11. Bone marrow c-Kit$^+$ Sca-1$^+$ Lin$^-$ cells were fractionated according to the extent of mCD34 expression. One hundred Fr. 1 or Fr. 2 cells or 500 Fr. 3 cells (Fig. 2A) from Ly5.1 mice were sorted into a well containing 2×10^4 lineage-depleted BM cells from C57BL/6-Ly5.2 mice. In a preliminary experiment, we determined that 2×10^4 lineage-depleted BM cells were the minimum number of cells required for the survival of lethally irradiated recipients and reconstitution of their hematopoiesis. The cells in each well were collected and transferred into lethally irradiated C57BL/6-Ly5.2 mice. Several weeks to months after the transplantation, peripheral blood mononuclear cells were collected from the retro-orbital sinus and stained with FITC–anti-Ly5.1, PE–anti-myeloid (Mac-1 and Gr-1), and APC–anti-lymphoid (Thy1.2 and B220). The proportion of myeloid and lymphoid cells that originated from Ly5.1 cells was estimated by flow cytometry as described [S. Okada *et al.*, *Blood* **81**, 1720 (1993)].
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14. One thousand Fr. 1, Fr. 2, or Fr. 3 cells (Fig. 2A) were sorted and lysed in 50 μl of Isogen-LS (Nippon Gene, Tokyo). Total RNA was isolated according to the manufacturer's protocol. The first-strand cDNA reaction was carried out with 100 U of Molony murine leukemia virus reverse transcriptase (Gibco-BRL) in 20 mM tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, 0.5 mM deoxynucleoside triphosphate (dNTP), 10 mM DTT, and 0.25 μg of oligo$(dT)_{12-18}$. For the analysis of mCD34 and hypoxanthine phosphoribosyl transferase (HPRT) gene expression, the following specific primers were used to amplify fragments from one-half of the cDNAs obtained from each of the fractions: mCD34 [5′-ATGCAGGTCCACAGGGACACG-3′ and 5′-CTGTCCTGATAGATCAAGTAG-3′, generating a 221–base pair (bp) fragment]; and HPRT (5′-GCTGGTGAAAAGGACCTCTCG-3′ and 5′-GCAGATGGCCACAGGACTAGA-3′, generating a 258-bp fragment). PCR conditions were as follows: 20 mM tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 2.5 U of Ex Taq (Takara Ohtsu, Japan), and 50 pmol of each primer for 30 cycles (94°C, 30 s; 55°C, 30 s; 72°C, 45 s) followed by 5 min at 72°C in a Perkin-Elmer thermocycler. Each PCR product was electrophoresed through 2% agarose, transferred to a nylon membrane, and hybridized with a biotinylated internal oligonucleotide. Hybridized probes were detected with an ECL system (Amersham Life Sciences) according to the manufacturer's protocol.
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18. We thank H. Kodama for discussion, T. Toyoshima for FACS operation, M. Ito for secretarial assistance, and C. Tarlinton for reading the manuscript. Supported by grants from Fujisawa Pharmaceutical, Uehara Memorial Foundation, The Ministry of Education, Science, and Culture, and the Agency for Science and Technology, Japan.

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Regulation of Myosin Phosphatase by Rho and Rho-Associated Kinase (Rho-Kinase)

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The small guanosine triphosphatase Rho is implicated in myosin light chain (MLC) phosphorylation, which results in contraction of smooth muscle and interaction of actin and myosin in nonmuscle cells. The guanosine triphosphate (GTP)–bound, active form of RhoA (GTP·RhoA) specifically interacted with the myosin-binding subunit (MBS) of myosin phosphatase, which regulates the extent of phosphorylation of MLC. Rho-associated kinase (Rho-kinase), which is activated by GTP·RhoA, phosphorylated MBS and consequently inactivated myosin phosphatase. Overexpression of RhoA or activated RhoA in NIH 3T3 cells increased phosphorylation of MBS and MLC. Thus, Rho appears to inhibit myosin phosphatase through the action of Rho-kinase.

Stimulation of smooth muscle and nonmuscle cells by specific agonists induces Ca^{2+} mobilization and activation of MLC kinase, which phosphorylates MLC and activates the myosin adenosine triphosphatase. This sequence of events results in contraction of smooth muscle (*1*) and interaction of actin and myosin for stress fiber formation in nonmuscle cells (*2*). However, because the cytosolic concentration of Ca^{2+} is not always proportional to the extent of MLC phosphorylation and contraction, an additional mechanism to regulate the Ca^{2+} sensitivity of both processes has been proposed (*3*). Because agonists induce MLC phosphorylation and contraction in permeabilized smooth muscle at fixed submaximal concentrations of Ca^{2+} in a GTP-dependent manner, a GTP binding protein is thought to regulate the receptor-mediated sensitization of MLC phosphorylation to Ca^{2+} (*4*). The small guanosine triphosphatase (GTPase) Rho is implicated in the enhancement of Ca^{2+} sensitivity of smooth muscle contraction by GTP (*5*). In permeabilized smooth muscle cells, the nonhydrolyzable GTP analog guanosine 5′-O-(3-thiotriphosphate) (GTP-γ-S) increases MLC phosphorylation at submaximal Ca^{2+} concentrations by inhibiting dephosphorylation of MLC, presumably by activating Rho (*6*). Rho exhibits both GDP (guanosine diphosphate) and GTP binding and GTPase activities (*7*). GTP·Rho presumably binds to specific targets and thereby exerts its biological functions, which include regulation of the formation of stress fibers and focal adhesions, cell motility, cell aggregation, and cytokinesis (*7*). We have identified two putative targets for Rho, p128 and p164 (*8*). The p128 protein is a serine-threonine kinase, known as protein kinase N (PKN) (*8*, *9*). The p164 protein is a serine-threonine kinase termed Rho-kinase (*10*). Activated Rho directly interacts with PKN and Rho-kinase, and stimulates their kinase activities (*8*, *10*).

To identify Rho targets other than PKN and Rho-kinase, we applied crude extracts of bovine brain membranes to glutathione-Sepharose (Pharmacia) affinity columns to which glutathione-S-transferase (GST), a GDP-bound GST-RhoA fusion protein (GDP·GST-RhoA), GTP-γ-S·GST-RhoA, GTP-γ-S·GST-$RhoA^{A37}$, GDP·GST-Rac1, or GTP-γ-S·GST-Rac1 was immobilized. $RhoA^{A37}$ is structurally equivalent to H-Ras^{A35}, which has a mutation in the effector-interacting domain (substitution of threonine by alanine) (*7*, *11*). In addition to p128 and p164, a 138-kD protein (p138) was eluted from the GTP-γ-S·GST-RhoA affinity column, but not from the GST or GDP·GST-RhoA columns (Fig. 1A), indicating that p138 specifically interacted either directly or indirectly with the GTP-γ-S–bound form of GST-RhoA. The p138 protein was eluted from neither the GTP-γ-S·GST-$RhoA^{A37}$ nor the GTP-γ-S·GST-Rac1 affinity columns.

The sequences of nine peptides derived from p138 were determined: RWIGSE, SLLQM, GYTEVL, ETLIIEPEK, DESPA, AYVAPTV, SLQGI, AQLHDTNMAL, and DENGALIRVIS (*12*). These sequences were almost identical to that of the 110-kD regulatory subunit of rat smooth muscle protein phosphatase 1M (*13*), which is a homolog of the myosin-binding subunit (MBS) of myosin phosphatase from chicken (*14*). We further confirmed that p138 is MBS by immunoblot analysis. The p138 protein was recognized by antibodies to MBS; an immunoreactive band was specifically detected in the eluate from the GTP-γ-S·GST-RhoA affinity column (Fig. 1B). Therefore, we concluded that p138

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is the bovine counterpart of MBS and hereafter refer to it as MBS. Myosin phosphatase is composed of MBS, a 37-kD type 1 phosphatase catalytic subunit, and a 20-kD regulatory subunit (*13*, *14*). We examined whether the other subunits were retained on the GTP-γ-S·GST-RhoA affinity column. An immunoreactive band corresponding to the catalytic subunit was specifically detected in the eluate from the GTP-γ-S·GST-RhoA affinity column (Fig. 1B). An immunoreactive band corresponding to the 20-kD regulatory subunit was detected in the membrane extract but not in any of the column eluates, suggesting that interaction of RhoA with myosin phosphatase may result in dissociation of the 20-kD regulatory subunit from the holoenzyme. Alternatively, RhoA may interact specifically with myosin phosphatase composed of MBS and the catalytic subunit. MLC phosphatase activity was specifically detected in the eluate from the GTP-γ-S·GST-RhoA affinity column (Fig. 1C).

To address whether recombinant MBS interacts with GTP-γ-S·RhoA, we mixed in vitro–translated rat MBS with affinity beads coated with GST-RhoA. MBS was retained on the affinity beads coated with GTP-γ-S·GST-RhoA and was eluted by the addition of glutathione (Fig. 2A). The interaction of MBS with GDP·GST-RhoA and GTP-γ-S·GST-RhoA^{A37} was less marked than that with GTP-γ-S·GST-RhoA; a background interaction of MBS with GST was also apparent.

We showed that the COOH-terminal domain of rat MBS (amino acids 699 to 976) (rMBS-C) interacted with dominant activated RhoA (RhoA^{V14}), but not with wild-type RhoA, in a yeast two-hybrid system (*15*) (Fig. 2B). RhoA^{V14} is structurally equivalent to H-Ras^{V12} (substitution of glycine by valine) (*7*). Because RhoA^{V14} exhibits reduced GTPase activity and is insensitive to the Rho GTPase-activating protein (GAP), it exists mainly in a GTP-bound form in intact cells (*16*). The NH_2-terminal domain of rat MBS (amino acids 1 to 707) (rMBS-N) interacted weakly with RhoA^{V14} but not with RhoA. The COOH-terminal domain of MBS contains a polybasic region followed by a leucine zipper–like motif (*13*). PKN contains a similar polybasic region followed by a leucine zipper–like motif in the domain that interacts with Rho (*8*, *9*), suggesting that the shared structure mediates interaction with Rho. We did not detect any sequence similarity between the NH_2-terminal domain of MBS and the Rho-interacting sites of PKN and Rho-kinase (*8–10*, *13*).

When COS-7 cells were transfected with a plasmid encoding Myc epitope–tagged MBS, ~40% of the Myc-MBS was present in the cytosolic fraction and ~60% in the particulate fraction (Fig. 2C). The amount of Myc-MBS in the cytosolic fraction decreased and that in the particulate fraction increased when the cells were cotransfected with a plasmid encoding RhoA, and this effect was even more marked in cells expressing RhoA^{V14}. Thus, RhoA appears to promote the association of Myc-MBS with membranes, presumably by forming a complex with Myc-MBS as described for Rho-kinase (*10*).

GTP-γ-S·GST-RhoA did not modulate the activity of MLC phosphatase (*17*). Thiophosphorylation of MBS is associated with inhibition of MLC phosphatase activity in permeabilized smooth muscle (*18*). These observations prompted us to examine whether PKN and Rho-kinase phosphorylate MBS. Rho-kinase phosphorylated the COOH-terminal domain of chicken MBS (amino acids 753 to 1004) (cMBS-C) (Fig. 3A) but did not phosphorylate the NH_2-terminal domain of chicken MBS (amino acids 1 to 721) (cMBS-N), whereas PKN catalyzed little phosphorylation of either fragment (*19*). GTP-γ-S·GST-RhoA stimulated the activity of Rho-kinase toward cMBS-C, whereas GDP·GST-RhoA, GTP-γ-S·GST-RhoA^{A37}, or GTP-γ-S·GST-Rac1 did not (Fig. 3A). To examine whether Rho-kinase regulates MLC phosphatase activity through the phosphorylation of MBS, we tested whether Rho-kinase would thiophosphorylate the native holoenzyme in the presence of adenosine 5′-O-(3-thiotriphosphate) (ATP-γ-S); we measured thiophosphorylation because it is resistant to

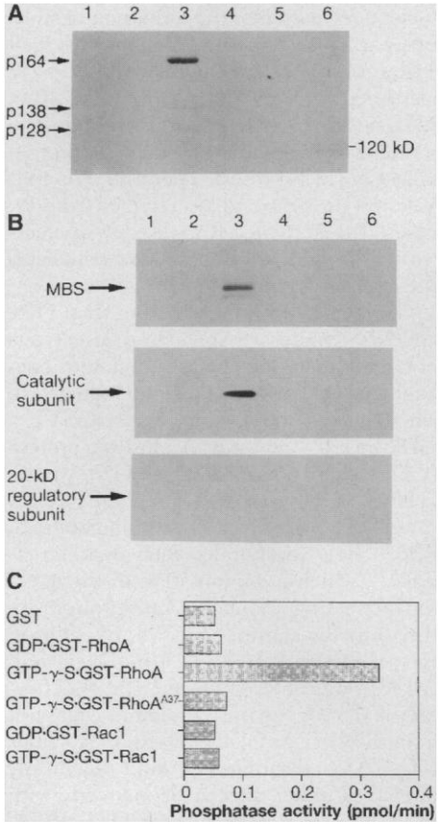

Fig. 1. Specific interaction of myosin phosphatase with activated RhoA. (**A**) Membrane extracts were applied to a glutathione–Sepharose 4B column containing either GST (lane 1), GDP·GST-RhoA (lane 2), GTP-γ-S·GST-RhoA (lane 3), GTP-γ-S·GST-RhoA^{A37} (lane 4), GDP·GST-Rac1 (lane 5), or GTP-γ-S·GST-Rac1 (lane 6) (*21*). Bound proteins were eluted, and portions (40 μl) of the eluates were subjected to SDS–polyacrylamide gel electrophoresis (PAGE) and silver staining. Arrows indicate specific eluted proteins, and the position of a 120-kD molecular size marker is indicated on the right. (**B**) Portions (40 μl) of the eluates from the affinity columns were subjected to immunoblot analysis with antibodies to MBS, the catalytic subunit, or the 20-kD regulatory subunit of myosin phosphatase (*22*). Lanes are as in (A). (**C**) Portions (20 μl) of the eluates from the affinity columns were assayed for MLC phosphatase activity (*23*). The results are representative of three independent experiments.

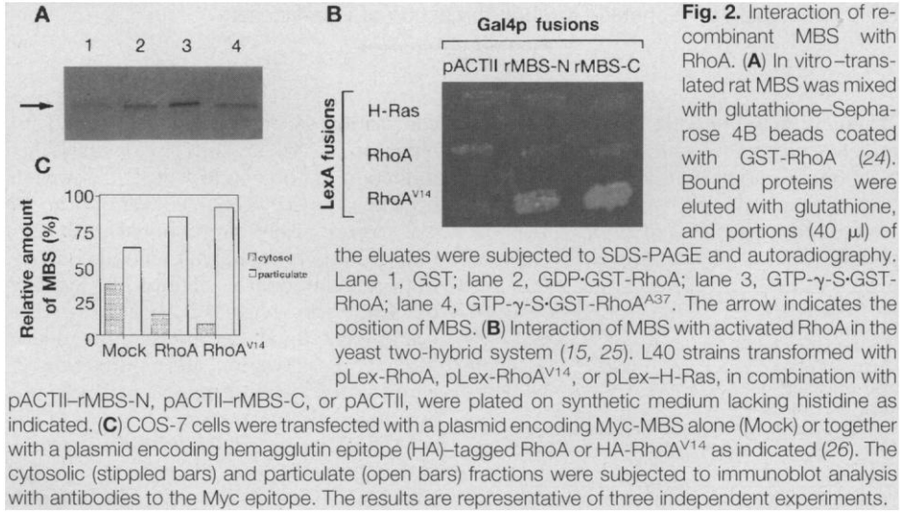

Fig. 2. Interaction of recombinant MBS with RhoA. (**A**) In vitro–translated rat MBS was mixed with glutathione–Sepharose 4B beads coated with GST-RhoA (*24*). Bound proteins were eluted with glutathione, and portions (40 μl) of the eluates were subjected to SDS-PAGE and autoradiography. Lane 1, GST; lane 2, GDP·GST-RhoA; lane 3, GTP-γ-S·GST-RhoA; lane 4, GTP-γ-S·GST-RhoA^{A37}. The arrow indicates the position of MBS. (**B**) Interaction of MBS with activated RhoA in the yeast two-hybrid system (*15*, *25*). L40 strains transformed with pLex-RhoA, pLex-RhoA^{V14}, or pLex–H-Ras, in combination with pACTII–rMBS-N, pACTII–rMBS-C, or pACTII, were plated on synthetic medium lacking histidine as indicated. (**C**) COS-7 cells were transfected with a plasmid encoding Myc-MBS alone (Mock) or together with a plasmid encoding hemagglutin epitope (HA)–tagged RhoA or HA-RhoA^{V14} as indicated (*26*). The cytosolic (stippled bars) and particulate (open bars) fractions were subjected to immunoblot analysis with antibodies to the Myc epitope. The results are representative of three independent experiments.

phosphatase activity and the holoenzyme exhibits phosphatase activity toward itself (*18*). The MBS of the holoenzyme was slightly thiophosphorylated in the absence of Rho-kinase, presumably because of contamination with an unidentified kinase. Rho-kinase thiophosphorylated MBS in a dose-dependent manner (Fig. 3B). Thiophosphorylation of MBS was associated with a decrease in MLC phosphatase activity (Fig. 3B). The decrease in MLC phosphatase activity was dependent on the simultaneous presence of ATP-γ-S and Rho-kinase. GTP-γ-S·GST-RhoA enhanced both the thiophosphorylation of MBS and the inhibition of phosphatase activity by Rho-kinase.

To test whether activated RhoA can stimulate the phosphorylation of MBS in intact cells, we established NIH 3T3 cell lines that expressed RhoA (RhoA-5, RhoA-24) or $RhoA^{V14}$ ($RhoA^{V14}$-7, $RhoA^{V14}$-25) under the control of an inducible promoter. Treatment of the cell lines with an inducer, isopropyl-β-D-thiogalactopyranoside (IPTG), resulted in a 2- to 5.4-fold increase in RhoA or $RhoA^{V14}$ expression, depending on the cell line, although they all showed basal expression of these proteins (*20*). Large numbers of stress fibers and focal adhesions were observed in these cell lines in the presence or absence of IPTG (*16*, *20*). The extent of phosphorylation of MBS in the RhoA-5, RhoA-24, $RhoA^{V14}$-7, and $RhoA^{V14}$-25 cell lines was greater than that in the parental NIH 3T3 cells when the cells were grown in the presence of IPTG (Fig. 4A), whereas the phosphorylation of other major proteins (*19*) and the amounts of MBS (Fig. 4A) were similar among these cell lines. Treatment of the $RhoA^{V14}$-7 cell line with IPTG induced a 5.4 ± 0.5–fold increase in $RhoA^{V14}$ expression and a 2.0 ± 0.3–fold increase in MBS phosphorylation (means ± SEM, $n = 3$) (Fig. 4B). IPTG did not affect MBS phosphorylation in the parental NIH 3T3 cells and induced only small increases in MBS phosphorylation in the RhoA-5, RhoA-24, and $RhoA^{V14}$-25 cell lines. IPTG increased RhoA or $RhoA^{V14}$ expression ~2- to 2.5-fold in the RhoA-5, RhoA-24, and $RhoA^{V14}$-25 cell lines; however, the basal expression of RhoA or $RhoA^{V14}$ in these cell lines may be sufficient to induce MBS phosphorylation.

Treatment of the parental NIH 3T3 cells with calyculin A (a phosphatase inhibitor) increased phosphorylation of MLC (Fig. 4C). The extent of phosphorylation of MLC in the RhoA-24 and $RhoA^{V14}$-7 cell lines in the presence of IPTG was greater than that in the parental NIH 3T3 cells. Essentially identical results were obtained with the RhoA-5 and $RhoA^{V14}$-25 cell lines (*20*).

Activated Rho apparently interacts with PKN, Rho-kinase, and MBS. The activated Rho-kinase then phosphorylates MBS, thereby inhibiting myosin phosphatase and resulting in an increase in MLC phosphorylation and consequent contraction of smooth muscle or interaction of actin and myosin leading to stress fiber formation in nonmuscle cells. Our results thus help explain how activated Rho increases the Ca^{2+} sensitivity of both MLC phosphorylation and contraction in smooth muscle.

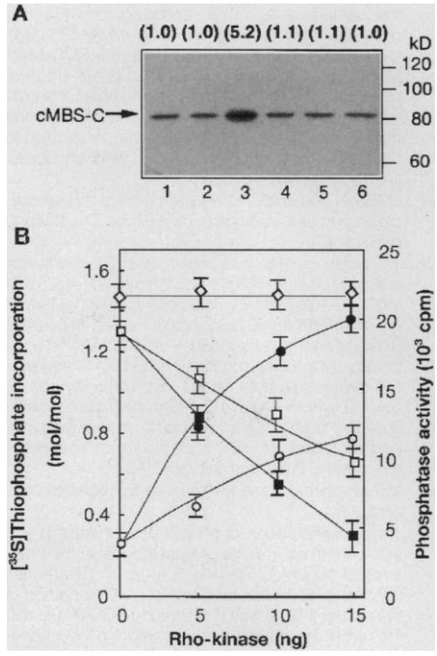

Fig. 3. Phosphorylation of MBS and inhibition of MLC phosphatase activity by Rho-kinase. (**A**) Phosphorylation of cMBS-C (*27*) by Rho-kinase in the presence of various small GTPases (1 μM each) (*28*). Lane 1, GST; lane 2, GDP·GST-RhoA; lane 3, GTP-γ-S·GST-RhoA; lane 4, GTP-γ-S·GST-$RhoA^{A37}$; lane 5, GDP·GST-Rac1; lane 6, GTP-γ-S·GST-Rac1. The values in parentheses represent fold stimulation relative to Rho-kinase incubated with GST. The results are representative of three independent experiments. The positions of molecular size standards (in kilodaltons) are indicated on the right. (**B**) MBS of native myosin phosphatase (*29*) was thiophosphorylated (circles) in the presence of the indicated amounts of Rho-kinase with (closed symbols) or without (open symbols) GTP-γ-S·GST-RhoA (1 μM) (*30*). Under similar conditions, MLC phosphatase activity (squares) was measured after MBS was thiophosphorylated (*30*). MLC phosphatase activity was also measured in the absence of ATP-γ-S (◇). Data are means ± SEM of triplicate determinations.

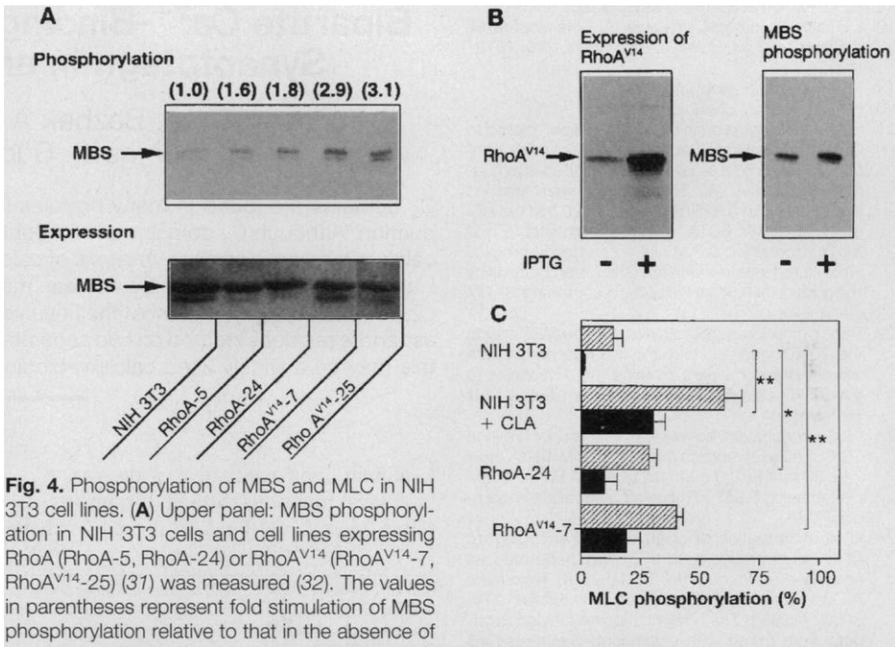

Fig. 4. Phosphorylation of MBS and MLC in NIH 3T3 cell lines. (**A**) Upper panel: MBS phosphorylation in NIH 3T3 cells and cell lines expressing RhoA (RhoA-5, RhoA-24) or $RhoA^{V14}$ ($RhoA^{V14}$-7, $RhoA^{V14}$-25) (*31*) was measured (*32*). The values in parentheses represent fold stimulation of MBS phosphorylation relative to that in the absence of transfected RhoA. Lower panel: Expression of MBS in NIH 3T3 cells and the RhoA-5, RhoA-24, $RhoA^{V14}$-7, and $RhoA^{V14}$-25 cell lines. The results are representative of three independent experiments. (**B**) $RhoA^{V14}$ expression and MBS phosphorylation in the $RhoA^{V14}$-7 cell line were determined in the absence or presence of IPTG (*32*). The results are representative of three independent experiments. (**C**) MLC phosphorylation in NIH 3T3 cells and the RhoA-24 and $RhoA^{V14}$-7 cell lines (*33*). CLA, calyculin A. Data represent the percentage of monophosphorylated (striped bars) and diphosphorylated (solid bars) MLC in total MLC and are means ± SEM of triplicate determinations. The values for total MLC phosphorylation were compared by Dunnett's t test (*$P < 0.01$, **$P < 0.001$).

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12. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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21. Extracts of bovine brain membranes were applied to 0.25-ml glutathione-Sepharose 4B columns containing GST-linked small GTPases loaded with guanine nucleotides (*8*). The columns were washed three times with 0.825 ml of buffer A [20 mM tris-HCl (pH 7.5), 1 mM EDTA, 1 mM dithiothreitol, 5 mM $MgCl_2$] containing 0.2 M NaCl, and bound proteins were eluted together with the GST fusion proteins by the addition of 0.825 ml of buffer A containing 10 mM glutathione.
22. Rabbit polyclonal antibodies to chicken MBS and to the 20-kD regulatory subunit were generated with the use of recombinant proteins (*14*). Antibodies to the 37-kD catalytic subunit were from Santa Cruz Biotechnology.
23. MLC phosphatase activity was assayed for 6 min at 30°C in 50 μl of reaction mixture [30 mM tris-HCl (pH 7.5), 30 mM KCl, 3 mM $MgCl_2$, 5 mM EDTA, 1 mM dithiothreitol, 5 μM ^{32}P-labeled MLC, and test samples] (*14*).
24. In vitro translation of plasmid pCRII encoding rat MBS (amino acids 1 to 976) was performed as described [T. Yamamoto, T. Matsui, M. Nakafuku, A. Iwamatsu, K. Kaibuchii, *J. Biol. Chem.* **270**, 30557 (1995)]. The [^{35}S]methionine-labeled products were mixed with glutathione–Sepharose 4B beads coated with GST-RhoA loaded with guanine nucleotides.
25. To express fusion proteins of small GTPases with the LexA DNA-binding domain, we constructed plasmids pLex–H-Ras, pLex-RhoA, and pLex-RhoA^{V14} (*15*). To express fusion proteins of rMBS-N and rMBS-C with the Gal4p acidic activation domain, we constructed pACTII–rMBS-N and pACTII–rMBS-C, respectively (*15*). The *Saccharomyces cerevisiae* reporter strain L40 [*MAT***a**, *trp1*, *leu2*, *his3*, *LYS2::(lexAop)$_4$-HIS3*, *URA::(lexAop)$_8$-lacZ*] was transformed with plasmids encoding the LexA fusion proteins and plasmids encoding the Gal4p fusion proteins (*15*). The transformants were plated on synthetic medium lacking histidine. If the LexA fusion protein interacts with the Gal4p fusion protein, the transformant can express the *HIS3* reporter gene and therefore grow in the absence of histidine.
26. To express Myc epitope–tagged MBS, we cloned the complementary DNA encoding rat MBS into pEF-BOS–Myc to yield pEF-BOS–Myc-MBS (*10*). COS-7 cells were transfected with pEF-BOS–Myc-MBS alone or together with pEF-BOS–HA-RhoA or pEF-BOS–HA-RhoA^{V14} (*10*). After 40 hours, the cells were collected and homogenized. The cytosolic and particulate fractions were prepared and subjected to quantitative immunoblot analysis with antibodies (9E10) to the Myc epitope (*10*).
27. cMBS-N and cMBS-C were expressed as maltose-binding protein fusion proteins in *Escherichia coli* and purified (*15*).
28. The kinase reaction was performed in 50 μl of kinase buffer [50 mM tris-HCl (pH 7.5), 2.8 mM EDTA, 6.5 mM $MgCl_2$, 0.16% CHAPS detergent] containing 10 μM [γ-^{32}P]ATP (60 to 80 GBq/mmol), purified Rho-kinase (10 ng of protein), and cMBS-N or cMBS-C (450 ng of protein). After incubation for 10 min at 30°C, the reaction mixture was boiled in SDS sample buffer and resolved by SDS-PAGE. The ^{32}P-labeled bands corresponding to MBS were visualized by autoradiography. Fold stimulation of MBS phosphorylation was determined with a Fuji image analyzer (BAS-2000).
29. Myosin phosphatase was purified from chicken gizzard (*14*).
30. Thiophosphorylation of myosin phosphatase (1 μg) was performed in 50 μl of kinase buffer in the presence of 10 μM [^{35}S]ATP-γ-S (8 to 10 GBq/mmol) (*28*). After incubation for 6 min at 30°C, a portion of the mixture (40 μl) was subjected to SDS-PAGE and the remaining portion was assayed for MLC phosphatase activity (*23*).
31. NIH 3T3 cells were stably transfected with p3′SS (repressor plasmid) (Stratagene) and pOPRSVI–HA-RhoA or pOPRSVI–HA-RhoA^{V14} to establish cell lines overexpressing RhoA (RhoA-5 and RhoA-24 cell lines) or RhoA^{V14} (RhoA^{V14}-7 and RhoA^{V14}-25 cell lines) under the control of IPTG [D. L. Wyborski and J. M. Short, *Nucleic Acids Res.* **19**, 4647 (1991)].
32. Confluent NIH 3T3 cells (parental and RhoA-5, RhoA-24, RhoA^{V14}-7, and RhoA^{V14}-25 cell lines) in 35-mm dishes were treated with 5 mM IPTG for 24 hours. During the last 12 hours, the cells were deprived of serum, and during the last 2 hours they were labeled with 9.25 MBq of [^{32}P]orthophosphate. The cells were then lysed and MBS was immunoprecipitated. The washed immunoprecipitates were subjected to SDS-PAGE and autoradiography. Fold stimulation of MBS phosphorylation was determined with a Fuji BAS-2000 image analyzer. Under similar conditions, with the exception that the cells were not labeled, cells were lysed and the lysates were subjected to immunoblot analysis with antibodies to MBS and RhoA.
33. IPTG-treated, serum-deprived NIH 3T3 cell lines (100-mm dishes) were treated with 10% (w/v) trichloroacetic acid. The resulting precipitates were subjected to glycerol-urea gel electrophoresis, and the relative amounts of nonphosphorylated and phosphorylated forms of MLC were determined by immunoblot analysis [D. A. Taylor and J. T. Stull, *J. Biol. Chem.* **263**, 14456 (1988)]. NIH 3T3 cells were treated with 0.1 μM calyculin A for 10 min.

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Bipartite Ca^{2+}-Binding Motif in C_2 Domains of Synaptotagmin and Protein Kinase C

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C_2 domains are found in many proteins involved in membrane traffic or signal transduction. Although C_2 domains are thought to bind calcium ions, the structural basis for calcium binding is unclear. Analysis of calcium binding to C_2 domains of synaptotagmin I and protein kinase C–β by nuclear magnetic resonance spectroscopy revealed a bipartite calcium-binding motif that involves the coordination of two calcium ions by five aspartate residues located on two separate loops. Sequence comparisons indicated that this may be a widely used calcium-binding motif, designated here as the C_2 motif.

Calcium ions regulate a wide variety of biological functions through binding to proteins. Most Ca^{2+}-binding proteins can be grouped into families with common structural motifs such as the EF-hand motif (*1*). A structural unit called the C_2 domain, first defined in protein kinase C (PKC) (*2*), has recently been recognized as a widespread domain that may participate in numerous Ca^{2+}-regulatory roles. More than 50 C_2 domains have been identified in various proteins, many of which participate in signal transduction or membrane traffic (*3*). Although Ca^{2+}-dependent binding of some of these proteins to phospholipids or to other proteins has been shown (*4–7*), the generality and structural basis of Ca^{2+} binding to C_2

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