Characterization of Ca^{2+}/calmodulin-dependent protein kinase I as a myosin II regulatory light chain kinase *in vitro* and *in vivo*

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Ca^{2+}/calmodulin (CaM)-dependent protein kinase I (CaM-KI), which is a member of the multifunctional CaM-K family, is thought to be involved in various Ca^{2+}-signalling pathways. In this report, we demonstrate that CaM-KI activated by an upstream kinase (CaM-K kinase), but not unactivated CaM-KI, phosphorylates myosin II regulatory light chain (MRLC) efficiently (K_{cat}, 1.7 s^{-1}) and stoichiometrically ($\approx$ 0.8 mol of phosphate/mol) in a Ca^{2+}/CaM-dependent manner *in vitro*. One-dimensional phosphopeptide mapping and mutational analysis of MRLC revealed that the activated CaM-KI monophosphorylates only Ser-19 in MRLC. Transient expression of the Ca^{2+}/CaM-independent form of CaM-KI (CaM-KI_{1-293}) in HeLa cells induced Ser-19 phosphorylation of myosin, II accompanied by reorganization of actin filaments in the peripheral region of the cells. CaM-KI-induced reorganization of actin filaments was suppressed by co-expression of non-phosphorylatable MRLC mutants (S19A and T18AS19A). Furthermore, a kinase-negative form of CaM-KI (CaM-$KI_{1-293,K49E}$) significantly reduced reorganization of actin filaments, indicating a dominant negative effect. This is the first demonstration that the activation of the CaM-KI cascade induces myosin II phosphorylation, resulting in regulation of actin filament organization in mammalian cells.

Key words: actin filament, phosphorylation, stress fibre.

INTRODUCTION

Ca^{2+} is an important intracellular second messenger for various physiological processes such as growth factor and hormone signalling, cell cycle regulation, gene expression and apoptosis (reviewed in [1,2]). A large part of this cellular signalling is mediated by calmodulin (CaM), which is a ubiquitous intracellular Ca^{2+} receptor. The Ca^{2+}/CaM complex allosterically activates numerous proteins including a diverse family of Ca^{2+}/CaM-dependent protein kinases (CaM-Ks), protein phosphatase calcineurin, phosphodiesterases and adenylate cyclases (reviewed in [3,4]). Recently, two members of the multifunctional CaM-Ks (CaM-KI and CaM-KIV) were found to be regulated by phosphorylation by the activator, CaM-K kinase (CaM-KK) α and β [5,6]. Recombinant CaM-KK phosphorylates Thr residues in the activation loops of CaM-KI and CaM-KIV, resulting in 10–20-fold increases in their catalytic activities. Therefore, these activated CaM-Ks are considered to be involved in cell function mediated by the CaM-K cascade in Ca^{2+} signalling. A recent study demonstrated that CaM-KIV participates in Ca^{2+}-dependent regulation of transcriptional activation through phosphorylation of cAMP-response-element-binding protein ('CREB'), which is consistent with nuclear localization of CaM-KIV [7]. On the other hand, the physiological function(s) of the CaM-KI remains to be elucidated, although it is known that CaM-KI has a wide tissue distribution and can phosphorylate a number of substrates *in vitro*, including synapsin I [8], cAMP-response-element-binding protein [9] and cystic fibrosis transmembrane conductance regulator (CFTR) [10].

Stimulation by growth factors or cytokines induces motile events in cultured mammalian cells, which is often accompanied by intracellular Ca^{2+} oscillation, suggesting that Ca^{2+} signalling evoked by these stimuli finally leads to activation of the cell motility machinery. The motogenic signals impact the actomyosin system, which is critical for cell movement (reviewed in [11]). Myosins are actin-activated ATPases capable of generating force by promoting translational movement along actin cables. While several classes of myosins have been identified, myosin II is best characterized as a motor protein to promote smooth-muscle contraction, or locomotion and cytokinesis of non-muscle cells (reviewed in [12]). The phosphorylation of myosin II regulatory light chain (MRLC) is an important factor for the regulation of myosin activity (reviewed in [13]). A recent study demonstrated that CaM-KII, a subfamily of multifunctional CaM-Ks, phosphorylated MRLC at Ser-19 mediated by myosin light-chain kinase (MLCK) *in vitro* [14]. The result suggested that a CaM-K family, including CaM-KII and MLCK, participates in MRLC phosphorylation through Ca^{2+} signal transduction in response to extracellular signals.

In this study, we demonstrated that MRLC is a good substrate for CaM-KI, especially CaM-KI that is activated by CaM-KK phosphorylation, using both isolated myosin II light chains (MLCs) and intact myosin II. Overexpression of a Ca^{2+}/CaM-independent form of CaM-KI in HeLa cells resulted in the induction of monophosphorylation of myosin II and ultimately the filamentous rearrangement of actin filaments. These were accompanied by the phosphorylation of myosin II and occurred in the peripheral regions of the cells, suggesting that CaM-KI

Abbreviations used: CaM, calmodulin; CaM-K, Ca^{2+}/CaM-dependent protein kinase; CaM-KK, CaM-K kinase; CPITC, coumarin phenyl isothiocyanate; DTT, dithiothreitol; GFP, green fluorescent protein; GST, glutathione S-transferase; ML-7, 1-(5-iodonapthalene-1-sulphonyl) homopiperazine hydrochloride; MLC, myosin II light chain; MLCK, myosin light-chain kinase; MRLC, myosin II regulatory light chain; ZIP kinase, zipper-interacting protein kinase.

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might be involved in the reorganization of actin filaments through the phosphorylation of myosin II in mammalian cells.

EXPERIMENTAL

Materials

Smooth muscle MLCK and myosin II were purified from chicken gizzard as described previously [15]. CaM from bovine brain and coumarin phenyl isothiocyanate (CPITC)-labelled phalloidin were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The MLCK inhibitor 1-(5-iodonapthalene-1-sulphonyl) homopiperazine hydrochloride (ML-7) was from Calbiochem (La Jolla, CA, U.S.A.). Calyculin A was from Wako Pure Chemical Industries (Osaka, Japan). Anti-green fluorescent protein (GFP) mouse monoclonal antibody, anti-Myc monoclonal antibody and anti-pan myosin II polyclonal antibody were from Clontech (Palo Alto, CA, U.S.A.), Invitrogen (Carlsbad, CA, U.S.A.) and Amersham Bioscience (Little Chalfont, Bucks., U.K.), respectively. Anti-Myc polyclonal antibody and anti-His_6 polyclonal antibody were from Santa Cruz Biotechnology (Delaware, CA, U.S.A.). Secondary antibodies of Alexa Fluor 568 goat anti-mouse IgG (H + L) and Alexa Fluor 680 goat anti-rabbit IgG (H + L) were from Molecular Probes (Eugene, OR, U.S.A.). Other materials and chemicals were from commercial sources. HeLa cells (RCB0007; similar to ATCC CCL2, HeLa) were obtained from the Riken Cell Bank (Tsukuba, Japan) and grown as described previously [16].

Plasmid construction and expression of recombinant protein

Glutathione S-transferase (GST)-CaM-KI was constructed and expressed in *Escherichia coli* JM109 as described previously [17]. GST-CaM-KI mutants (CaM-$KI_{1\text{-}293}$ and CaM-$KI_{1\text{-}293,K49E}$) were expressed as described previously [18]. CaM-KKα cDNA (GenBank accession no. L42810) was from a rat brain cDNA library [19]. For the GFP-CaM-KI construct, rat CaM-KI C-terminal deletion (residues 294–374) mutant cDNA (CaM-$KI_{1\text{-}293}$) was amplified by PCR to introduce convenient restriction sites using the following sense primer, 5′-GAATTC-TATGCCAGGGGCAGTGGAAGGC-3′; and antisense primer, 5′-GGATCCAACTGCTCACTCACTGACTGG-3′. The PCR products were digested with *Eco*RI and *Bam*HI, respectively (sites are underlined above), and then ligated into pEGFP-C1 (Clontech). GFP-tagged wild-type CaM-KI (GFP-CaM-KIwt) expression construct was generated as described previously [20].

For expression of 6 × His-tagged CaM-KKα, rat CaM-KKα C-terminal deletion (residues 435–505) mutant cDNA (CaM-$KK\alpha_{1\text{-}434}$) [21] was amplified by PCR to introduce convenient restriction sites using the sense primer 5′-GGATCCTTATG-GAGCGCAGTCCAGCCGTC-3′ and the antisense primer 5′-GAATTCTCACACCTCCTCCTCAGTCAC-3′. The PCR products were digested with *Bam*HI and *Eco*RI, respectively (underlined), and then ligated into pcDNA3.1/HisA vector (Invitrogen). For Myc-tagged MRLC constructs, wild-type MRLC and its three mutants, which are substituted at both Thr-18 and Ser-19 (T18AS19A), only Thr-18 (T18A) or only Ser-19 (S19A), by Ala, were subcloned in-frame with the C-terminal Myc peptide of the pcDNA3.1/Myc-HisA vector (Invitrogen) as described previously [22].

Protein kinase assays

Recombinant kinases were assayed in a reaction mixture containing 25 mM Hepes (pH 7.5), 4 mM $MgCl_2$, 1.0 mM EGTA, 1.2 mM $CaCl_2$, 1.2 mM dithiothreitol (DTT), 0.1 μM calyculin A, 3 μg/ml GST-CaM-KI, 50 μg/ml CaM and 0.9 mM [γ-^{32}P]ATP. Myosin II from chicken gizzard, MLCs (essential and regulatory light chains) [23] or the GST fusion proteins of HeLa MRLC (wild-type, S19A and T18AS19A), which were generated as described previously [23], were used as substrates at the concentrations indicated in the Figure legends. The reaction solution (total volumes of 20 μl) was incubated at 30 °C for the times indicated in the Figure legends, and the reaction was terminated by addition of 5 μl of 5 × sample buffer [23]. The proteins of the samples were separated by SDS/PAGE. ^{32}P-Labelled proteins were detected with a Bio Imaging Analyser (BAS2000; Fuji Photo Film Co., Tokyo, Japan). The radioactivity was determined by Cerenkov counting with a liquid-scintillation counter (LSC 4100; Aloka Co., Tokyo, Japan).

Phosphopeptide mapping analysis

Phosphorylation of MRLC by CaM-KI was performed as described above. As a control, mono- (at Ser-19) and di- (at both Thr-18 and Ser-19) phosphorylation of MRLC were performed by 1 μg/ml and 100 μg/ml MLCK, respectively, in a kinase mixture containing 30 mM Tris/HCl (pH 7.5), 45 mM NaCl, 0.1 mM DTT, 0.9 mM EGTA, 1.2 mM $CaCl_2$, 50 μg/ml CaM, 3.7 mM $MgCl_2$, 0.1 μM calyculin A, 0.9 mM [γ-^{32}P]ATP (0.1 mCi/ml) and 63 μg/ml MLCs. ^{32}P-Labelled MRLC was separated by SDS/PAGE and excised from the polyacrylamide gel. One-dimensional phosphopeptide mapping analysis of tryptic peptide was performed as described previously [23]. Autoradiography was performed using a Bio Imaging Analyser.

Transfection and indirect immunofluorecence

HeLa cells were plated at a density of 10^4 cells per 3.5 cm dish with coverslips. After culturing for 2 days, cells were transfected with each expression plasmid by the application of a LipofectAMINE (Gibco BRL)–DNA complex. After 4 h of incubation, minimal essential medium containing 10 % fetal bovine serum was added and the cells were cultured for an additional 18 h. For immunofluorescence, cells on coverslips were fixed for 15 min in 3.7 % formaldehyde in PBS containing 1 mM EGTA at room temperature. After fixation, the cells were made permeable by incubating with 0.2 % Triton X-100 in PBS for 15 min, and then washed with PBS. After blocking with PBS including 1 % BSA, antibodies were applied directly to the coverslips. The coverslips were incubated for 1 h at 37 °C in a moist chamber and then washed for 15 min with several changes of PBS. The cells were then stained with secondary antibodies in a manner identical to that of the first staining. After washing with PBS, the coverslips were mounted on a microscope slide with a drop of PermaFluor™ Aqueous Mounting Medium for preserving fluorescence (ThermoShandon, Pittsburgh, PA, U.S.A.).

Quenching assays were done as follows: pLC1 and PP1, which are antibodies that specifically recognize MRLC monophosphorylated at Ser-19 [24,25] or diphosphorylated at both Thr-18 and Ser-19 [22,26], respectively, were preincubated for 2 h at 37 °C with 1.4 mM of mono- and di-phosphorylated MRLC peptides (Lys-Arg-Pro-Gln-Arg-Ala-Thr-phosphoSer-Asn-Val-Phe and Lys-Arg-Pro-Gln-Arg-Ala-phosphoThr-phosphoSer-Asn-Val-Phe, respectively), corresponding to residues 12–22 of HeLa MRLC [27]. Imaging was performed under a Nikon ECLIPSE TE300 microscope. All images were acquired with a digital CCD camera (ORCA, C4742-95-12; Hamamatsu Photonics) and processed with custom software.

Transfection and immunoprecipitation

HeLa cells were plated at a density of 10^5 cells/10 cm dish for immunofluorecence and immunoprecipitation studies. After culturing for 2 days, cells were transfected with each construct by the application of LipofectAMINE–DNA complex. After 4 h of incubation, minimal essential medium containing 10 % fetal bovine serum was added and the cells were cultured for an additional 18 h. For immunoprecipitation, 2.5×10^6 transfected cells were washed twice with PBS (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4 and 8 mM Na_2HPO_4, pH 7.3) containing 5 mM $MgCl_2$, 0.2 mM EGTA and 1 mM PMSF and lysed with RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 % Nonidet P-40, 0.5 % deoxycholic acid and 0.1 % SDS) containing protease inhibitors (10 μM leupeptin, 1 μM pepstatin A, 1 mM PMSF and 10 mM EGTA) and phosphatase inhibitors (0.1 μM calyculin A, 10 mM β-glycerophosphate, 10 mM NaF, 1 mM Na_3VO_4 and 0.1 mM Na_2MoO_4). The cell lysate was precleared with Protein G–Sepharose beads (Amersham Bioscience) for 1 h and then incubated with 2 μg of anti-GFP antibody or anti-Myc monoclonal antibody for 1 h by rotation. Protein G–Sepharose beads were added to the lysate and incubated for an additional 1 h by rotation. The beads were washed three times with cold PBS and immunoprecipitates were eluted with 5 × sample buffer, subjected to SDS/PAGE and transferred on to a PVDF membrane (Immobilon-P; Millipore) as described previously [16].

Kinase assay for immunoprecipitates

Immunoprecipitation was performed using HeLa cell lysates that were prepared as described above. Immunoprecipitated complexes were washed twice with the reaction mixture (20 mM Hepes, pH 7.5, 1 mM DTT, 1 mM EGTA, 5 mM $MgCl_2$, 4 μg/ml leupeptin, 1 mM PMSF and 0.1 μM calyculin A). Phosphorylation of MRLC by immunoprecipitates was performed for 10 min at 30 °C in a reaction mixture (total volumes of 20 μl) containing 0.9 mM [γ-^{32}P]ATP. To examine whether the immunoprecipitates are activated by CaM-KKα, immunoprecipitated complexes were incubated with or without 0.28 μg/ml CaM-KKα for 20 min at 30 °C prior to the initiation of the kinase assay, and the reaction was stopped by the addition of 5 μl of 5 × sample buffer. The proteins of these samples were separated by SDS/PAGE. ^{32}P-Labelled proteins were detected with a Bio Imaging Analyser.

Effect of ML-7 on the phosphorylation of MRLC induced by CaM-KI

In vitro analysis of inhibitory effect with ML-7 was carried out as described previously [28] with minor modifications. ML-7 was stored as a 10 mM stock solution in DMSO at −20 °C before use. In brief, the kinase assay was performed in a solution containing 25 mM Tris/HCl (pH 7.2), 1 mM EGTA, 1.2 mM $CaCl_2$, 2.3 mM $MgCl_2$, 30 μM [γ-^{32}P]ATP, 63 μg/ml MLCs, 28 nM CaM, ML-7 at concentrations as indicated in the Figure legends and 8.3 nM MLCK or 9 nM GST-CaM-KI in a final volume of 70 μl. Assays were performed for 8 min at 30 °C and aliquots (20 μl) of the reaction mixture were withdrawn and mixed with 5 μl of 5 × sample buffer to stop the reaction. The proteins of the samples were separated by SDS/PAGE. ^{32}P-Labelled proteins were detected with a Bio Imaging Analyser. The radioactivity was determined by Cerenkov counting with a liquid-scintillation counter. The kinase activity was calculated by analysis of the initial linear phase of the time course data. In order to treat cells transfected with CaM-$KI_{1\text{-}293}$ with ML-7, transfection of GFP-CaM-$KI_{1\text{-}293}$ into HeLa cells was carried out as described above. After transfection, cells were treated with ML-7 at the concentration indicated in the Figure legend for 0–120 min. After treatment with ML-7, the cells were fixed for indirect immunofluorecence as described above.

Other procedures

Determination of protein concentration was performed by Lowry's method [29] with slight modifications [30] using BSA as a standard. SDS/PAGE was carried out as described previously [23]. Western blotting was carried out as described elsewhere [16].

RESULTS

Activated CaM-KI efficiently phosphorylates MRLC

Previously, in order to search for protein kinase(s) which phosphorylate MRLC in mammalian cells, we had screened a HeLa cell cDNA library with a partial cDNA fragment including kinase (catalytic) and regulatory domains, including a CaM-binding site, of the bovine stomach MLCK cDNA as a probe [31]. As a result, several clones encoding protein kinases were obtained. One of them was identified as the zipper-interacting protein (ZIP) kinase and it was characterized as a novel non-muscle-type protein kinase to diphosphorylate MRLC at both Thr-18 and Ser-19 of the MLCK site [31]. Another clone (clone no. 3-3-1-1) is highly homologous to CaM-KI and has been shown to share $\approx$ 70 % identity with CaM-KI derived from rat fetal lung (accession no. L26288; results not shown). Therefore we first examined whether CaM-KI has an ability to phosphorylate MRLC by using recombinant wild-type rat CaM-KI (CaM-KIwt) as a GST fusion protein with or without activation by CaM-KKα (Figure 1). Although CaM-KIwt very weakly phosphorylated both MRLC from isolated MLCs and MRLC in intact myosin II (0.02 mol of phosphate/mol of protein) without activation by CaM-KK (Figures 1A and 1B), activated CaM-KI by phosphorylation with CaM-KK exhibited stoichiometrical phosphorylation of MRLC (approx. 0.8 mol/mol). Based on the kinetic analysis of MRLC phosphorylation by activated CaM-KIwt (inset in Figure 1B), the K_m and molecular activity (K_{cat}) values of activated CaM-KIwt for MRLC were estimated to be 26 μM and 1.7 s^{-1}, respectively.

CaM-KI monophosphorylates MRLC at Ser-19

In order to analyse the phosphorylation site(s) of MRLC by activated CaM-KI, one-dimensional phosphopeptide mapping was performed (Figure 1C). The mobility of the phosphopeptide spot recovered from MRLC phosphorylated by activated CaM-KI was identical to that of MRLC monophosphorylated at Ser-19 by 1 μg/ml MLCK. Phosphorylation of MRLC at Thr-18 by activated CaM-KI has not been observed. Phosphorylation of MRLC (a primary site, Ser-19, and a secondary site, Thr-18) by MLCK has been shown to activate actin-activated MgATPase activity of myosin II [32]. To confirm the phosphorylation of Ser-19 by CaM-KI, two MRLC mutants (S19A and T18AS19A) as GST-fusion proteins were used for a CaM-KI phosphorylation assay (Figure 1D). As a result, mutation of Ser-19 completely abol- ished MRLC phosphorylation by activated CaM-KI, which is consistent with the phosphopeptide mapping shown in Figure 1(C).

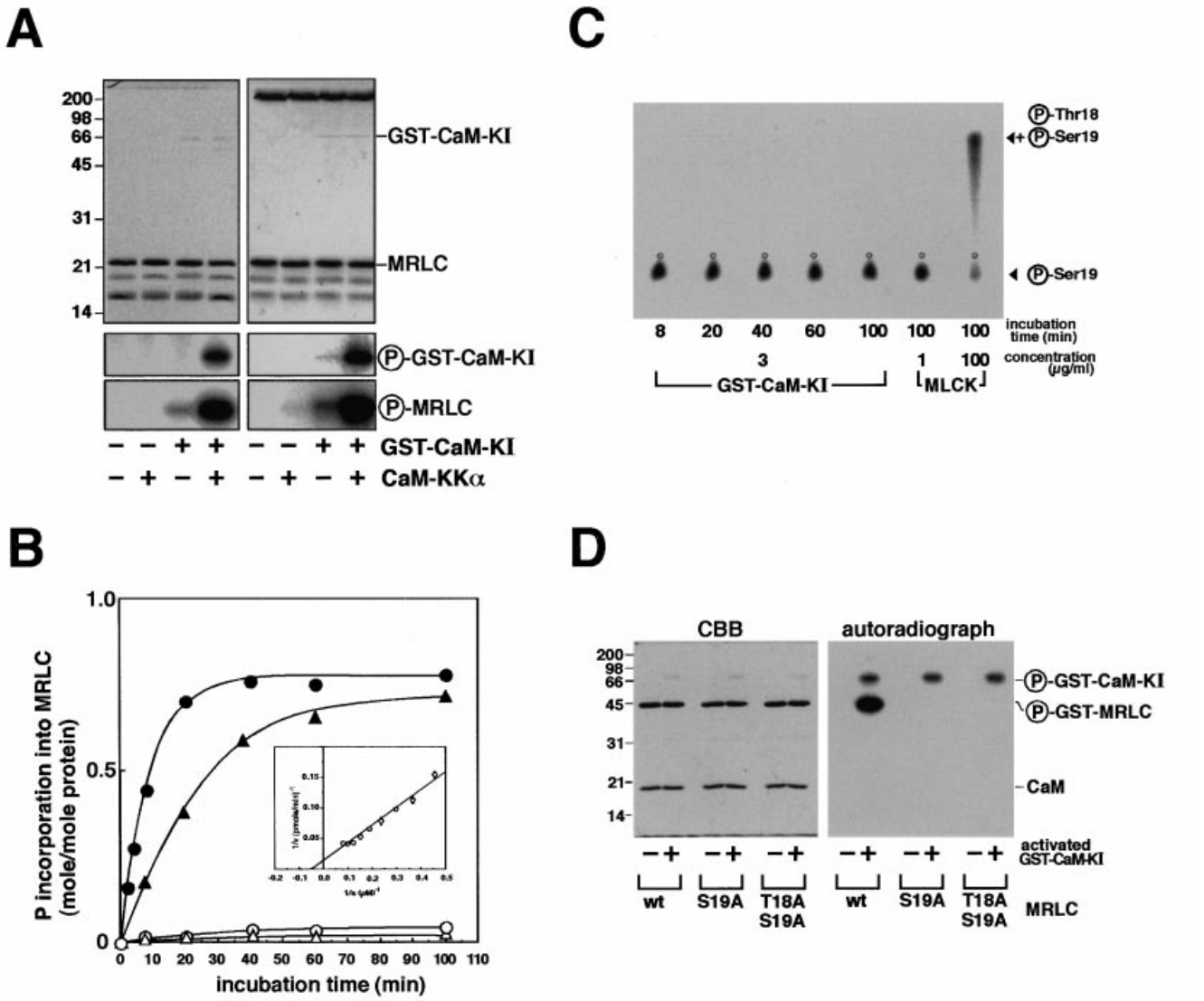

Figure 1 Characterization of MRLC phosphorylation by CaM-KI

(**A**) Isolated MLCs (left-hand panel) and intact myosin II (right-hand panel) from chicken gizzard were used as substrates for the phosphorylation by GST-CaM-KIwt. CaM-KIwt was incubated with (+) or without (−) CaM-KKα prior to the initiation of the kinase assay as described in the Experimental section. Samples were analysed by SDS/PAGE followed by either Coomassie Brilliant Blue (CBB) staining (upper panel) or autoradiography (middle and lower panels). Phosphorylation of GST-CaM-KIwt by CaM-KKα is indicated in the middle panel. Phosphorylation of MRLC is indicated in the lower panel. Molecular mass markers (in kDa) are indicated. (**B**) Time course of phosphorylation of MRLC by CaM-KI. Isolated MLCs (●, ○) and intact myosin II (▲, △) from chicken gizzard were incubated with GST-CaM-KIwt for the indicated times. CaM-KIwt was incubated with (●, ▲) or without (○, △) CaM-KKα prior to the initiation of the kinase assay. Aliquots (20 μl) of the reaction mixture were withdrawn at the indicated times for quantification of protein-bound ^{32}P. The incorporation of phosphate is represented as mol of phosphate/mol of MRLC. Means from two independent experiments are shown. Kinetic analysis of MRLC phosphorylation by activated CaM-KIwt is shown in the inset. After recombinant CaM-KIwt was incubated with CaM-KKα at 30 °C for 30 min (see the Experimental section), the kinetic properties of the enzyme were analysed and presented as double-reciprocal (Lineweaver–Burk) plots. For the titration of MRLC, 3 μg/ml GST-CaM-KI and 0.2–56 nM MLCs were used. The experiments were performed in triplicate for each point and the values are indicated as the means ± S.D. from three experiments. (**C**) Identification of the phosphorylation site of MRLC by CaM-KI. A one-dimensional phosphopeptide map of MRLC phosphorylated by activated GST-CaM-KIwt (3 μg/ml) or MLCK (1 or 100 μg/ml) using isolated MLCs from chicken gizzard as substrate. MRLC was phosphorylated at 30 °C for the indicated times. The phosphorylated MRLC bands excised from the polyacrylamide gel were digested with trypsin and processed for electrophoresis in the vertical dimension as described in the Experimental section. Arrowheads show the phosphopeptide spots of the MRLC phosphorylated at Ser-19 and at both Thr-18 and Ser-19. The circles indicate the origins. (**D**) The phosphorylation assay of recombinant GST-MRLC fusion proteins by CaM-KI. GST-MRLCs (wt, S19A or T18AS19A) at a final concentration of 0.18 mg/ml were incubated with (+) or without (−) activated GST-CaM-KIwt (3 μg/ml) as described in the Experimental section. After phosphorylation, samples were analysed by SDS/PAGE with CBB (left-hand panel) and by autoradiography (right-hand panel). The GST-CaM-KIwt phosphorylated by CaM-KKα, GST-MRLCs phosphorylated by CaM-KI and Ca^{2+}/CaM are indicated. Molecular mass markers (kDa) are indicated.

CaM-KI induced monophosphorylation *in vivo* of MRLC and reorganization of actin filaments in peripheral regions of the cells

It has been reported that the phosphorylation of MRLC has an important role in the regulation of actomyosin contractility in smooth muscle and in many non-muscle cells. Therefore, we tried to investigate whether CaM-KI phosphorylates MRLC *in vivo* and is involved in the regulation of cytoskeletal proteins through the phosphorylation of MRLC. In order to examine *in vivo* the function of CaM-KI, first we performed characterization of

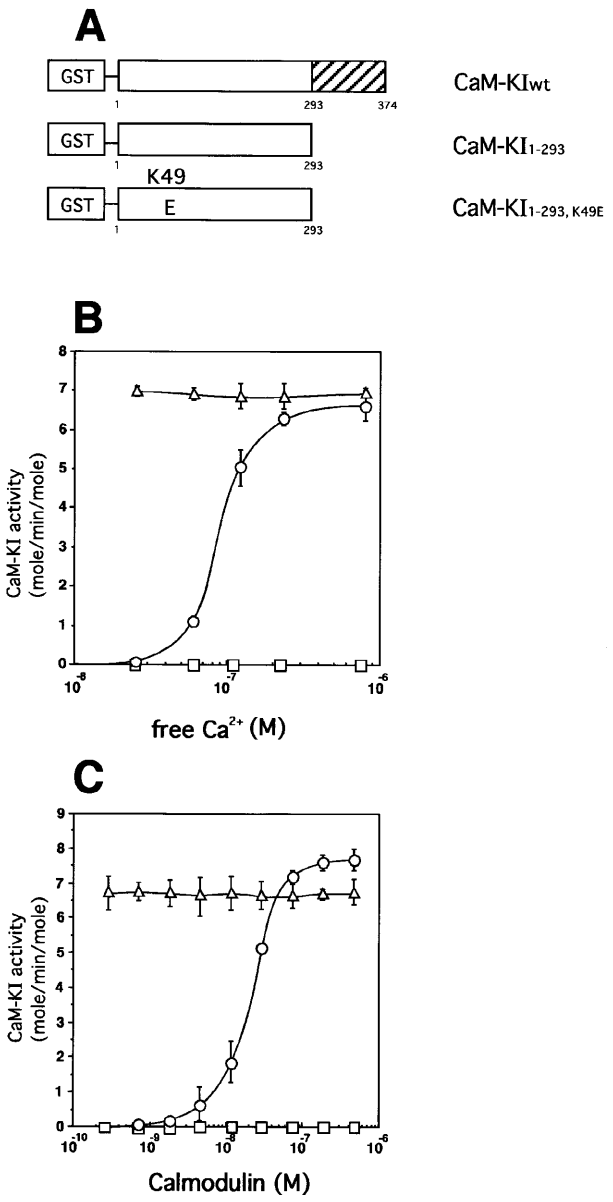

Figure 2 Characterization of CaM-KI mutants activated by CaM-KK

(**A**) Schematic diagram of CaM-KIwt and two mutants of truncated CaM-KI (CaM-$KI_{1\text{-}293}$ and CaM-$KI_{1\text{-}293,K49E}$), which were constructed as GST fusion proteins. The hatched box corresponds to a regulatory region containing autoinhibitory and CaM-binding domains. (**B**) Activation of GST-CaM-KI by Ca^{2+} was performed in a solution containing 25 mM Hepes (pH 7.5), 4 mM $MgCl_2$, 2.0 mM EGTA, $CaCl_2$ at concentrations calculated to produce the required free Ca^{2+} concentrations (0.02–0.64 μM), 1.2 mM DTT, 0.1 μM calyculin A, 3 μg/ml GST-CaM-KIwt (○), GST-CaM-$KI_{1\text{-}293}$ (△) and GST-CaM-$KI_{1\text{-}293,K49E}$ (□), 2.8 μM CaM, 63 μg/ml MLCs and 0.9 mM [γ-^{32}P]ATP. These GST-CaM-Ks were incubated with CaM-KKα prior to kinase reaction as described in the Experimental section. (**C**) Ca^{2+}/CaM-dependent phosphorylation of MRLC by GST-CaM-KIwt (○), GST-CaM-$KI_{1\text{-}293}$ (△) and GST-CaM-$KI_{1\text{-}293,K49E}$ (□) was carried out in a mixture as described above except for addition of 2.0 mM $CaCl_2$ (free Ca^{2+} concentration, 7.8 μM) and CaM at the indicated concentrations (0.18–100 nM). The values shown are means ± S.D. of triplicate determinations.

the activated forms of CaM-KIs, including mutants, by using MRLC as a substrate (Figure 2 and Table 1). As shown in Figure 2, the MRLC phosphorylation activity of activated CaM-KIwt was completely dependent on Ca^{2+} and CaM, whereas activated CaM-$KI_{1\text{-}293}$ was independent of Ca^{2+} and CaM. Apparent

Table 1 Comparison of specific activity of wild-type and mutant enzymes

Values shown are ± S.D. of triplicate determinations.

	Specific activity (mol/min per mol)			
	− CaM-KK		+ CaM-KK	
CaM-KI	$-Ca^{2+}$/CaM	$+Ca^{2+}$/CaM	$-Ca^{2+}$/CaM	$+Ca^{2+}$/CaM
Wild type	0	0.26 ± 0.08	0	7.8 ± 0.63
$Mutant_{1\text{-}293}$	0.89 ± 0.09	0.84 ± 0.12	7.0 ± 0.13	7.0 ± 0.8
$Mutant_{1\text{-}293,K49E}$	0	0	0	0

K_{Ca} and K_{CaM} values for activated CaM-KIwt by CaM-KK were 0.1 μM and 22 nM, respectively. Unlike CaM-$KI_{1\text{-}293}$, CaM-$KI_{1\text{-}293,K49E}$ could no longer phosphorylate MRLC (Figures 2B and 2C, and Table 1), indicating a kinase-negative mutant. As shown in Table 1, protein kinase activity of CaM-KIwt was enhanced ≈ 30-fold by the phosphorylation with CaM-KKα. Full activities of both activated CaM-KIwt and CaM-$KI_{1\text{-}293}$ were indistinguishable. These enzymic features of CaM-KI by using MRLC as a substrate are well correlated with previous reports using synthetic peptide as a substrate [33,34]. Consequently, either constitutively active or dominant negative effects are expected from overexpression of CaM-$KI_{1\text{-}293}$ or CaM-$KI_{1\text{-}293,K49E}$ in the cells, respectively.

In order to examine the function of CaM-KI *in vivo*, we transfected an expression plasmid carrying either CaM-KI1-293, CaM-$KI_{1\text{-}293,K49E}$ or CaM-KIwt into HeLa cells (Figure 3). These CaM-KI constructs were tagged with GFP to identify the cells expressing CaM-KIs following transfection. After transfection, these cells were stained with pLC1 (Figures 3B, 3E, 3H and 3K) or PP1 (Figure 3N), antibodies specifically recognizing MRLC monophosphorylated at Ser-19 [24,25] or diphosphorylated at both Thr-18 and Ser-19 [22,26], respectively. Monophosphorylation (Figure 3K) but not diphosphorylation (Figure 3N) of MRLC was remarkably induced in the GFP-CaM-$KI_{1\text{-}293}$-expressing cells (Figures 3J and 3M), and the monophosphorylated MRLC was found to be strongly accumulated along the thick actin bundles (Figure 3L) in the peripheral region of the cells. Weak staining with pLC1 was observed in most of the cells transfected with CaM-$KI_{1\text{-}293,K49E}$ (Figures 3G–3I). Furthermore, the filamentous structures of the monophosphorylated MRLC were observed in 54 ± 2.4 % of cells expressing GFP-CaM-$KI_{1\text{-}293}$, 22.3 ± 3.0 % expressing GFP-CaM-KIwt, 13.9 ± 2.0 % expressing GFP (control) and 8.1 ± 2.6 % expressing GFP-CaM-$KI_{1\text{-}293,K49E}$ (Figure 4). As shown by the arrowheads in Figure 5, the phosphorylated MRLC (Figure 5B; red) co-localized with total myosin II (Figure 5C; purple) and cortical bundles of actin filaments (Figure 5D; blue), indicating that contractility of actomyosin is enhanced in these places.

The MRLC phosphorylation activity of CaM-$KI_{1\text{-}293}$ has shown to be enhanced ≈ 9-fold by *in vitro* phosphorylation with CaM-KKα (Table 1). Thus, it can be expected that co-expression of CaM-$KI_{1\text{-}293}$ with CaM-KKα probably causes more remarkable MRLC phosphorylation in the cells than expression of just CaM-$KI_{1\text{-}293}$. Therefore we co-transfected a constitutively active CaM-KK (CaM-KK1-434 [21]) with GFP-CaM-$KI_{1\text{-}293}$ in HeLa cells (Figure 6). Unexpectedly, no obvious change was observed between singly and doubly transfected cells with respect to the percentage of cells (53 ± 3.8 % and 48 ± 7.1 %, respectively) in the peripheral regions, where thick bundles of actin filaments formed (as shown in Figure 5D), out of the total number of co-transfected cells.

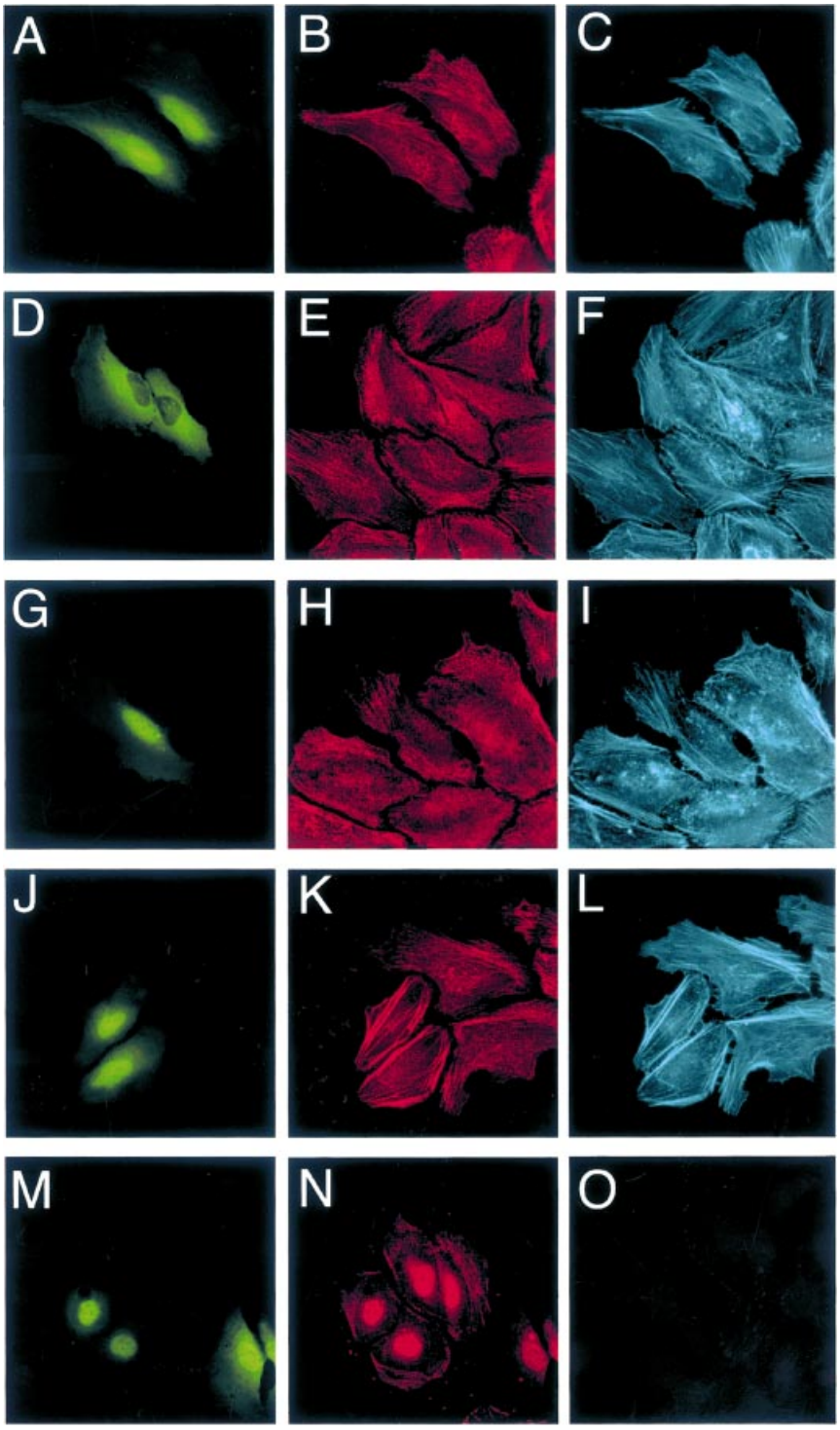

Figure 3 Induction of MRLC monophosphorylation by CaM-KI resulted in the assembly of actin filaments in transfected HeLa cells

HeLa cells were transiently transfected with 1 μg of plasmid DNA of GFP (control; **A**–**C**), GFP-CaM-KIwt (**D**–**F**), GFP-CaM-$KI_{1\text{-}293,K49E}$ (**G**–**I**) and GFP-CaM-$KI_{1\text{-}293}$ (**J**–**N**). After transfection, the cells were fixed with 3.7% formaldehyde, made permeable with 0.2% Triton X-100 and then stained with pLC1 (Alexa 568; red; **B**, **E**, **H** and **K**), PP1 (Alexa 568; red; **N** and **O**) and CPITC-phalloidin (blue; **C**, **F**, **I** and **L**). Transfected cells in panels **A**, **D**, **G**, **J** and **M** are shown with GFP colour (green). As a control, preincubated PP1 with diphosphorylated MRLC peptides was used for immunofluorescent staining of intact HeLa cells in panel **O**. The sample is different from that in panels **M** and **N**. Note that the bundling of thick actin filaments is accompanied by induction of monophosphorylation of MRLC in the peripheral region of CaM-$KI_{1\text{-}293}$-expressed cells (**J**–**L**).

We examined whether immunoprecipitated GFP-CaM-$KI_{1\text{-}293}$ (Figure 6A, lanes 3 and 4) and $6\times$His-tagged CaM-$KK\alpha_{1\text{-}434}$ (Figure 6A, lane 5) from HeLa cell extracts have the ability to phosphorylate each substrate, or whether GFP-CaM-$KI_{1\text{-}293}$ is activated by co-transfected His6-CaM-$KK\alpha_{1\text{-}434}$ or not (Figure 6B). Unlike purified GST-CaM-$KI_{1\text{-}293}$

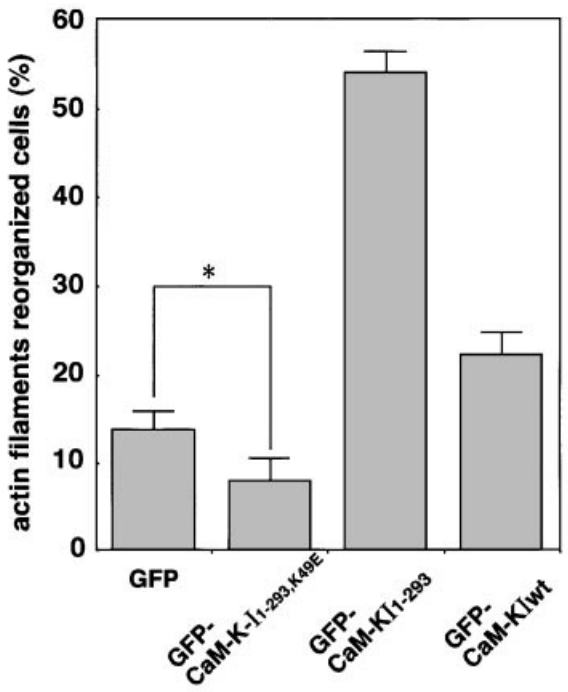

Figure 4 Quantitative analysis of actin filament reorganization in HeLa cells expressing various CaM-KI mutants

Shown is the percentage of cells in cell peripheral regions of which thick bundles of actin filaments including phosphorylated MRLC are formed (as panel K in Figure 3), from the total number of the transfected (green) cells. More than 100 individual cells were counted in three independent transfection experiments. The means ± S.D. of triplicates are shown. Data were compared by Student's *t* test: GFP versus GFP-CaM-$KI_{1\text{-}293,K49E}$, $P < 0.025$ (*); GFP versus CaM-$KI_{1\text{-}293}$, $P < 0.005$; GFP versus CaM-KIwt, $P < 0.025$.

(control), both immunoprecipitated GFP-CaM-$KI_{1\text{-}293}$ from singly and doubly transfected cells were no longer phosphorylated by recombinant wild-type CaM-KKα (CaM-KKαwt). We confirmed that immunoprecipitated His6-CaM-KK$\alpha_{1\text{-}434}$ (Figure 6A, lane 5) had an activity to phosphorylate CaM-KI in a Ca^{2+}/CaM-independent manner (Figure 6C). Taken together, GFP-CaM-$KI_{1\text{-}293}$ expressed in HeLa cells was already activated by endogenous CaM-KK. This is in good agreement with a recent report indicating that unlike CaM-KKα, Ca^{2+}/CaM-independent CaM-KK activity (60–70 % of autonomous activity) was observed in the CaM-KKβ isoform in rat brain [35].

To determine whether monophosphorylation of MRLC by CaM-KI is responsible for induction of actin filament reorganization in the cells, we co-transfected CaM-KI with MRLC mutants (S19A or T18AS19A) or the wild-type into HeLa cells (Figure 7). Strikingly, non-phosphorylatable mutants (S19A and T18AS19A) decreased the percentage of cells including filamentous structures with phosphorylated myosin II. In contrast, co-transfection of wild-type MRLC, whose Ser-19 was intact, resulted in no obvious effect on actin filament organization. From these results, we conclude that monophosphorylation of MRLC at Ser-19 by CaM-KI is involved in the reorganization of actin filaments in these cells.

MLCK may not participate in the organization of filamentous structures of phosphorylated myosin II evoked by expression of CaM-KI

Previously it has been reported that MLCK contributed to the organization of actin filaments [36], which may be accompanied by the phosphorylation of myosin II in the peripheral regions of cultured mammalian cells [37]. Consequently, we examined whether MLCK is involved in the organization of filamentous structures including phosphorylated myosin II induced by expression of CaM-$KI_{1\text{-}293}$. We treated HeLa cells with ML-7, a specific inhibitor of MLCK, to abolish the MLCK activity in the cells. First, the effect of ML-7 against CaM-KI was confirmed at a concentration that is known to inhibit MLCK [28]. We examined the inhibitory effect of ML-7 against MLCK and GST-CaM-$KI_{1\text{-}293}$ (Figure 8A). Under our *in vitro* experimental conditions, IC_{50} values of ML-7 against MLCK were 0.3–0.4 μM (see Figure 8A), which is consistent with a previous report [28]. The IC_{50} value of ML-7 against CaM-KI was 6 μM, which was 15–20 fold higher than that of MLCK. Therefore, we chose the concentration of 10 μM ML-7 for treatment of cells transfected with GFP-CaM-KI1-293, in which MLCK activity was less than 5 %, although CaM-KI still had 37 % of its activity to phosphorylate MRLC, as shown by the arrows in Figure 8(A). Several reports also showed that phosphorylation of MRLC by endogenous MLCK in human fibroblasts [36], bovine pulmonary endothelial cells [38] and human leucocytes [39] was inhibited in the presence of 3–25 μM ML-7. As a result, 10 μM ML-7 showed a less inhibitory effect on the organization of actin filaments in the GFP-CaM-KI1-293-transfected cells (Figure 8B). Furthermore, even in the presence

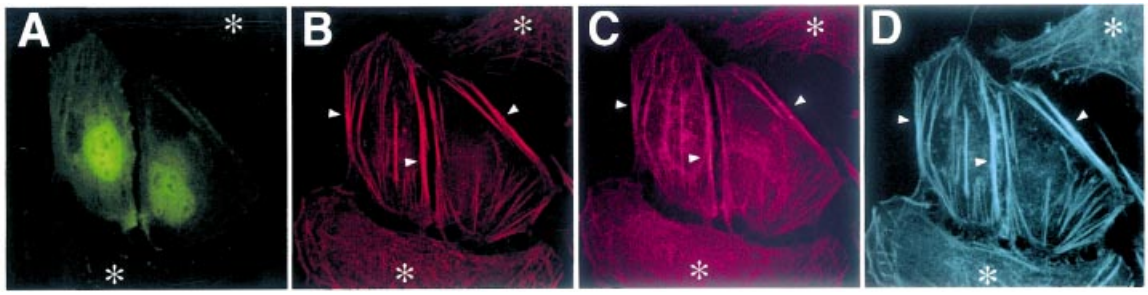

Figure 5 Co-localization of myosin II heavy chain with monophosphorylated MRLC and actin filaments

HeLa cells were transiently transfected with 1 μg of plasmid DNA of GFP-CaM-$KI_{1\text{-}293}$ and were fixed in 3.7% formaldehyde, made permeable with 0.2% Triton X-100 and then triple-stained with pLC1 (Alexa 568; red; **B**), anti-pan myosin II (Alexa 680; purple; **C**) and CPITC-phalloidin (blue; **D**). Two GFP-CaM-$KI_{1\text{-}293}$-expressed cells in panel **A** are shown with GFP (green). Untransfected cells were shown by asterisks. As shown with arrowheads, myosin II co-localized well with monophosphorylated MRLC and thick bundles of actin filaments were well induced in the peripheral regions of cells transfected with CaM-$KI_{1\text{-}293}$ but not in untransfected cells.

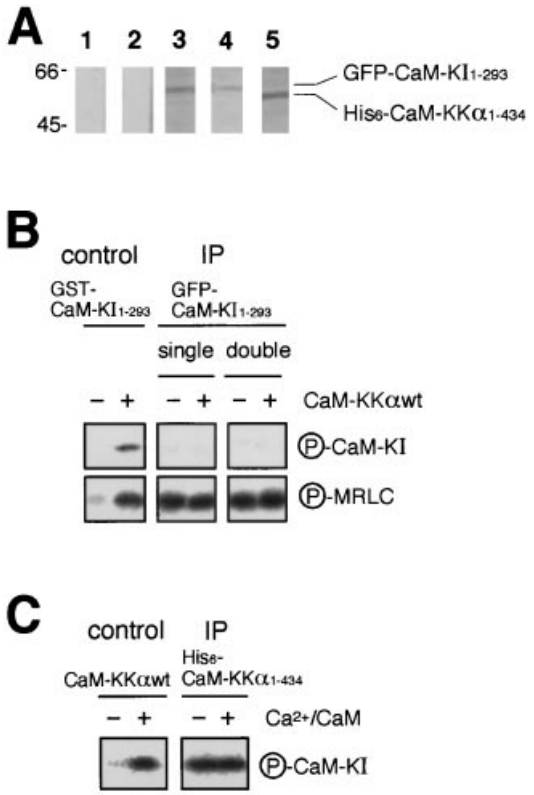

Figure 6 GFP-CaM-KI1-293 is activated by endogenous CaM-KK in HeLa cells

(**A**) Western blotting of immunoprecipitated GFP-CaM-KI$_{1-293}$ or His6-CaM-KKα_{1-434}. Total lysates of cells transfected with pEGFP (lane 1) or pcDNA3.1/HisA (lane 2) were used for immunoblotting using anti-GFP antibody or anti-His$_6$ antibody, respectively. GFP-CaM-KI$_{1-293}$ in lane 3 was immunoprecipitated from the cell lysates of singly transfected cells, and GFP-CaM-KI1-293 in lane 4 and His$_6$-tagged CaM-KKα in lane 5 were immunoprecipitated from the cell lysates of doubly transfected cells. Total cell lysates or immunoprecipitates eluted with 5 × sample buffer were subjected to SDS/PAGE and transferred on to a PVDF membrane as described in the Experimental section. Western blotting of the immunoprecipitates was performed using anti-GFP (lanes 1, 3 and 4) or anti-His$_6$ (lanes 2 and 5) antibodies. The positions of molecular mass markers (kDa) are indicated. (**B**) Activation of GFP-CaM-KI$_{1-293}$ in HeLa cells. To examine whether expressed GFP-CaM-KI$_{1-293}$ is activated in HeLa cells, kinase assay for the immunoprecipitated GFP-CaM-KI$_{1-293}$ (IP) from cell lysates of transfected cells or recombinant GST-CaM-KI$_{1-293}$ (control) were performed using isolated MLCs as substrates. Immunoprecipitation of GFP-CaM-KI$_{1-293}$ was performed from cell lysates of singly (single) or doubly (double) transfected cells. These kinases were preincubated with (+) or without (−) recombinant wild-type CaM-KKα (CaM-KKαwt) in the presence of Ca^{2+}/CaM, followed by kinase assay using MLCs as substrates. (**C**) To test whether immunoprecipitated His6-CaM-KKα_{1-434} from the cell lysates of doubly transfected cells can phosphorylate CaM-KI, a kinase assay of immunoprecipitates (IP) or CaM-KKαwt (control) was carried out. A kinase-negative form of CaM-KI (CaM-KI$_{1-293,K49E}$) was used as a substrate of CaM-KKα, because autophosphorylation of CaM-KI is often observed in *in vitro* experiments (results not shown).

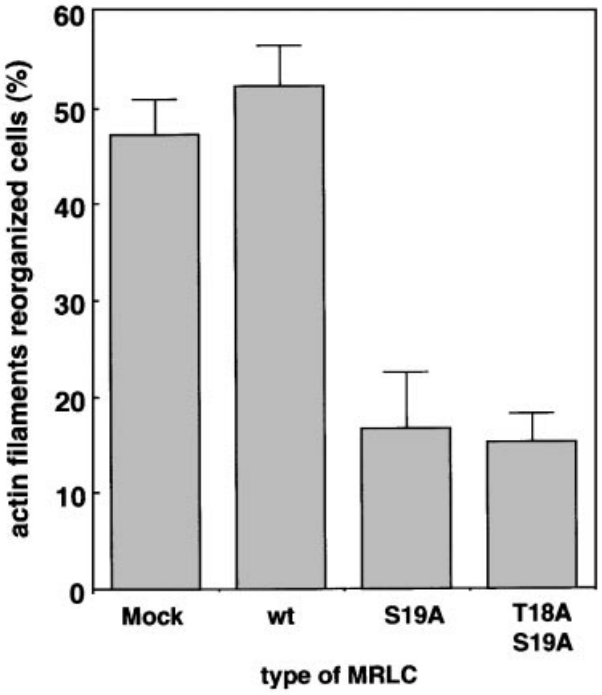

Figure 7 Suppression of CaM-KI$_{1-293}$-mediated actin filament reorganization by non-phosphorylatable MRLC mutant

HeLa cells were co-transfected with GFP-CaM-KI$_{1-293}$ and various types of MRLC (wild-type, S19A or S19AT18A) or pcDNA3.1/HisA vector (Mock) as a control. After transfection, indirect immunofluorescence was performed using anti-Myc antibody and CPITC-phalloidin as described in Figure 3. Shown is the percentage of cells in peripheral regions in which thick bundles of actin filaments are formed (as shown in Figure 5D), out of the total number of co-transfected cells. More than 100 co-transfected cells were counted in three independent transfection experiments. The means ± S.D. from triplicates are shown. Note that S19A and S19AT18A remarkably inhibited the reorganization of actin filaments induced by CaM-KI$_{1-293}$.

of 30 μM ML-7, a significant inhibitory effect on filamentous structures of the cells was not observed. Under these conditions, although GFP-CaM-KI$_{1-293}$ still has 21 % of its full activity, only 2 % MLCK activity remained (arrowheads in Figure 8A). Immunofluorescent images (insets of Figure 8B) also revealed that accumulation of phosphorylated myosin II (right-hand inset) in peripheral regions of the cells was not inhibited in the presence of 30 μM ML-7. Therefore, the filamentous accumulation of phosphorylated myosin II along the cortical actin bundles is probably caused by direct phosphorylation of MRLC with expressed CaM-KI but not MLCK.

DISCUSSION

Although several *in vitro* substrates of CaM-KI have been reported [8–10], the kinetic analysis of phosphorylation has not been carried out and the physiological significance of phosphorylation by the CaM-KI cascade remains unknown. Therefore, this is the first demonstration that a signalling cascade mediated by CaM-KI participates in phosphorylation of myosin II both *in vitro* and in intact cells, and could be involved in reorganization of actin filaments in the peripheral regions of mammalian cells.

Recent evidence indicates that CaM-KI is phosphorylated at Thr-177 by CaM-KK, resulting in a large increase in its catalytic efficiency [33]. Kinetic parameters of MRLC phosphorylation by CaM-KI activated with CaM-KK revealed that the specificity constant, K_{cat}/K_m, of CaM-KI was calculated to be 0.07 $\mu M^{-1} \cdot s^{-1}$, which is comparable with that of Rho kinase (0.1 $\mu M^{-1} \cdot s^{-1}$) [40] or sea urchin CaM-K (0.04 $\mu M^{-1} \cdot s^{-1}$) [41]. This indicates that CaM-KI efficiently phosphorylates MRLC, as did the other MRLC kinases. The site of phosphorylation of MRLC by activated CaM-KI was identified as Ser-19, which is known to be responsible for the increase in the actin-activated Mg-ATPase activity of myosin II [32]. Activation of myosin ATPase activity is thought to be important for the reorganization of actin filaments in cells, since an inhibitor of myosin ATPase activity (3-butanedione monoxime) inhibits actomyosin con-

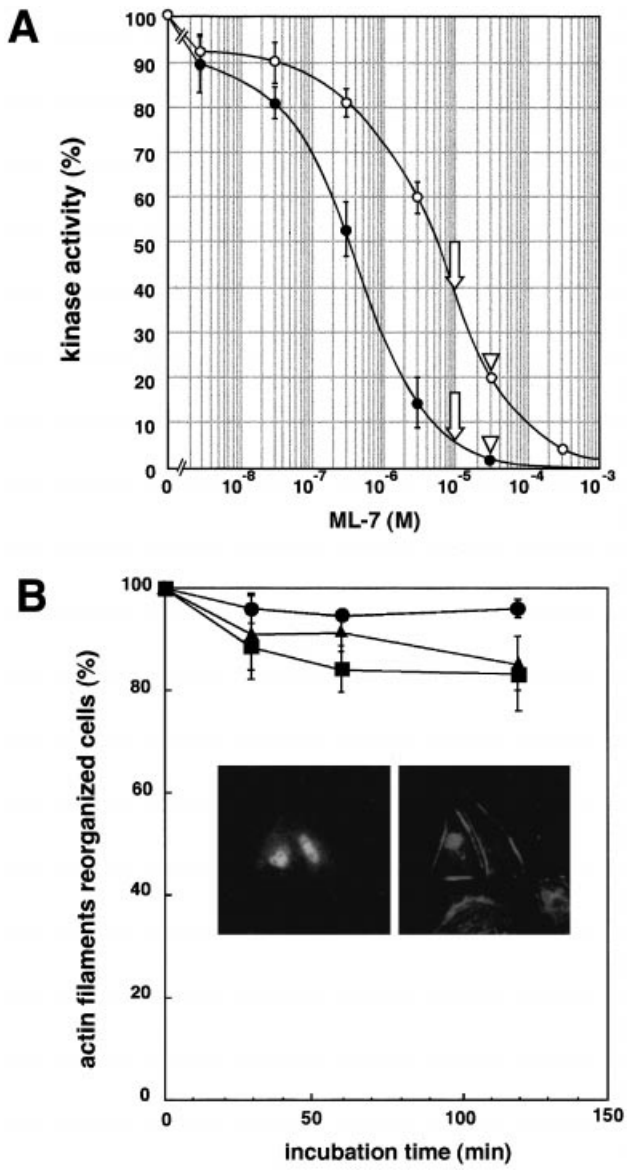

Figure 8 Effect of ML-7, a specific inhibitor of MLCK, on filamentous structures of phosphorylated myosin II

(**A**) Kinetic analysis of inhibition of MLCK and CaM-KI by an MLCK inhibitor. Specific activities of MLCK (●) and CaM-KI (○) were analysed in the presence of ML-7 at concentrations of 0–300 μM. The IC_{50} values of ML-7 against MLCK and GST-CaM-$KI_{1\text{-}293}$ were 0.3–0.4 μM and 6 μM, respectively, indicating that sensitivity of CaM-KI to ML-7 was $\approx$ 15–20-fold lower than that of MLCK. Arrows (10 μM) and arrowheads (30 μM) indicate the concentrations of ML-7 used to treat cells shown in the insets of (**B**). (**B**) Effect of ML-7 on the organization of filamentous structures of phosphorylated myosin II induced by expression of CaM-$KI_{1\text{-}293}$. After transfection, the cells were incubated in the culture medium in the presence of 10 (▲) or 30 μM (■) ML-7, or 0.3% DMSO (●) as a control. After incubation for the indicated times, cells were fixed and immunofluorescence was performed as described in Figure 3. We established the number of the cells in peripheral regions in which thick bundles of actin filaments, including phosphorylated myosin II, were formed, taking the level at 0 min as 100%, and monitored them at the indicated times. Values are the means $\pm$ S.D. from triplicates. Insets show indirect immunofluorescence images stained by pLC1 (right-hand panel) after treatment of 30 μM ML-7 for 120 min, indicating that filamentous structures were still observed in GFP-CaM-$KI_{1\text{-}293}$-expressing cells (left-hand panel) after ML-7 treatment.

tractility and assembly of stress fibres during serum stimulation of mammalian cells [42].

Localization of expressed GFP-CaM-KIwt was more or less diffusely dispersed in the cytoplasm of interphase cells. In contrast, two truncated mutants of the C-terminal end of CaM-KI (GFP-CaM-$KI_{1\text{-}293}$ and GFP-CaM-$KI_{1\text{-}293,K49E}$) were localized not only in the cytoplasm but also in the nucleus. It is reasonable that GFP-CaM-KIwt localizes in the cytoplasm and is not translocated to the nucleus, since CaM-KI does not have a signal sequence corresponding to a nuclear localization signal. On the other hand, it is possible that nuclear accumulation of two truncated mutants is induced by both GFP tagging and downsizing by C-terminal deletion of CaM-KI. These possibilities are supported by two findings. (i) A previous report demonstrated that GFP has a tendency to accumulate in the nucleus [43]. This result is also confirmed in Figure 3(A). (ii) Small molecules (< 40–60 kDa), such as two truncated mutants of CaM-KI (putative molecular mass, 57 kDa), can passively diffuse through the nuclear pore complexes (reviewed in [44]). Therefore, it might be hypothesized that only GFP-CaM-KIwt is excluded from the nucleus. Myosin II localized along actin filaments rather than in the nucleus, or dispersed diffusely in the cytoplasm (Figure 5C). This is consistent with a previous report demonstrating that myosin II exists in the cytoplasm but not in the nucleus of mammalian cells [45]. Also it has been shown that CaM-KK isoforms (CaM-KKα and CaM-KKβ) localized primarily in the cytoplasm of mammalian cells [46]. Therefore, it is considered that cytoplasmic GFP-CaM-$KI_{1\text{-}293}$ rather than the nuclear-localized form could phosphorylate myosin II along cortical actin filaments of the cells. In addition, expressed GFP-CaM-$KI_{1\text{-}293}$ in HeLa cells has been shown to be an activated form by phosphorylation with endogenous CaM-KK (Figure 6), which has the ability to phosphorylate MRLC efficiently, as demonstrated in Table 1.

We have also observed that overexpression of a kinase-negative mutant of CaM-KI (GFP-CaM-$KI_{1\text{-}293,K49E}$) in HeLa cells reduced the proportion of cells, including the thick bundles of actin filaments, containing phosphorylated myosin II, which was approximately half of that of control cells expressing only GFP tag (Figure 4). This may indicate that the endogenous CaM-KI has a role in reorganization of actin filaments through phosphorylation of myosin II in the cells.

Since CaM-KI is likely to have additional substrates, it is important to determine that the monophosphorylation of MRLC at Ser-19 by CaM-KI is mainly responsible for the reorganization of actin filaments. This notion is strongly supported by the co-expression of a non-phosphorylatable MRLC, at Ser-19 (S19A) or both Thr-18 and Ser-19 (T18AS19A), with CaM-$KI_{1\text{-}293}$, which effectively inhibited the formation of filamentous structures of actin filaments accompanied by phosphorylation of myosin II. Our recent report [27] demonstrated that transfection of two mimic forms of mono- and di-phosphorylated MRLC (T18AS19D, substitution of Thr-18 by Ala and Ser-19 by Asp; T18DS19D, substitution of both Thr-18 and Ser-19 by Asp) into HeLa cells caused an increase in the number and the thickness of actin filament bundles. In contrast, induction of non-phosphorylatable MRLC mutant (T18AS19A; substitution of both Thr-18 and Ser-19 by Ala) resulted in a decrease in the number of actin filament bundles. Consequently, it was found that phosphorylation of MRLC has an important role in the reorganization of actin filaments. As shown in Figure 1(D), since CaM-KI is not able to phosphorylate two MRLC mutants (S19A and T18AS19A), co-transfection of the mutants with GFP-CaM-$KI_{1\text{-}293}$ could abolish the phosphorylation of endogenous MRLC by expressed GFP-CaM-$KI_{1\text{-}293}$. It is reasonable that the co-transfection with MRLC mutants suppresses reorganization of actin filaments by GFP-CaM-$KI_{1\text{-}293}$, as shown in Figure 7. In a recent report, stellar aggregation of actin filaments, which has been shown to be mediated by diphos-

phorylation (at both Thr-18 and Ser-19) of myosin II by ZIP kinase [22], was not evoked by CaM-KI-induced monophosphorylation of myosin II at Ser-19 in HeLa cells. These results suggest that mono-(at Ser-19) and di- (at both Thr-18 and Ser-19) phosphorylation of myosin II differentially regulate dynamic reorganization of actin filaments in the cells.

As well as the results *in vitro* indicated in Figure 1(C), it seems that GFP-CaM-KI$_{1-293}$ is also not involved in diphosphorylation of MRLC *in vivo* (Figure 3N). Interestingly, diphosphorylation of MRLC is observed not only in the cytoplasm but also in the nucleus. Recently, we identified ZIP kinase and Rho kinase as kinases to diphosphorylate MRLC *in vitro* and *in vivo* [22,31,47]. They are found to be primarily localized in the cytoplasm. Since the kinase (or possibly kinases) used to diphosphorylate MRLC is not isolated from the nucleus, it is possible that MRLC diphosphorylated by such kinases in the cytoplasm is transported to the nucleus, although its mechanism has not been elucidated.

Our present study also suggested that CaM-KI participates in the reorganization of peripheral stress fibre-like structures through the phosphorylation of myosin II, which was less affected by an MLCK inhibitor. Therefore, CaM-KI also may have a role in the regulation of cell spreading in an MLCK-independent manner. It has also been reported that monophosphorylation of MRLC concomitantly with reorganization of actin filaments was evoked by inhibition of myosin phosphatase by injection of the antibody against its myosin-binding subunit into mouse 3T3 fibroblasts [37]. Taking these observations together, it is considered that the formation of actin filaments could be strictly regulated by various MRLC kinases and myosin phosphatase in mammalian cells.

Several lines of evidence show that the phosphorylated MRLC at Ser-19 is localized in the contractile ring of dividing mammalian cells, suggesting that phosphorylation of MRLC plays an important role during cytokinesis [16]. Further, it has been indicated that spatiotemporal Ca^{2+} transients were evoked during mitosis, which was thought to be essential for the progression of mitosis [48,49]. Joseph and Means [50] suggested that the CaM-K cascade in *Aspergillus nidulans* is required for cell-cycle progression during nuclear division. According to these observations, the CaM-KK/CaM-K cascade could have a potentially important role in the regulation of progression of mitosis in the cells. Our present studies also suggested that CaM-KI is activated by endogenous CaM-KK and functions as an MRLC kinase, at least in interphase cells. Therefore, one possibility could be that the CaM-KI cascade participates in the progression of mitosis through the phosphorylation of myosin II in mammalian cells. In future, it will be necessary to investigate how the phosphorylation of myosin II by CaM-KI is regulated and participates in cellular function in response to a Ca^{2+}-signalling pathway via the CaM-KK/CaM-KI cascade.

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