ZIP kinase identified as a novel myosin regulatory light chain kinase in HeLa cells

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Received 4 March 1999; received in revised form 4 April 1999

Abstract A novel myosin light chain kinase (MLCK) cDNA was isolated from a HeLa cell cDNA library. The deduced amino acid sequence was identical to that of a zipper-interacting protein kinase (ZIPK) which mediates apoptosis [Kawai et al. (1998) Mol. Cell. Biol. 18, 1642–1651]. Here we found that HeLa ZIPK phosphorylated the regulatory light chain of myosin II (MRLC) at both serine 19 and threonine 18 in a Ca^{2+}/calmodulin independent manner. Phosphorylation of myosin II by HeLa ZIPK resulted in activation of actin-activated MgATPase activity of myosin II. HeLa ZIPK is the first non-muscle MLCK that phosphorylates MRLC at two sites.

Key words: Regulatory light chain of myosin II; Zipper-interacting protein kinase; Phosphorylation; Myosin light chain kinase; Actin-activated MgATPase activity; Cytokinesis

1. Introduction

In most eukaryotic cells, it is considered that myosin II plays an important role during cytokinesis [1–3]. However, the molecular mechanisms for controlling of myosin II still remain unknown. Phosphorylation of the regulatory light chain of myosin II (MRLC) regulates positively and negatively both smooth muscle and non-muscle myosin. The actin-activated MgATPase activity of myosin II is increased by phosphorylation of MRLC by Ca^{2+}/calmodulin-dependent myosin light chain kinase (MLCK) [4]. The phosphorylation sites of MRLC by smooth muscle (sm) MLCK are mainly serine 19 and, under some conditions, threonine 18 and serine 19. This phosphorylation also promotes assembly of myosin filament, at least in vitro [5]. MRLC was also phosphorylated by protein kinase C (PKC) at serine 1 and/or serine 2 and threonine 9 [6,8]. Phosphorylation by PKC inhibits the increase in MgATPase activity of myosin II phosphorylated by smMLCK [7,8].

Recent evidence showed that phosphorylated MRLC at serine 19 localized in the contractile ring of dividing mammalian cells, suggesting that phosphorylation of MRLC plays an important role during cytokinesis [9,10]. Consequently, we tried to investigate the kinase(s) which phosphorylate MRLC during cytokinesis. In order to search for such kinase(s), we screened a HeLa cell cDNA library with the fragment of the bovine stomach MLCK gene including kinase and calmodulin regulatory domains as a probe. We first found that one serine/threonine kinase, HeLa zipper-interacting protein kinase (ZIPK), from non-muscle cells phosphorylated MRLC at both serine 19 and threonine 18. Furthermore, phosphorylation of intact myosin II by HeLa ZIPK enhanced the actin-activated MgATPase activity of myosin II. HeLa ZIPK is a candidate for phosphorylation of MRLC at both serine 19 and threonine 18 in non-muscle cells.

2. Materials and methods

2.1. Materials and chemicals

Myosin II and smMLCK were purified from chicken gizzard [11,12]. Light chains from myosin II (MLCs) were obtained as described [13]. Actin was prepared from rabbit skeletal muscle by the method of Spudich and Watt [14]. G-actin was further purified by gel filtration. Calmodulin from bovine brain was purchased from Sigma. Other materials and chemicals were obtained from commercial sources.

2.2. Cell culture

HeLa cells (RCB007; just like ATCC CCL2, HeLa) were obtained from the Riken Cell Bank (Tsukuba, Japan) and were grown as described previously [15].

2.3. Construction of HeLa cell cDNA library

The HeLa cell cDNA library was constructed using lambda ZAPII as a vector. Messenger RNA was extracted from exponentially growing cells using a messenger RNA isolation kit (Stratagene). The cDNA synthesis was performed using a ZAP cDNA library synthesis kit (Stratagene). The first strand was synthesized by priming an oligo(dT) linker-primer carrying a *Xho*I adapter. After the second strand was synthesized, the cDNA termini were blunted by Klenow fragments and ligated with *Eco*RI adapters. *Xho*I digestion was performed to generate a *Xho*I site at the 5′ end. The synthesized cDNAs were ligated into *Xho*I-*Eco*RI sites of the lambda ZAPII vector.

Packaging of lambda DNA in vitro was performed with GIGA PACK II Gold (Stratagene) according to the manufacturer's instructions.

2.4. Screening of HeLa cell cDNA library

The bovine stomach MLCK cDNA clone (pSL17), kindly provided by Dr. H. Kobayashi [16], was digested with *Sal*I and *Hin*dIII. The obtained 1.0 kb fragment was used as a probe. Screening of the HeLa cell cDNA library in lambda ZAPII was performed using the ECL direct nucleic acid labeling and detection system (Amersham Pharmacia Biotech). Recombinant phages of 4×10^5 pfu were plated with *Escherichia coli* XL1 Blue and blotted onto Hybond N^+ membranes (Amersham Pharmacia Biotech). The cDNA fragments prepared above were labeled with horseradish peroxidase (HRP) and used as probes. Hybridization using 10 ng/ml of HRP-labeled probes in the recommended hybridization buffer and washing and detection procedures was performed according to the manufacturer's instructions. The membranes were used to expose to X-ray film.

2.5. Determination of complete cDNA sequences

The cDNA inserts were subcloned into pBluescript SK^- from the ZAPII vector in vivo using an ExAssist/SOLR system (Toyobo). A series of deletion mutants of subclones were prepared for sequencing using exonuclease III. Sequencing was done with the Model 373S DNA sequencing system (Perkin-Elmer) and ALFexpress II (Amer-

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0014-5793/99/$20.00
PII: S0014-5793(99)00550-5

sham Pharmacia Biotech). Sequences were assembled with the software DNASIS (Hitachi Software Engineering).

2.6. Plasmid construction and expression of recombinant kinase

The cDNA clones encoding the complete amino acids of HeLa ZIPK were in-frame inserted into vector pGEX-5X-3 (Amersham Pharmacia Biotech) in order to express the glutathione *S*-transferase (GST) fusion protein. *E. coli* cells were cultured in 2×YT medium containing 10 mg/ml Bacto yeast extract (Difco), 16 mg/ml Bacto tryptone (Difco), 5 mg/ml NaCl and 100 μg/ml ampicillin at 37°C with vigorous agitation. After 2.5 h culture, protein expression was induced by adding isopropyl-β-D-thiogalactopyranoside to a final concentration of 1 mM. After an additional 2 h at 37°C, cells were harvested and lysed by sonication in cold phosphate-buffered saline containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mg/ml leupeptin, and 1 mM dithiothreitol (DTT), and GST-HeLa ZIPK fusion protein was purified with a glutathione-Sepharose 4B (Amersham Pharmacia Biotech) as described by the manufacturer.

2.7. Protein kinase assay

Phosphorylation of isolated MLCs by GST-HeLa ZIPK was carried out in a reaction mixture (27 mM Tris-HCl (pH 8.0), 1 mM EGTA, 8 mM $MgCl_2$, 10 mM NaCl, 0.01 mM DTT, 0.1 mM PMSF, 0.3 μg/ml leupeptin, 0.3 μg/ml pepstatin A, 0.5 μM calyculin A and 0.18 mM [γ-^{32}P]ATP) containing 0.11 mg/ml MLCs, 1.25 μg/ml GST-HeLa ZIPK and variable amounts of $CaCl_2$ to give the required pCa values with or without 50 μg/ml calmodulin (CaM). Phosphorylation of intact myosin II by GST-HeLa ZIPK was carried out in a reaction mixture (28.5 mM Tris-HCl (pH 7.5), 1 mM EGTA, 1.5 mM $MgCl_2$, 50 mM KCl, 40 μM PMSF, 0.5 μM calyculin A, 0.5 mM β-mercaptoethanol and 0.18 mM [γ-^{32}P]ATP) containing 3.5 mg/ml myosin II and 1.25 μg/ml GST-HeLa ZIPK. Both reaction mixtures for the isolated MLCs and myosin II were incubated for 30 min at 25°C and subjected to SDS-PAGE. The radiolabeled bands were visualized by the Bio Imaging Analyzer BAS 2000 (Fuji).

2.8. Two-dimensional phosphopeptide mapping analysis

Phosphorylation of isolated MLCs by GST-HeLa ZIPK was carried out in the same reaction mixture as described above containing 0.11 mg/ml MLCs and 125 μg/ml GST-HeLa ZIPK for 6 min at 25°C. Phosphorylation of MLCs by smMLCK was done in a buffer (20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EGTA, 2 mM $CaCl_2$, 3.8 mM $MgCl_2$, 20 μg/ml CaM, 0.3 mM DTT, 1 mM PMSF, 1.4 μg/ml leupeptin, 1.4 μg/ml pepstatin A and 1 mM [γ-^{32}P]ATP) containing 0.5 mg/ml MLCs and 0.1 mg/ml smMLCK for 30 min at 25°C. ^{32}P-labeled MRLC was separated by SDS-PAGE, and excised from the gel. Two-dimensional phosphopeptide mapping was done as described previously [17] except for using a cellulose plate (TLC precoated plates (glass) cellulose: Merck) instead of a silica gel plate (Merck).

2.9. Assay of MgATPase activity

The MgATPase activity of myosin II unphosphorylated or phosphorylated by GST-HeLa ZIPK was assayed in a reaction mixture containing 40 mM Tris-HCl (pH 7.4), 15 mM KCl, 2 mM $MgCl_2$, 1.5 mM EGTA, 1.2 μM reduced glutathione, 1.5 mM ATP, 10 μM PMSF, 0.2 mM β-mercaptoethanol, 0.2 mg/ml of F-actin and 0.24 mg/ml of myosin II with or without 50 μg/ml GST-HeLa ZIPK for 20 min at 25°C. Phosphorylation of myosin II was carried out by incubating 0.8 mg/ml of myosin II with 0.16 mg/ml GST-HeLa ZIPK in a buffer containing 40 mM Tris-HCl (pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 0.04 mM PMSF, 0.6 mM β-mercaptoethanol and 2 mM ATP for 1 h at 25°C. The actin-activated MgATPase reaction was started by addition of F-actin to the reaction mixture. Inorganic phosphate was determined by using a IATRON PiSET (IATRoN Lab.) according to the method of Fiske and Subbarow [18].

2.10. Other procedures

SDS-PAGE was carried out by the method of Blattler et al. [19] with 15% polyacrylamide slab gels, except that the buffer system of Laemmli [20] was used.

3. Results and discussion

In order to search for the kinase(s) that phosphorylate MRLC, we screened a HeLa cell cDNA library under low stringency conditions with a HRP-labeled partial cDNA fragment of the bovine stomach MLCK cDNA. This fragment was obtained by digestion with the restriction enzymes *Sal*I and *Hin*dIII and encoded the kinase and the calmodulin regulatory domains of smMLCK [16]. After screening 4×10^5 recombinant phages with this fragment as a probe, several clones encoding kinases were obtained. One of them was characterized by determination of the nucleotide sequence. The decided nucleotide sequence was 2079 bp and the deduced amino acid sequence was identical to that of ZIPK from human placenta in the protein sequence database [21]. ZIPK was first identified as a kinase which is a mediator of apoptosis [21]. However, the nucleotide sequence of our clone was slightly different from that of placenta ZIPK in both coding and untranslated regions (data not shown). The kinase domain of HeLa ZIPK had 48% amino acid sequence identity to that of smMLCK [16] (Fig. 1). Furthermore, it is also 81% identical to the kinase domain of death-associated protein

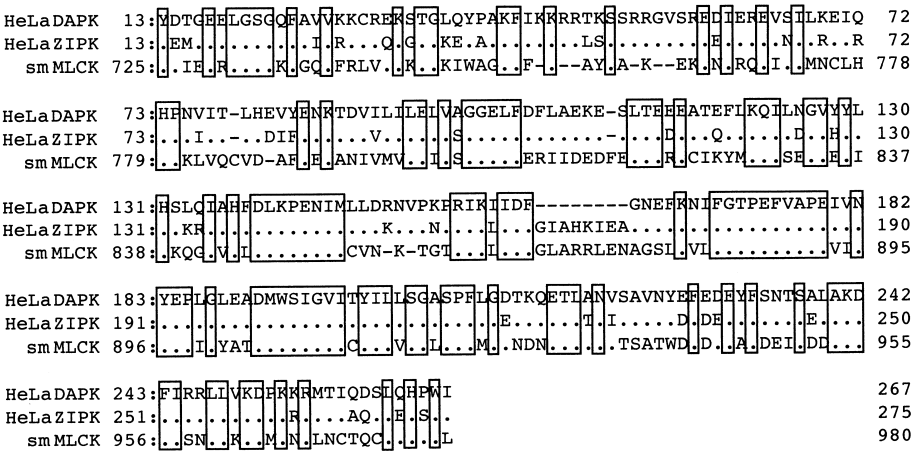

Fig. 1. Comparison of the kinase domain of HeLa ZIPK, HeLa DAPK and smMLCK. Identical amino acids are boxed. The nucleotide sequence data of HeLa ZIPK will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AB022341.

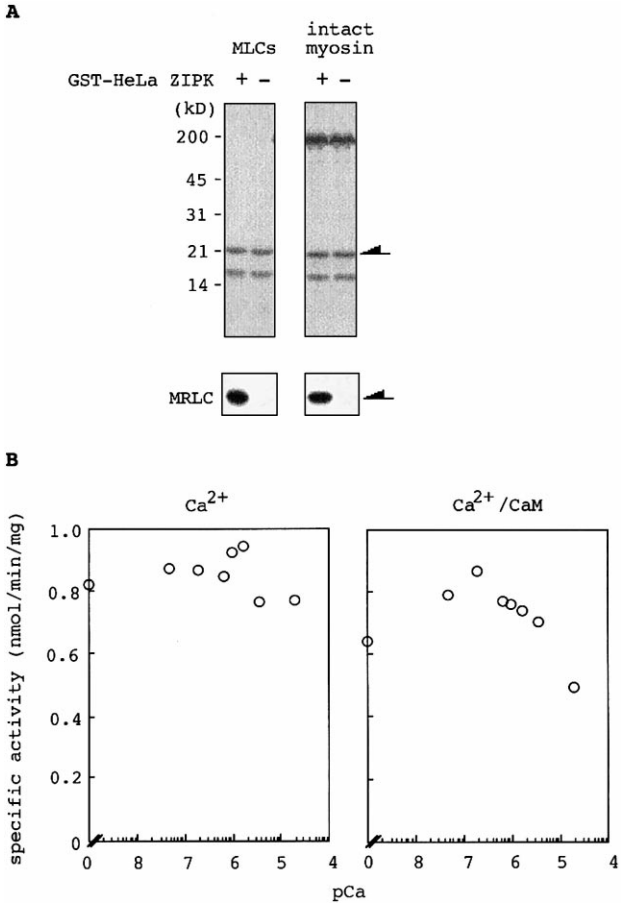

Fig. 2. Phosphorylation of MLCs of intact myosin II and isolated MLCs by HeLa ZIPK. A: MLCs of intact myosin II and isolated MLCs were phosphorylated by GST-HeLa ZIPK. GST-HeLa ZIPK phosphorylated both MRLC of intact myosin II and isolated MRLC in the absence of Ca^{2+}/CaM (arrows). Only GST did not phosphorylate MRLC in the same condition (data not shown). B: Ca^{2+} sensitivity of HeLa ZIPK activity. Isolated MLCs were phosphorylated by GST-HeLa ZIPK at various values of pCa in the absence (left panel) and presence (right panel) of calmodulin.

kinase (DAPK) from HeLa cells which plays an important role in γ-interferon-induced cell death [22]. Because both DAPK [23] and smMLCK phosphorylate MRLC, we investigated the ability of HeLa ZIPK to phosphorylate isolated MRLC or MRLC of intact myosin II (Fig. 2A). The cDNA encoded by HeLa ZIPK was expressed as GST fusion protein in *E. coli*. Affinity purified GST-HeLa ZIPK phosphorylated both MRLC of intact myosin II and isolated MRLC in the absence of Ca^{2+}/CaM (Fig. 2A). It is well known that both DAPK and smMLCK phosphorylate MRLC in a Ca^{2+}/CaM-dependent manner. Therefore, to know whether the activity of HeLa ZIPK is regulated by Ca^{2+}/CaM, various concentrations of $CaCl_2$ to give required values of pCa were added to the reaction mixture in the absence or presence of CaM (Fig. 2B). However, any concentrations of Ca^{2+} or Ca^{2+}/CaM did not influence the activity of GST-HeLa ZIPK (Fig. 2B). Furthermore, HeLa ZIPK lacked the CaM regulatory domain of DAPK or smMLCK as well as placenta ZIPK [21]. These data suggest that HeLa ZIPK phosphorylates MRLC in a Ca^{2+}/CaM-independent manner.

MRLC was phosphorylated first at serine 19 and second at threonine 18 by smMLCK [24]. To determine the phosphorylation sites of MRLC by HeLa ZIPK, two-dimensional phosphopeptide mapping of phosphorylated MRLC by either GST-HeLa ZIPK or smMLCK was performed (Fig. 3). In this condition, smMLCK phosphorylates MRLC at both serine 19 and threonine 18. The pattern of the mapping of phosphorylated MRLC by GST-HeLa ZIPK was identical to that by smMLCK. When GST-HeLa ZIPK phosphorylated MRLC at a low concentration (1.25 μg/ml), phosphorylation of MRLC still occurred at both residues. A phosphoamino acid analysis revealed that phosphorylation of MRLC by GST-HeLa ZIPK was at both serine and threonine residues. These results suggest that HeLa ZIPK phosphorylated MRLC first at serine 19 and second at threonine 18. Phosphorylation of MRLC at serine 19 and threonine 18 by smMLCK increased the actin-activated MgATPase activity of smooth muscle and non-muscle myosin [25,26]. Fig. 4 showed that phosphorylation of myosin II by GST-HeLa ZIPK also increased the actin-activated MgATPase activity.

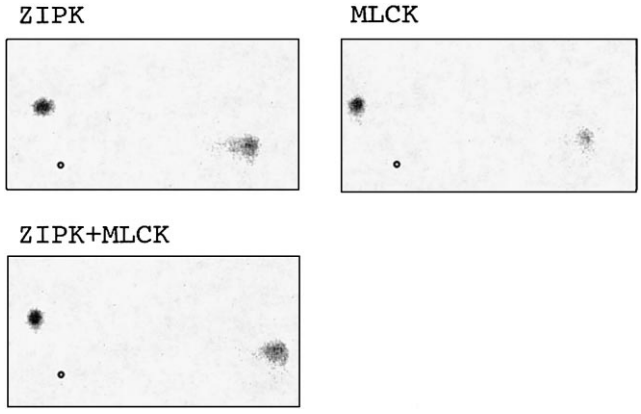

Fig. 3. Identification of the phosphorylation site of MRLC by HeLa ZIPK. MRLC was phosphorylated by GST-HeLa ZIPK or smMLCK. Phosphorylated MRLC was digested with trypsin and processed on a cellulose plate for electrophoresis (horizontal) followed by chromatography (vertical). Open circles denote origins.

In this paper, we showed that the serine/threonine kinase, HeLa ZIPK, phosphorylated MRLC at both serine 19 and threonine 18 as smooth muscle smMLCK did. Its activity for phosphorylation of MRLC was not regulated by Ca^{2+}/CaM. Furthermore, the phosphorylation of MRLC of intact

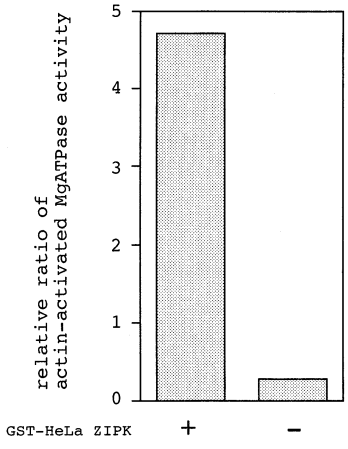

Fig. 4. Effect of phosphorylation of myosin II by HeLa ZIPK on the actin-activated MgATPase activity. Myosin II was incubated with (+) or without (−) GST-HeLa ZIPK.

myosin II enhanced the MgATPase activity. These data suggest that HeLa ZIPK phosphorylates MRLC of myosin II and increases its MgATPase activity in a Ca^{2+}/CaM-independent manner. Recently, it has been reported that two kinds of serine/threonine kinases phosphorylate MRLC predominantly at serine 19 in non-muscle cells. Phosphorylation of myosin II by these non-muscle (nm) MLCKs resulted in enhancement of the MgATPase activity of myosin II in vitro. One is mitogen-activated protein kinase-activated protein kinase-4 from sea urchin eggs [26] and another is Rho-kinase from bovine brain [27]. In contrast with these, nmMLCKs which phosphorylate MRLC at both serine 19 and threonine 18 from non-muscle cells have never been reported previously. Therefore, HeLa ZIPK is the first nmMLCK which can phosphorylate MRLC at two sites.

Recently, Kögel et al. reported that a DAPK homologue, Dlk, phosphorylates core histones H3 and H4 [28]. Whether MRLC and/or histones represent physiological substrates remains to be elucidated. However, it is attractive to speculate that these kinases are involved in regulation of nuclear functions through cytoskeletal structures including myosins. We are now investigating the localization of phosphorylated MRLC at both serine 19 and threonine 18 in HeLa cells. In future, it is necessary to investigate whether HeLa ZIPK contributes to phosphorylation of MRLC at two sites in cytokinesis of non-muscle cells.

Acknowledgements: We would like to thank H. Ohara, K. Ohno and N. Tanaka for help in screening the HeLa cell cDNA library. This work was supported in part by The Fugaku Trust For Medicinal Research.

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