRMP-2 was transfected together with the plasmids encoding indicated cDNAs in COS7 cells. As a negative control, pEF-BOS-HA-CRMP-2 T555A was also transfected with pEF-BOS-myc-CAT in COS7 cells. 24 h after transfection, the cells were cultured in serum-free medium for 24 h. The cells were then treated with 10% trichloroacetic acid. The resulting precipitates were subjected to SDS-PAGE, and immunoblot analysis with anti-pT555 antibody (*upper panel*) or anti-CRMP-2 antibody (*lower panel*) was carried out.

Fig. 12. Immunofluorescene labeling of phosphorylated CRMP-2 in COS7 cells. COS7 cells were transiently transfected with pEF-BOS-HA-CRMP-2 and either pEF-BOS-myc (a, b and c), pEF-BOS-HA-Rho^{V14} (d, e and f), or pEF-BOS-myc-CAT (g, h and i). 24 h after the transfection, the cells were cultured in serum-free medium for 24 h. The cells were then fixed, permeabilized and and stained with anti-pT555 antibody (a, d and g) and with anti-HA antibody (b, e and h). Merge: Phosphorylated HA-CRMP-2 in green and HA-CRMP-2 in red.

Fig. 13. Model for the regulation of axon guidance and neuronal cell morphology by Rho-kinase.

Abbreviations

The Abbreviations used are: Rho-kinase, Rho-associated kinase; MLC, myosin light chain; LPA, lysophosphatidic acid; CRMP-2, collapsin response mediator protein-2; DRG, dorsal root ganglion; MBS, myosin-binding subunit; GST, glutathione *S*-transferase; FITC, fluorescein isothiocyanate; PCR, polymerase chain reaction; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; HA, hemagglutinin; PBS, phosphate buffer saline; PH, pleckstrin-homology; MRCK, myotonic dystrophy kinase-related Cdc42-binding kinase.

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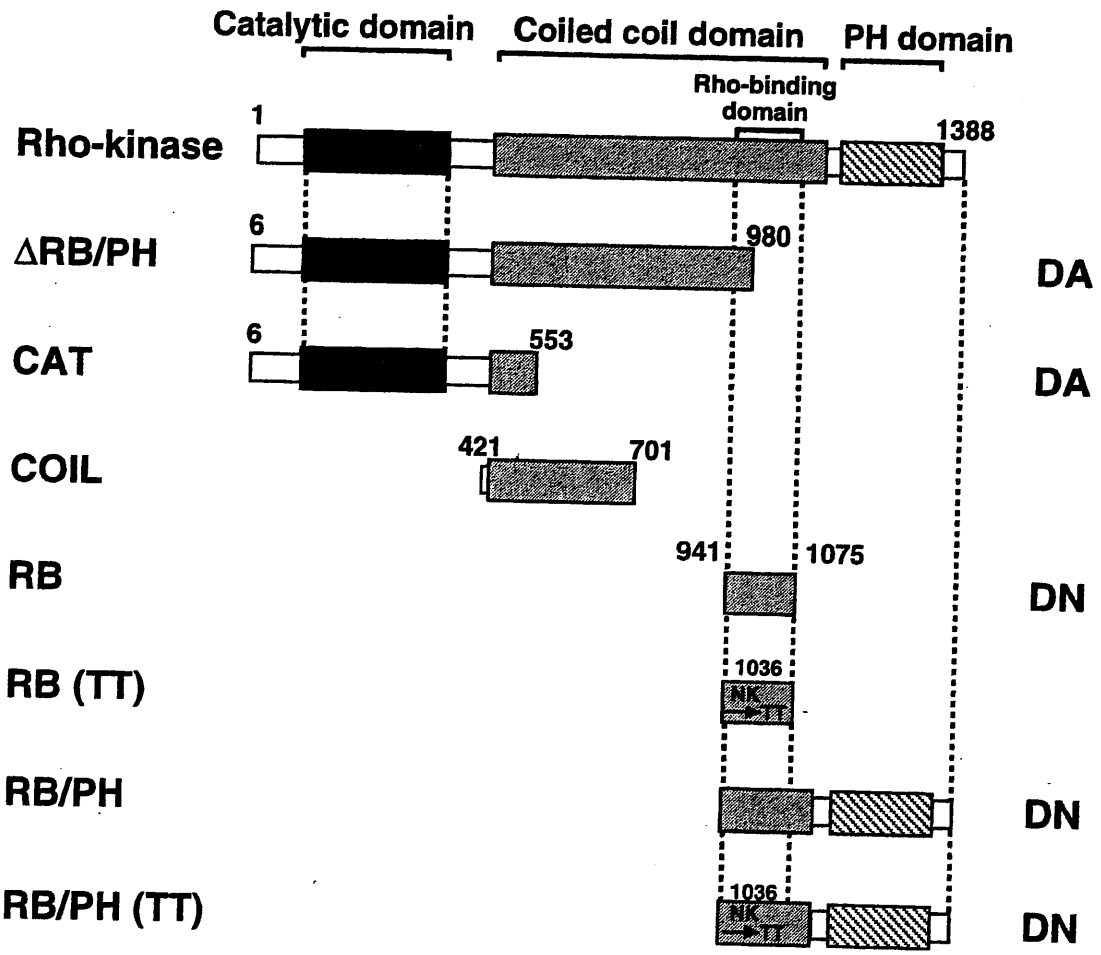
Fig. 1


Catalytic domain
Coiled coil domain
PH domain
Rho-binding domain
Rho-kinase
1
1388
ΔRB/PH
6
980
DA
CAT
6
553
DA
COIL
421
701
RB
941
1075
DN
RB (TT)
1036
NK→TT
RB/PH
DN
RB/PH (TT)
1036
NK→TT
DN

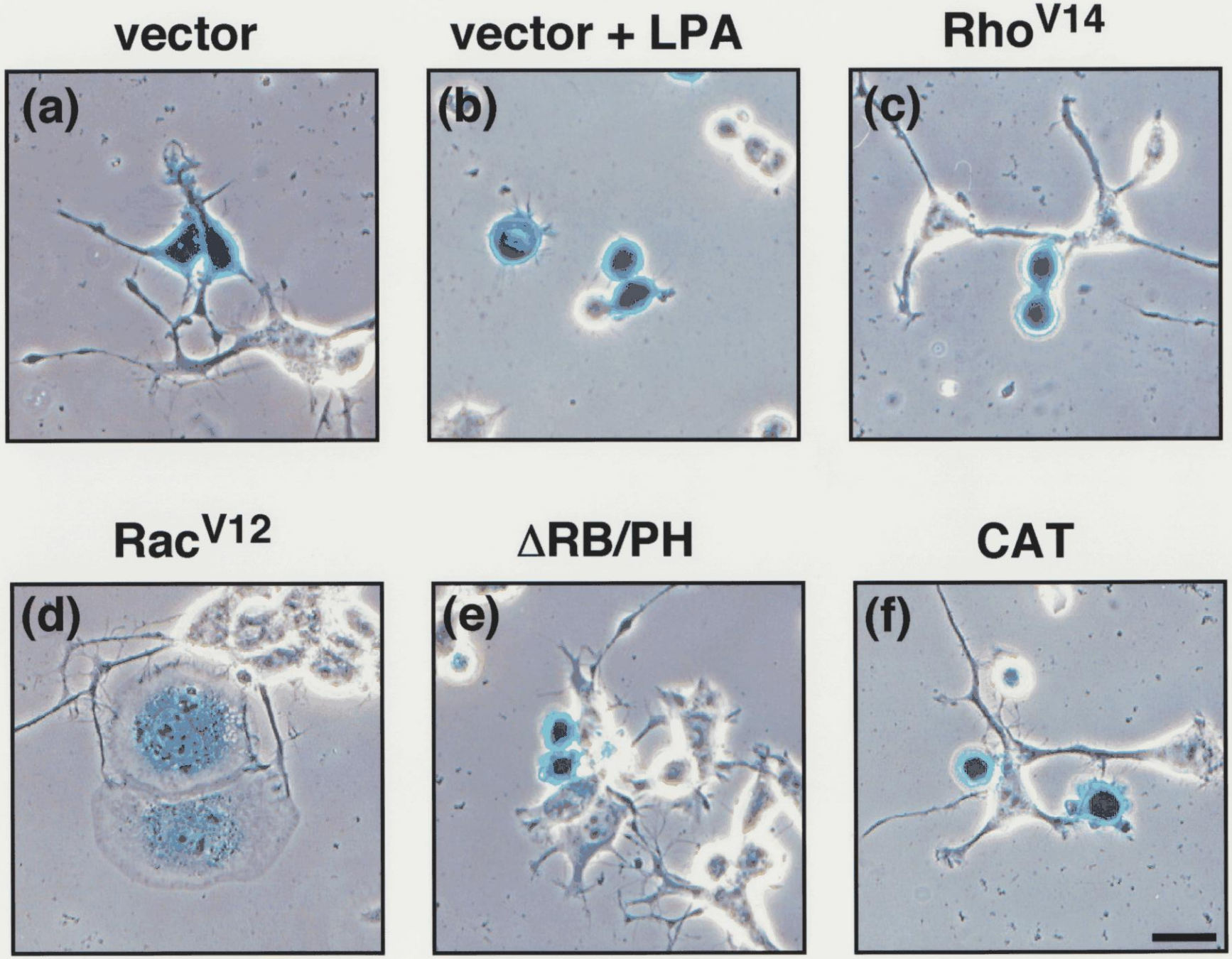
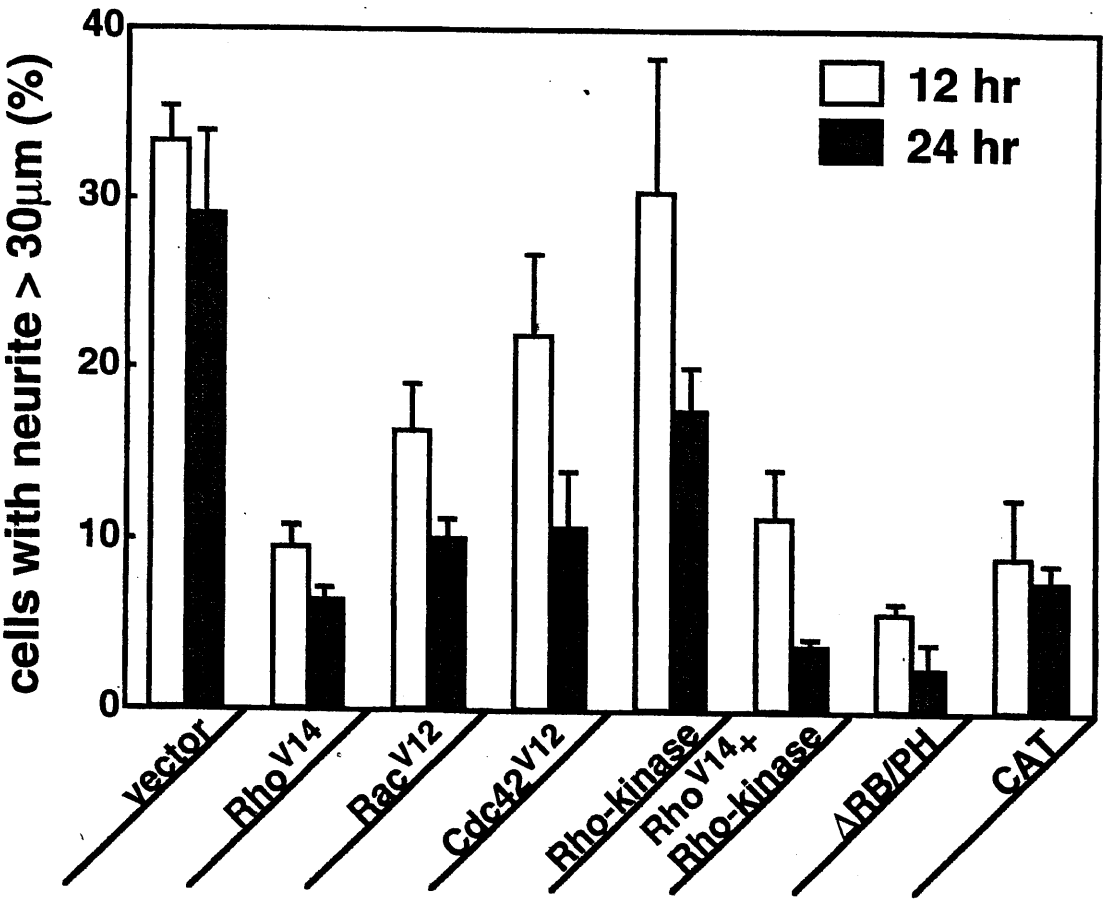


Fig. 2A

Fig. 2B


cells with neurite > 30μm (%)
40
30
20
10
0
12 hr
24 hr
vector
Rho V14
Rac V12
Cdc42 V12
Rho-kinase
Rho V14+ Rho-kinase
ΔRB/PH
CAT

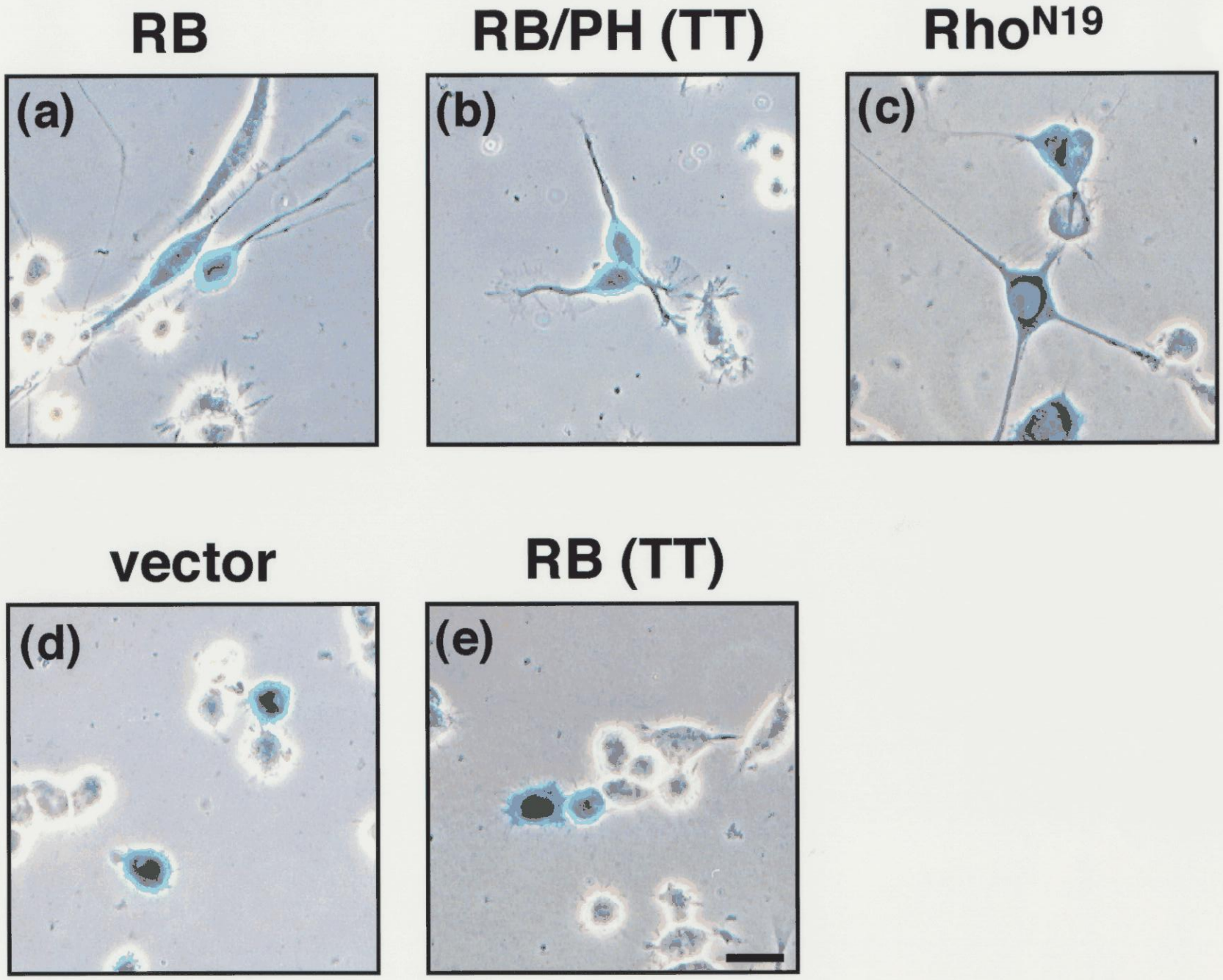
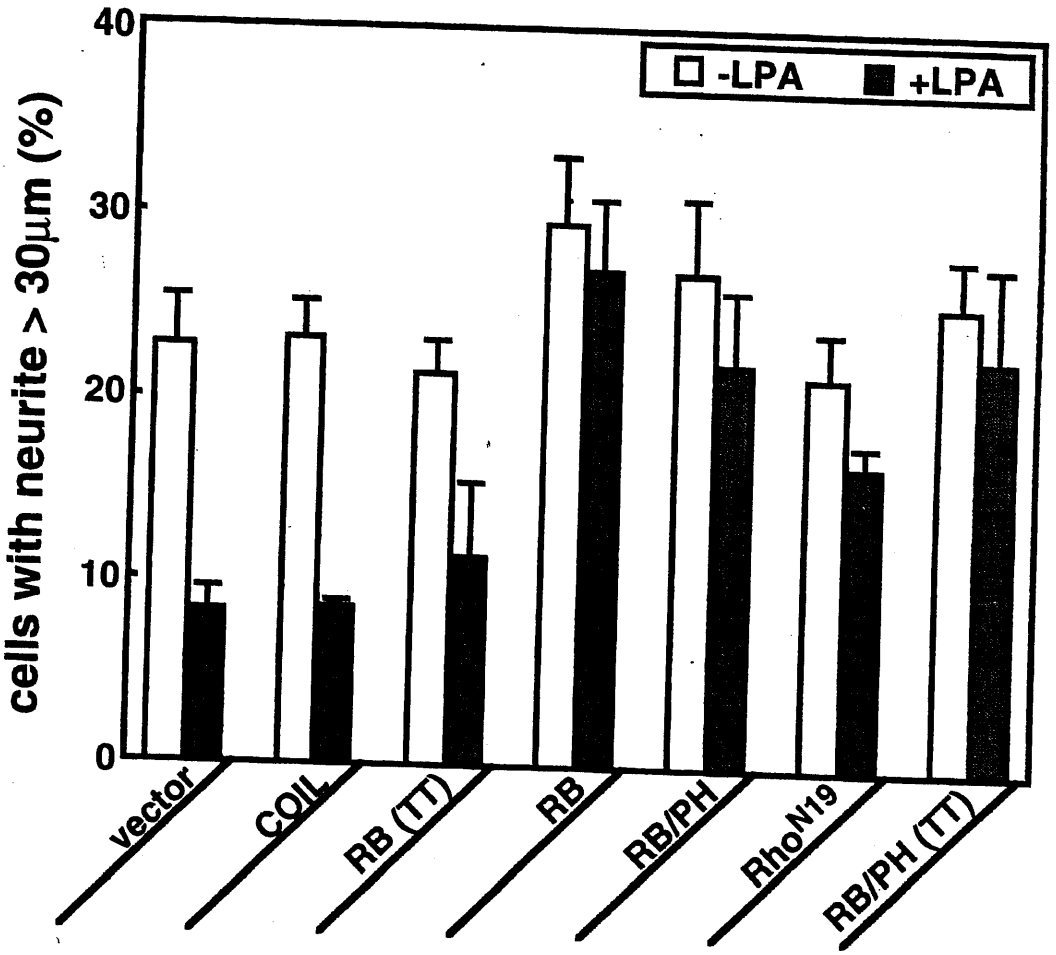


Fig. 3A

Fig. 3B


40
30
20
10
0
cells with neurite > 30μm (%)
-LPA
+LPA
vector
COIL
RB (TT)
RB
RB/PH
RhoN19
RB/PH (TT)

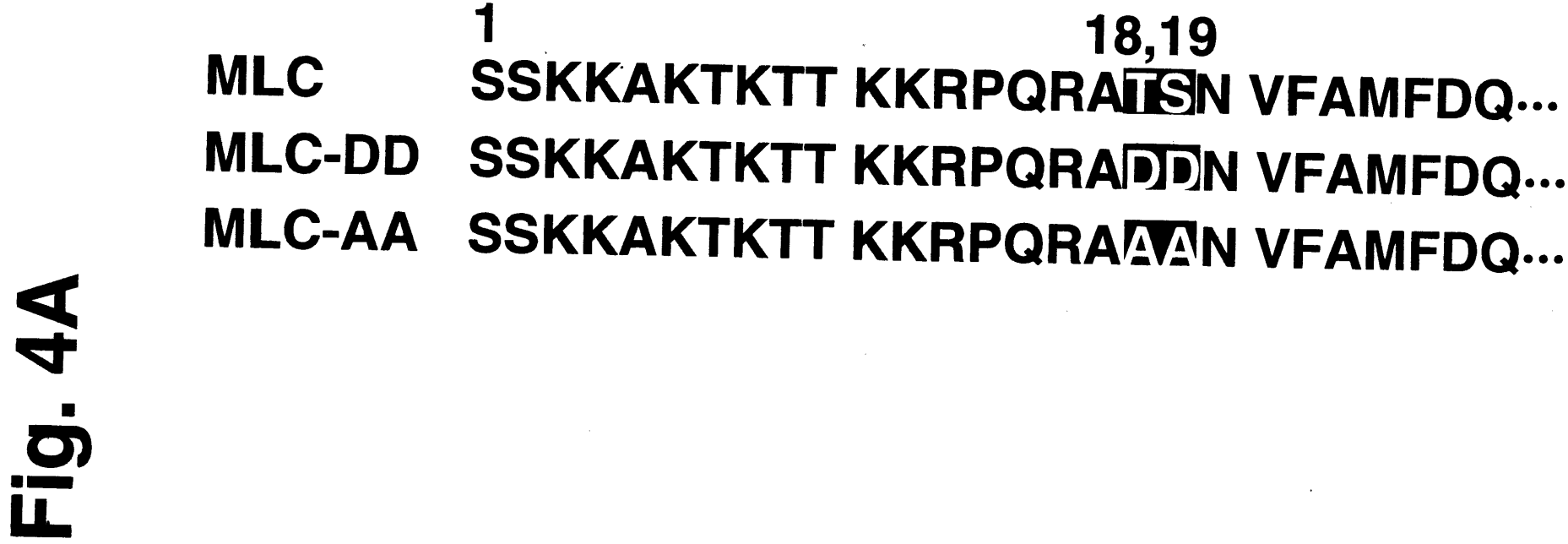

1
18,19
MLC SSKKAKTKTT KKRPQRATSN VFAMFDQ...
MLC-DD SSKKAKTKTT KKRPQRADDN VFAMFDQ...
MLC-AA SSKKAKTKTT KKRPQRAAAN VFAMFDQ...

Fig. 4A

Fig. 4B

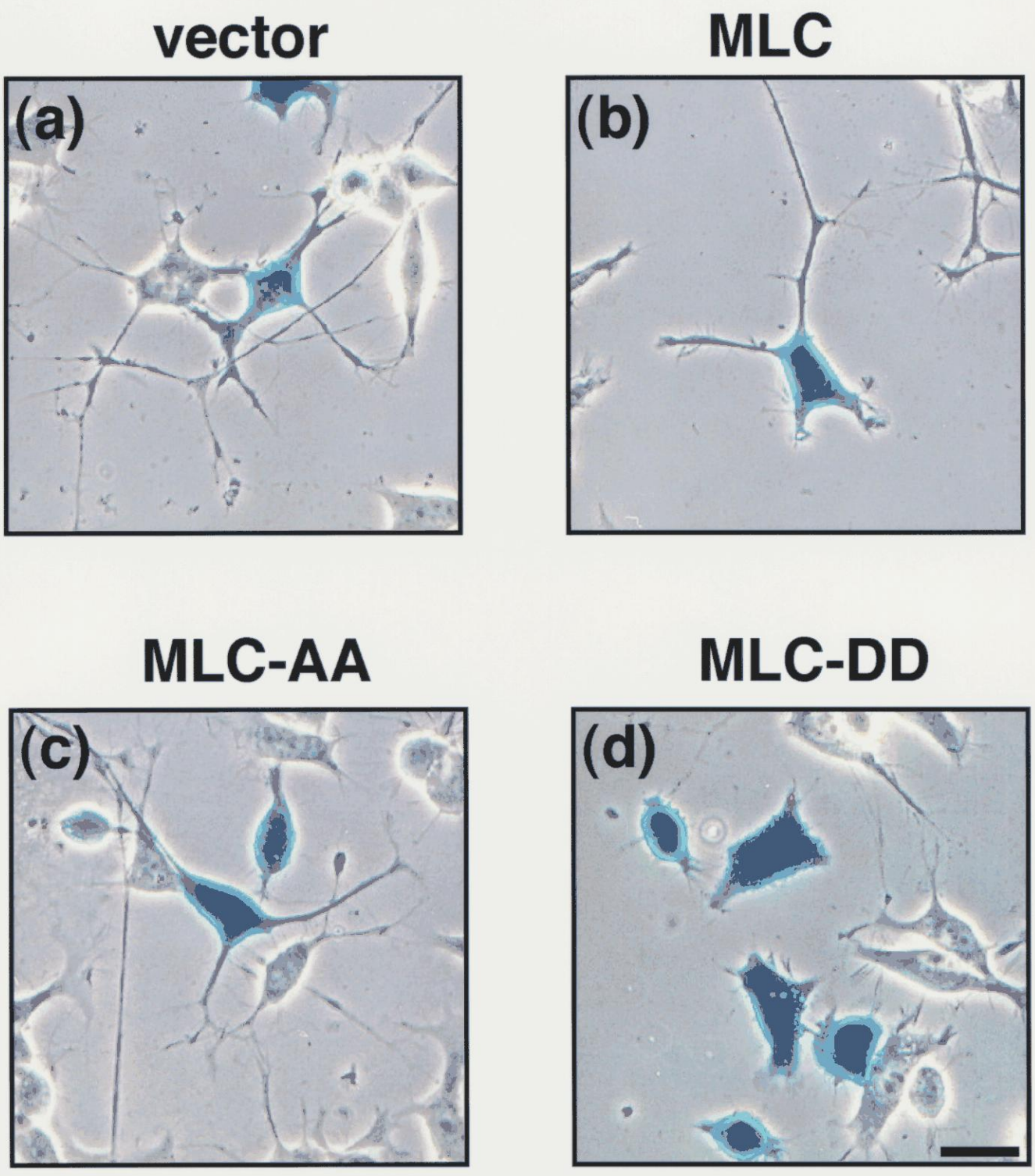


Fig. 4C

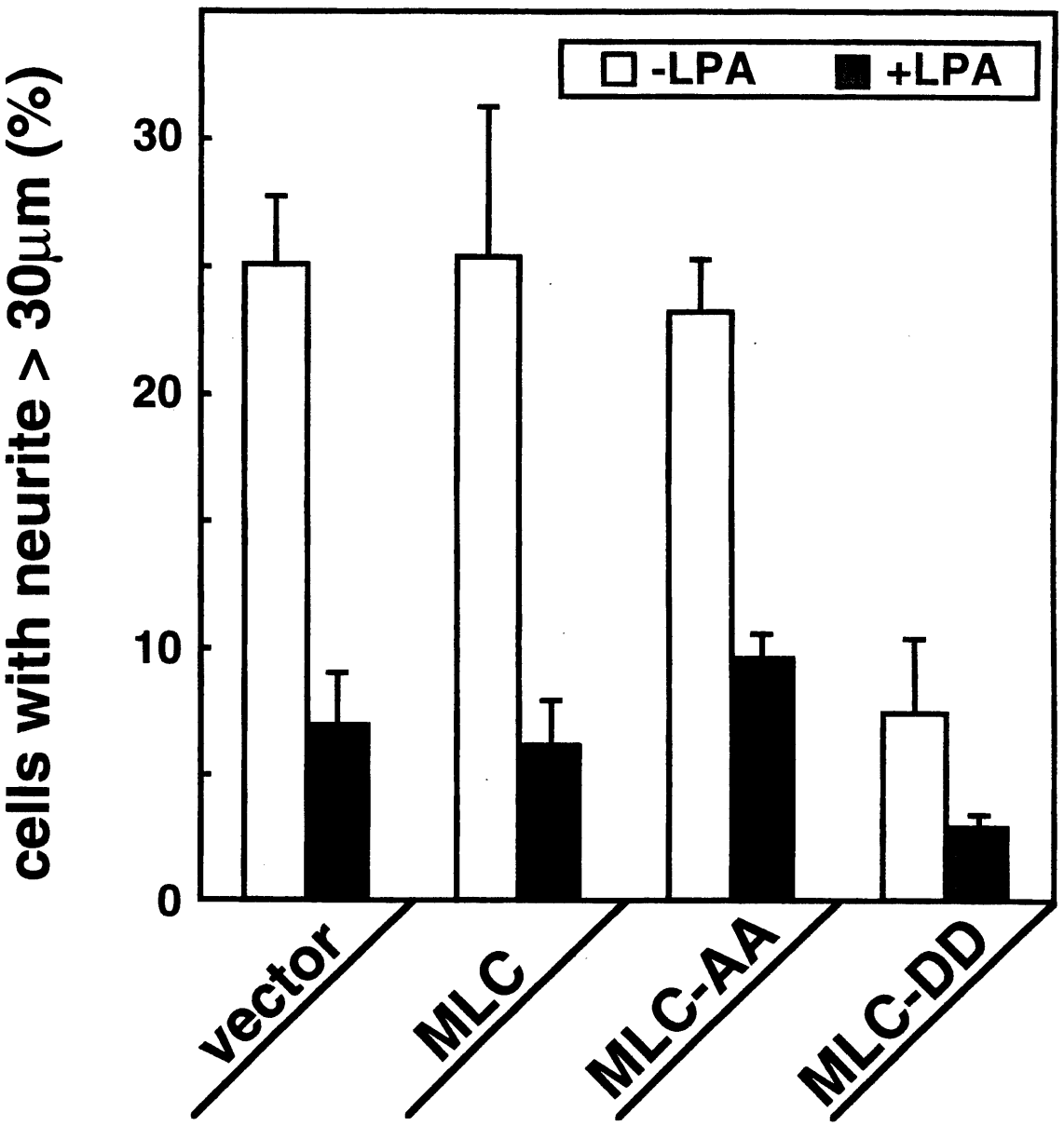

-LPA
+LPA
cells with neurite > 30μm (%)
30
20
10
0
vector
MLC
MLC-AA
MLC-DD

Fig. 5

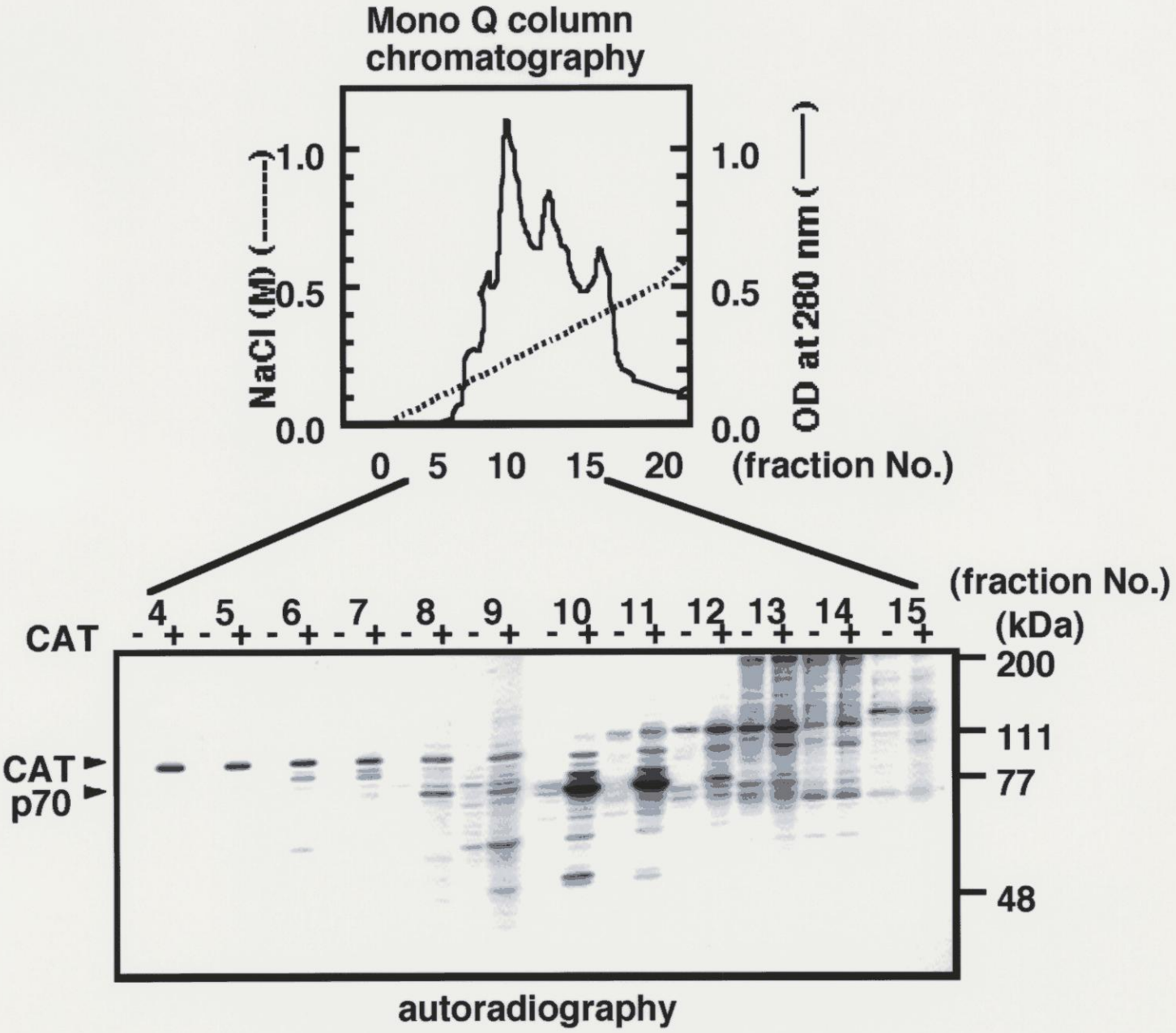

Mono Q column chromatography
NaCl (M) (------)
1.0
0.5
0.0
OD at 280 nm (——)
1.0
0.5
0.0
0 5 10 15 20
(fraction No.)
(fraction No.)
4 5 6 7 8 9 10 11 12 13 14 15
CAT
- + - + - + - + - + - + - + - + - + - + - + - +
(kDa)
200
111
77
48
CAT
p70
autoradiography

Fig. 6

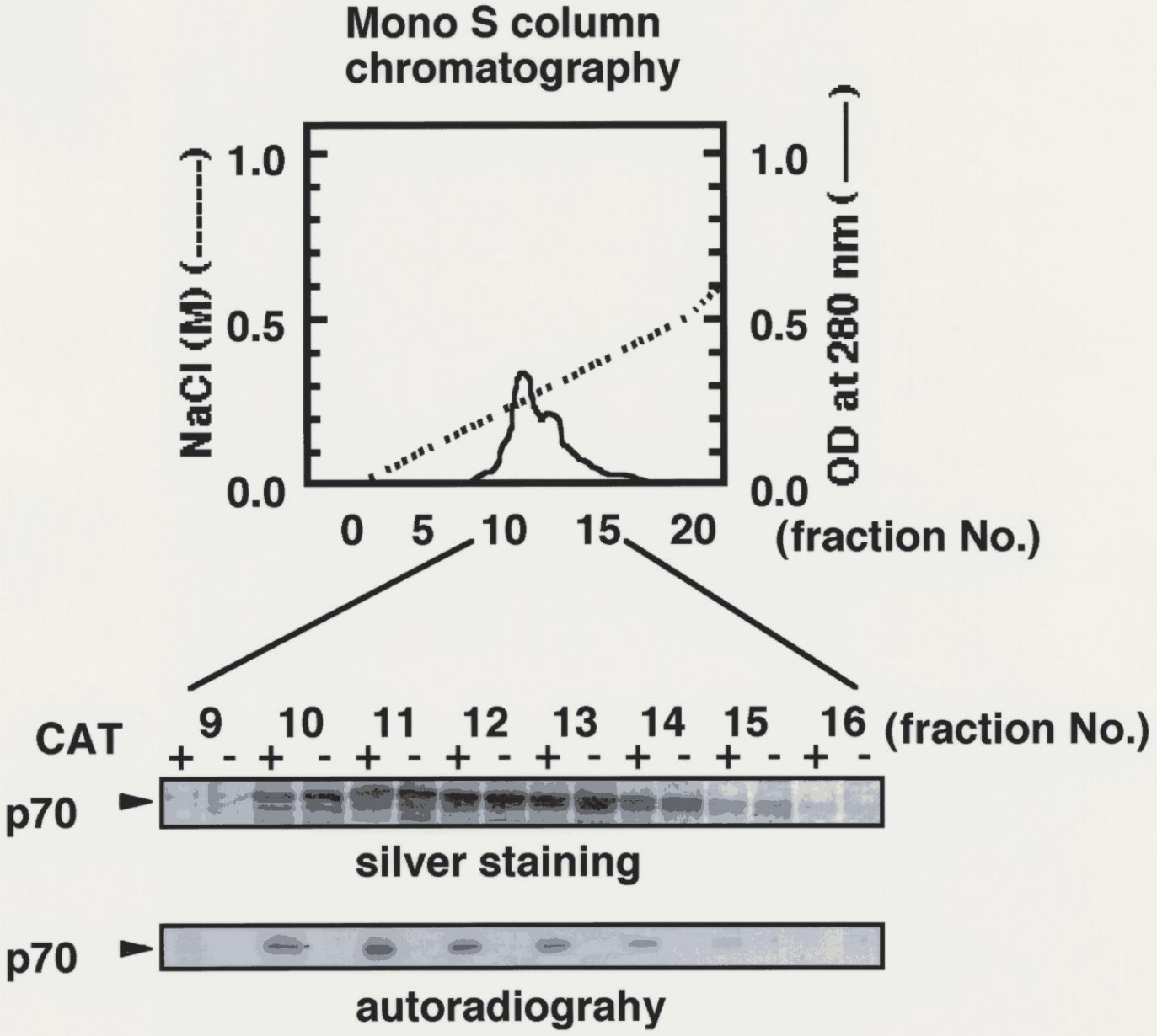

Mono S column chromatography
NaCl (M) (------)
1.0
0.5
0.0
OD at 280 nm (——)
1.0
0.5
0.0
0 5 10 15 20
(fraction No.)
CAT
9 10 11 12 13 14 15 16 (fraction No.)
+ - + - + - + - + - + - + - + -
p70
silver staining
p70
autoradiograhy

Fig. 7

A

```
MSYQGKKNIP RITSDRLLIK GGKIVNDDQS FYADIYMEDG LIKQIGENLI   50
VPGGVKTIEA HSRMVIPGGI DVHTRFQMPD QGMTSADDFF QGTKAALAGG  100
TTMIIDHVVP EPGTSLLAAF DQWREWADSK SCCDYSLHVD ISEWHKGIQE  150
EMEALVKDHG VNSFLVYMAF KDRFQLTDCQ IYEVLSVIRD IGAIAQVHAE  200
NGDIIAEEQQ RILDLGITGP EGHVLSRPEE VEAEAVNRAI TIANQTNCPL  250
YITKVMSKSS AEVIAQARRK GTVVYGEPIT ASLGTDGSHY WSKNWAKAAA  300
FVTSPPLSPD PTTPDFLNSL LSCGDLQVTG SAHCTFNTAQ KAVGKDNFTL  350
IPEGTNGTEE RMSVIWDKAV VTGKMDENQF VAVTSTNAAK VFNLYPRKGR  400
IAVGSDADLV IWDPDSVKTI SAKTHNSSLE YNIFEGMECR GSPLVVISQG  450
KIVLEDGTLH VTEGSGRYIP RKPFPDFVYK RIKARSRLAE LRGVPRGLYD  500
GPVCEVSVTP KTVTPASSAK TSPAKQQAPP VRNLHQSGFS LSGAQIDDNI  550
PRRTTQRIVA PPGGRANITS LG                                572
```

B

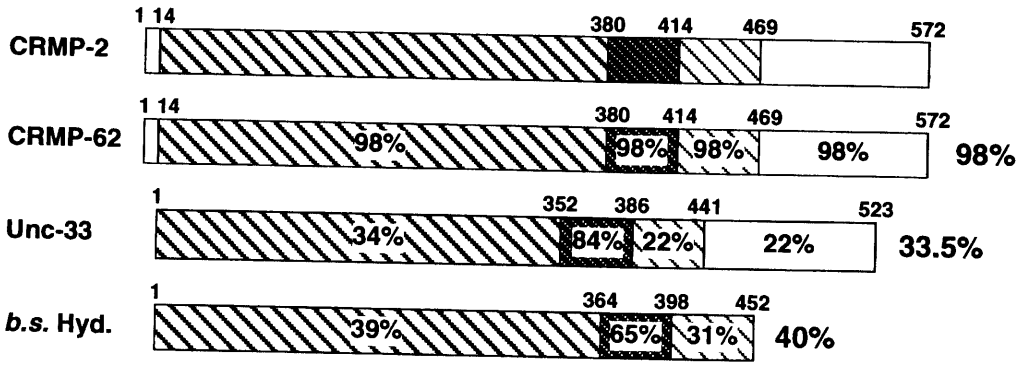
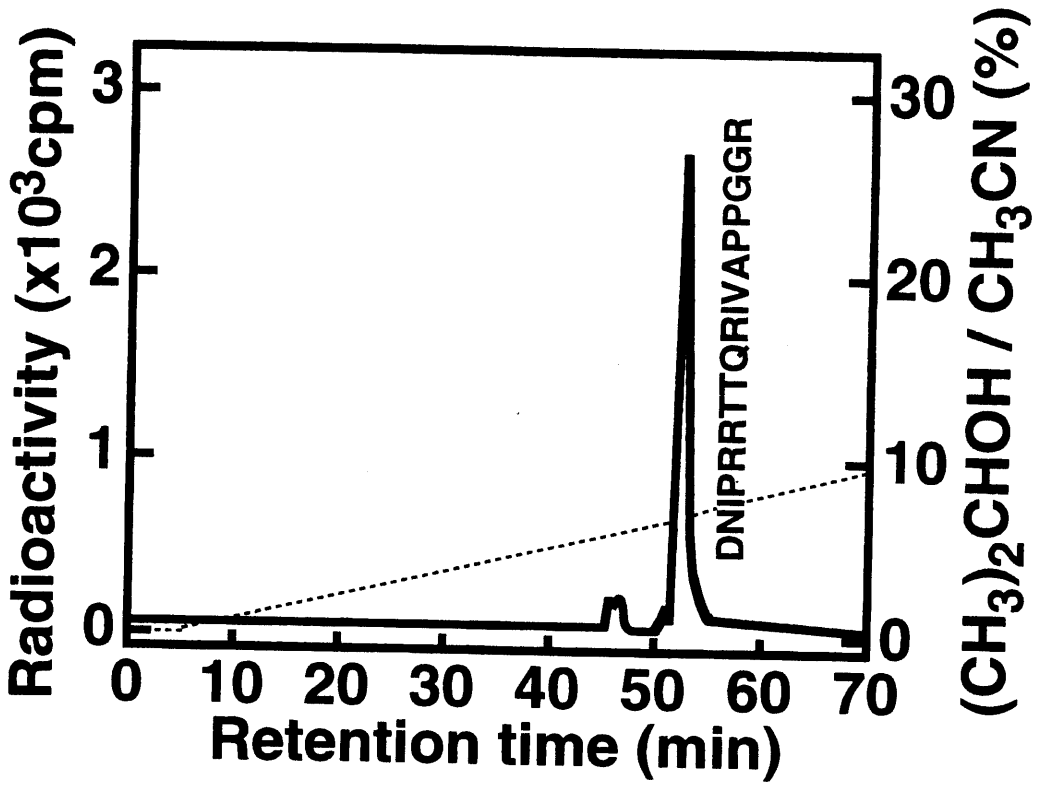


Fig. 8

A


Radioactivity (x10³cpm)
3
2
1
0
30
20
10
0
(CH₃)₂CHOH / CH₃CN (%)
DNIPRRTTQRIVAPPGGR
0 10 20 30 40 50 60 70
Retention time (min)

B

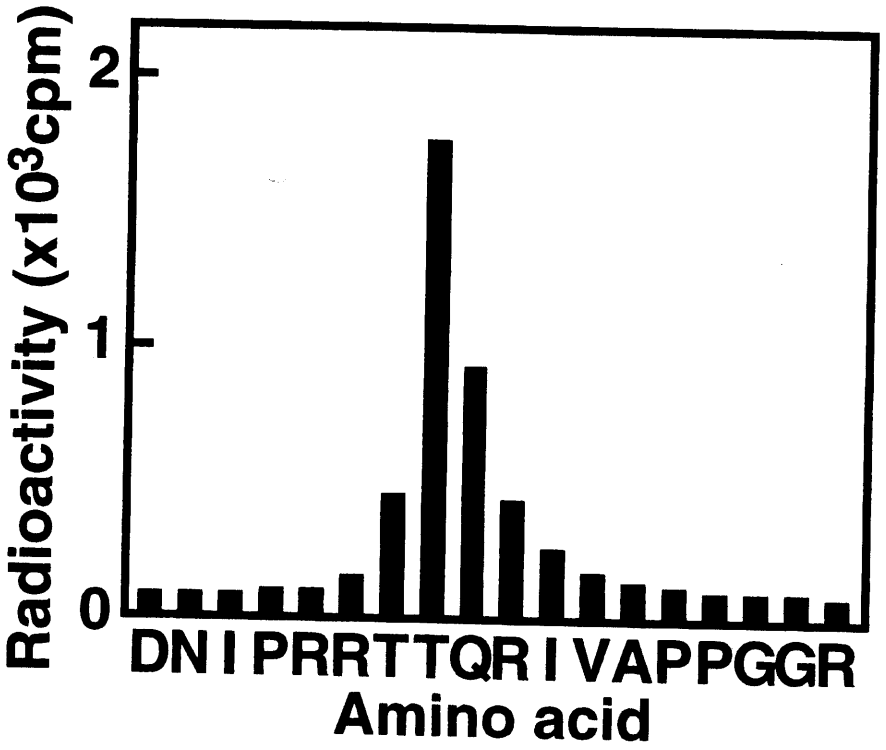

Radioactivity (x10³cpm)
2
1
0
DN I PRRTTQR I VAPPGGR
Amino acid

Fig. 9

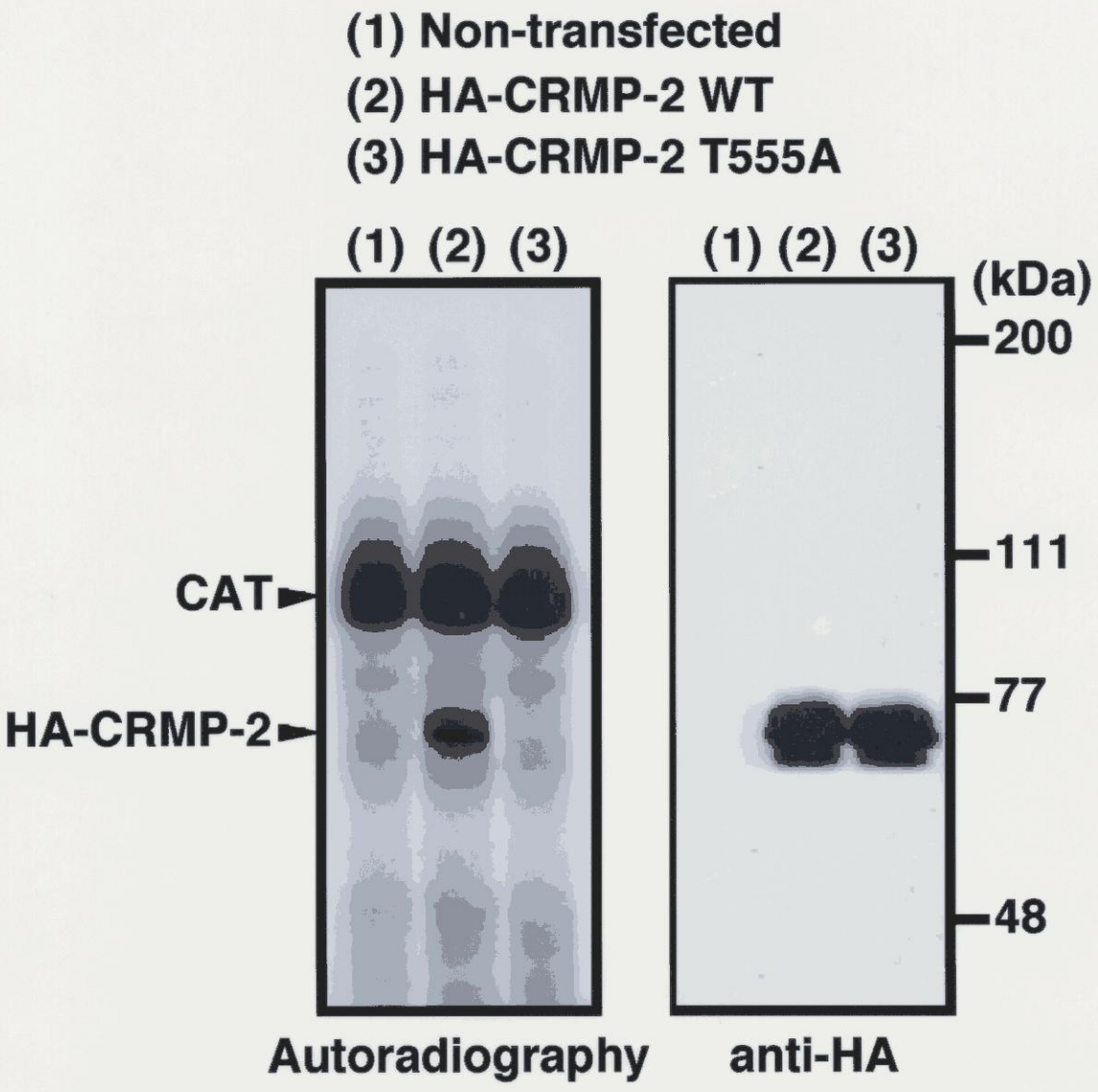

(1) Non-transfected
(2) HA-CRMP-2 WT
(3) HA-CRMP-2 T555A
(1) (2) (3)
(1) (2) (3)
(kDa)
200
111
77
48
CAT
HA-CRMP-2
Autoradiography
anti-HA

Fig. 10

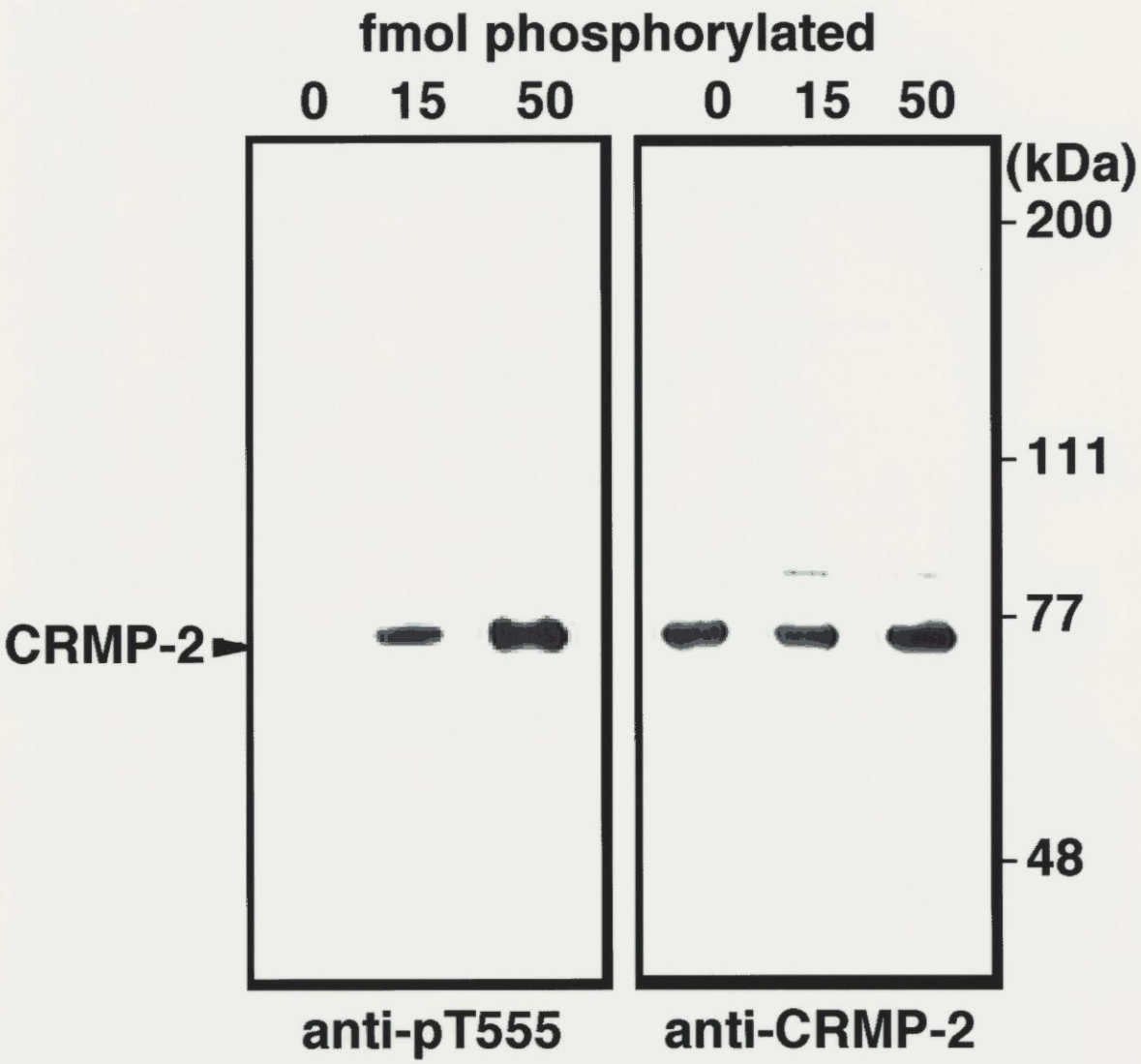

fmol phosphorylated
0
15
50
0
15
50
(kDa)
200
111
77
48
CRMP-2
anti-pT555
anti-CRMP-2

Fig. 11

(1) vector / CRMP-2 WT
(2) Rac^{V12} / CRMP-2 WT
(3) $Cdc42^{V12}$ / CRMP-2 WT
(4) Rho^{V14} / CRMP-2 WT
(5) Rho^{N19} / CRMP-2 WT
(6) Rho-kinase / CRMP-2 WT
(7) Rho^{V14}+Rho-kinase / CRMP-2 WT
(8) CAT / CRMP-2 WT
(9) CAT / CRMP-2 T555A

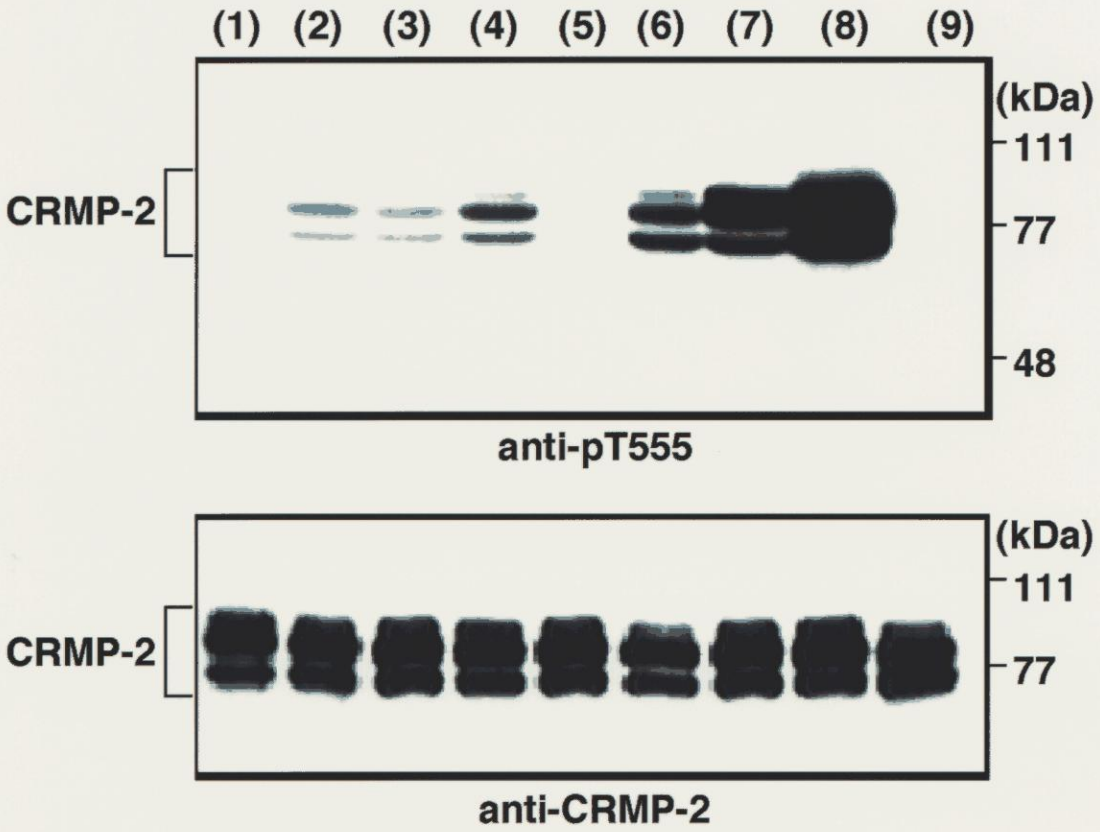


Fig. 12

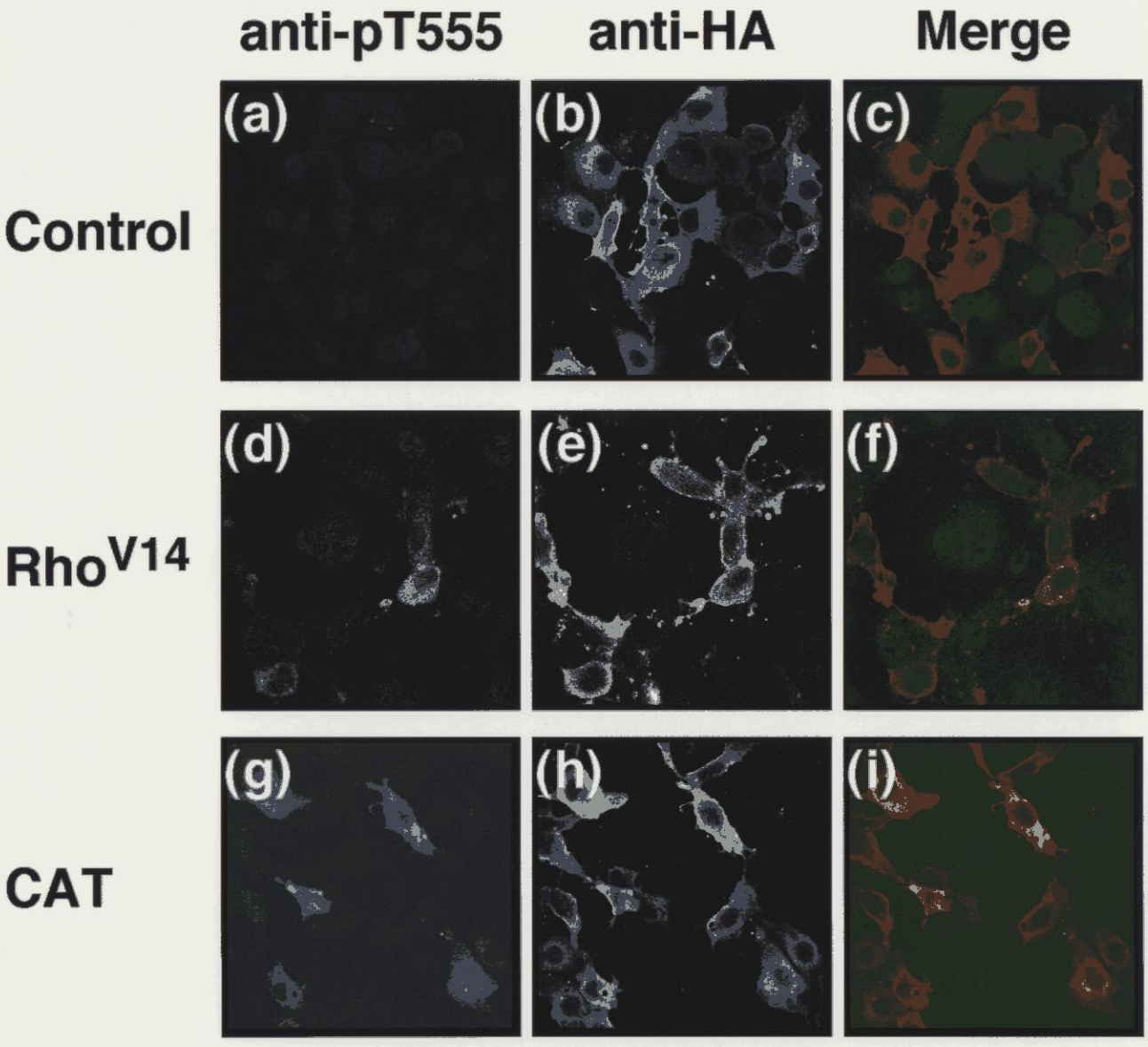
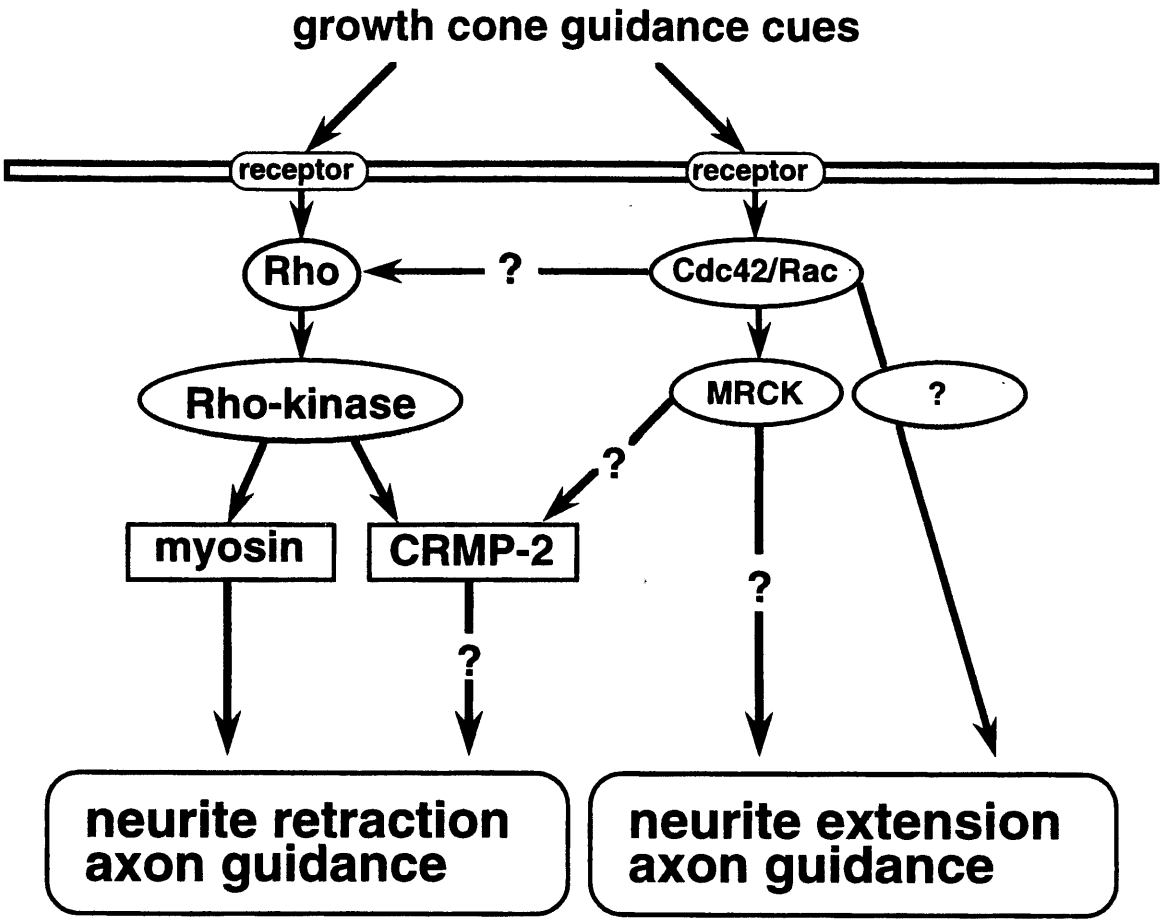


Fig. 13


growth cone guidance cues
receptor
receptor
Rho
?
Cdc42/Rac
Rho-kinase
MRCK
?
?
myosin
CRMP-2
?
?
neurite retraction
axon guidance
neurite extension
axon guidance