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# Mutation Analysis of the Non-Muscle Myosin Light Chain Kinase (MLCK) Deletion Constructs on CV1 Fibroblast Contractile Activity and Proliferation

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**Abstract** Smooth muscle myosin light chain kinase (MLCK) is a multifunctional molecule composed of an N-terminal actin binding domain, a central kinase domain, and C-terminal calmodulin- and myosin-binding domains. We previously cloned and characterized a novel MLCK isoform from endothelial cells (EC MLCK) consisting of 1,914 amino acids displaying a higher molecular weight (210 kDa) and a novel-amino-terminal stretch of 922 amino acids not shared by the smooth muscle isoform (smMLCK, 150 kDa). To further define the role of specific EC MLCK motifs in endothelial and non-muscle cells, we constructed two epitope-tagged EC MLCK deletion mutants in mammalian expression vectors lacking either the C-terminal auto-inhibitory and calmodulin-binding domain (EC MLCK1745) or the ATP-binding site (EC MLCKATPdel). Expression of EC MLCK1745 in CV1 fibroblasts showed increased basal actin stress fiber formation, which was markedly enhanced after tumor necrosis factor (TNF-$\alpha$) or thrombin treatment. Distribution of EC MLCK1745 was largely confined to stress fibers, cortical actin filaments, and focal adhesion contacts, and co-localized with myosin light chains (MLCs) diphosphorylated on $Ser^{19}$ and $Thr^{18}$. In contrast, immunofluorescence staining demonstrated that EC MLCKATPdel abolished thrombin- and TNF$\alpha$-induced stress fiber formation and MLC phosphorylation, suggesting this kinase-dead mutant functions as a dominant-negative MLCK construct, thereby confirming the role of EC MLCK in stress fiber formation. Finally, we compared the serum-stimulated growth rate of mutant MLCK-transfected fibroblasts to sham controls, and found EC MLCK1745 to augment thymidine incorporation whereas EC MLCKATPdel reduced CV1 growth rates. These data demonstrate the necessary role for MLCK in driving the contractile apparatus via MLC phosphorylation, which can alter fibroblast growth and contractility. J. Cell. Biochem. 88: 623–634, 2003. 

**Key words:** contraction; stress fibers; mitogenesis; actin polymerizaton; myosin phosphorylation

In muscle and non-muscle cells, phosphorylation of the regulatory myosin light chain (MLC) is catalyzed by $Ca^{2+}$/calmodulin-regulated myosin light chain kinases (MLCK) with tissue-specific contractile function and increase in actin-dependent myosin ATPase activity [Sellers and Pato, 1984; Kamm and Stull, 1985]. For example, in smooth muscle, phosphorylation of MLC by MLCK is necessary for the initiation of contraction whereas in skeletal muscle this phosphorylation has a modulatory role in contraction-induced potentiation of isometric twitch tension [Sweeney et al., 1993]. In cardiac muscle, MLCK is involved in sarcomere organization and may have a novel adaptive role in response to hypertrophic stimuli [Aoki et al., 2000]. In cardiac hypertrophy and heart failure subsequent to myocardial infarction, region-specific changes in the levels of MLCK and MLC phosphorylation have been reported [Liu et al., 1995]. Recent studies have shown that MLCK-dependent increases in myosin ATPase activity play a critical role in cell division [Poperechnaya et al., 2000], receptor capping [Kerrick and Bourguignon, 1984], platelet or endothelial cell contraction [Adelstein, 1975; Wysolmerski and Lagunoff, 1990], and lead to apoptotic pathways [Mills et al., 1998; Jin et al., 2001; Petrache et al., 2001].

MLCK proteins are muscle members of the immunoglobulin super family containing a catalytic core domain homologous to that of other protein kinases and a regulatory segment

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located C-terminal to the catalytic core. This segment consists of an auto-inhibitory domain, which connects the calmodulin-binding region to the catalytic core [Guerriero et al., 1986; Olson et al., 1990; Shoemaker et al., 1990; Gallagher and Herring, 1991] and can fold back on the catalytic domain to inhibit kinase activity by intrasteric regulation [Kamm and Stull, 2001]. Binding of $Ca^{2+}$/calmodulin activates MLCK by specific displacement of the autoinhibitory region from the catalytic clefts allowing MLC binding and phosphorylation on $Ser^{19}$ and $Thr^{18}$. The C-terminal region of the MLCK isoform contains a C2-type immunoglobulin-like domain known as kinase-related protein (KRP) or telokin which facilitates binding of the enzyme to the unphosphorylated MLC or myosin and consequently promotes MLCK contractile activities [Shirinsky et al., 1993; Silver et al., 1997; Kamm and Stull, 2001].

The human MLCK gene yields both smooth muscle and non-muscle cell isoforms, and is a single-copy gene located on chromosome 3qcen-q21 [Potier et al., 1995] with at least two promoters that initiate transcription of SM MLCK (5.8 kb), and non-muscle MLCK (8.1 kb) mRNAs [Watterson et al., 1995]. Our previous studies have shown that the endothelial MLCK isoform (EC MLCK) is distinct from smooth muscle MLCK, displaying a higher molecular weight (210–214 kDa) and a novel amino terminal 922 amino acids not shared by smooth muscle MLCK (150 kDa). Comparison of the 214 kDa EC MLCK isoform with non-muscle avian fibroblast MLCK sequence [Shoemaker et al., 1990; Garcia et al., 1997] revealed substantial homology in the C-terminal parts of the two molecules (amino acids #923–1,914), but much less homology between amino acids 1 through 922, suggesting that the novel N-terminal region may have a unique tissue-specific role in phosphorylated MLC-dependent endothelial cell contractile function. Our recent studies have underscored the multifunctional role of EC MLCK in vascular barrier regulation [Dudek and Garcia, 2001], leukocyte diapedesis, cell migration [Garcia et al., 1998], angiogenesis (26), and cellular apoptosis [Petrache et al., 2001]. Phosphorylation-dependent activation of kinase itself has become a subject of intense importance. Several studies from our laboratory have correlated Ser/Thr phosphorylation of MLCK with inhibition of kinase activity [Shi et al., 1998; Garcia et al., 1999] whereas both EC MLCK and MLCK isolated from fibroblasts [Lazar, 1999] contain an SH2 binding domain and a consensus tyrosine phosphorylation site for $p60^{src}$. We recently reported that the N-terminal tyrosine phosphorylation of EC MLCK by $p60^{Src}$ on $Y^{464}$ and $Y^{471}$ increases enzymatic activity. Furthermore, of the several splice variants identified, at least two have exon 8 deleted, which contains these SH2 domains and $Y^{464}$ and $Y^{471}$ [Birukov et al., 2001].

To further define the structure/function role of endothelial MLCK, we constructed two deletion mutants in mammalian expression vectors with transfection into CV1 fibroblasts. Our results indicate that an MLCK mutant construct lacking an auto-inhibitory domain and a calmodulin-binding site (EC MLCK1745) co-localizes with actomyosin stress fibers and increases actin stress fiber formation in the absence of agonist stimulation. A second kinase-dead EC MLCK construct lacking the ATP binding site (EC MLCKATPdel) functioned as a complete dominant-negative EC MLCK and abolished agonist-induced stress fiber formation and MLC phosphorylation. To understand the relation between cytoskeletal rearrangement and cell growth, we measured the thymidine incorporation and cellular enzyme activity in cells expressing these MLCK mutants. Our results imply a necessary role for MLCK in fibroblast contractility and growth.

## METHODS

### Cell Culture and Antibodies

CV-1 (African Green Monkey kidney fibroblast) cells were purchased from ATCC (Manasass, VA) and maintained in minimal essential medium containing 10% fetal bovine serum (ATCC). Smooth muscle MLC (*MY21-M4401*) and MLCK (K36) monoclonal antibodies were obtained from Sigma (St. Louis, MO). A polyclonal antibody directed against diphosphorylated MLC at $Ser^{19}$ and $Thr^{18}$, which we have characterized previously [Petrache et al., 2001] was a gift from Dr. Michael Crow. V5 epitope antibody was obtained from Invitrogen (Carlsbad, CA). Human thrombin was obtained from Sigma (T 6759). For thrombin treatment, CV-1 cells were pre-incubated in serum-free medium for 1 h and human thrombin was added (100 ng/ml) for 30 min.

### Construction of MLCK Deletion Mutants

The full length EC MLCK previously cloned from endothelial cDNA library [Garcia et al., 1997; Lazar and Garcia, 1999] was used as a template to obtain the deleted inserts by PCR analysis. To generate the EC MLCK-1745 construct which lack the EC MLCK C-terminus amino acids 1,745–1,914, forward and reverse PCR primers were synthesized from start codon and 1,745 amino acid as an end (A27F–ATGGGGATGTGTGAAGCTGGTTGCCTCGTCACACA, A28–GCCATTTCCTCTTGCCATGTACTTCTTCATCCGGTC). The PCR product was cloned into TOPO expression vector [Invitrogen] so that V5 and His tag are within the MLCK reading frame. To generate the ATP binding site deletion mutant (EC MLCKATPdel), the 1–1,580 amino acid product was amplified using A27F and A29R reverse primer (GCCCTGCTTGTGGATGTACTCCACTCCACTCCCTCCGA), cloned into TOPO expression vector followed by the addition of the C-terminal portion using forward A32F (GATATCATCTCAGGGCTCAGTGGCAGGAAATCCTCA), and reverse A31R (GATATCCATCGTTTCCACAATGAGCTCTGCTGT) of the C-terminal of MLCK was cloned into the EcoRV site of multiple cloning region.

### DNA Transfection Into Mammalian Cells

For transfection of mammalian expression vector into CV1 cells (50–60% confluent) in 100-mm dishes were incubated with DNA/FuGENE 6 (Roche, NJ) mixture prepared as follows: 50 μl FuGENE 6 was diluted in 1 ml OPTI-MEM (GIBCO-BRL; Grand Island, NY) incubated at room temperature for 15 min and added to 5 μg DNA containing 1 μg EC MLCK1745, EC MLCK ATPdel, or the TOPO empty vector control. The mixture was incubated at room temperature for 30 min and added to the dishes with 10 ml fresh complete medium. After incubation (48 h) cells were washed and harvested in lysis buffer and the total cell extract prepared for Western blotting or cells were fixed for immunofluorescence studies.

### Immunoblotting

For immunoblot analysis, fibroblasts were washed with PBS, scraped directly into ice-cold lysis buffer containing 0.2% NP-40, 10 mM MOPS, pH 7.0, 5 mM EDTA, 0.5 mM EGTA, 1 mM DTT, 50 mM $MgCl_2$, 100 mM NaCl, 1 mM PMSF, and 1 × protease inhibitor mix (Boehringer Mannheim Roche Applied Science; Indianapolis, IN), and centrifuged at 4°C for 5 min at 16,000*g*. SDS–polyacrylamide gel (PAGE) sample buffer was added to the supernatant, which was boiled and immediately loaded onto a 4–12% gradient SDS–PAGE for electrophoresis. Proteins were transferred to nitrocellulose and reacted with antibodies either against V5, MLC, MLCK, or diphosphorylated MLC (MLCpp). Immunoreactive proteins were detected using the ECL chemiluminescent detection system (Amersham-Pharmacia Biotech; Piscataway, NJ). To measure the relative amounts of the full length, and deletion mutants of MLCK and MLC in cells, autoradiograms were scanned on a Molecular Dynamics densitometer and quantified using the program ImageQuant v 5.0.

### Immunofluorescence Microscopy

Fibroblasts were transfected with the EC MLCK constructs or using empty vector controls using the Fugene (Roche) or lipofectin (GIBCO BRL) method. Cells were fixed 48 h after transfection in freshly prepared 4% formaldehyde in PBS for 15 min at room temperature. After rinsing the cells in an excess of PBS containing 0.02% sodium azide, the coverslips were mounted in Pro-Long Antifade (Molecular Probes; Eugene, OR). Transfected CV1 fibroblasts were permeabilized according to the method we have previously described [Liu et al., 2001]. In brief, cells were cooled to 4°C, washed with ice-cold PBS, and extracted for 10 min with ice-cold 10 mM Tris-HCl, pH 7.0, 60 mM KCl, 125 mM sucrose, and 0.05% Triton X-100. Cells were washed three times with ice-cold 10 mM Tris-HCl, pH 7.0, 30 mM KCl, 5 mM $MgCl_2$, and 1 μM $CaCl_2$. Images were acquired using Image Analysis software with an FKI 1000 interline 12-bit, cooled CCD camera mounted on an Olympus IX70 microscope with a PlanApo 60 ×, 1.4 N.A. objective (Olympus; Melville, NY) and HiQ bandpass filters (Chroma Technology Corp.; Brattleboro, VT). Images were processed using Adobe Photoshop® software (Adobe Systems; San Jose, CA).

### Cell Growth Assay

To determine the cell division time, EC MLCK1745-, MLCKATPdel-, and empty vector-transfected cells were synchronized by growing them in the presence of CV1 medium containing

0.5% FBS for 48 h. The cells were then trypsinized, washed, and 50,000 cells were plated in triplicate in complete CV1 medium containing 10% serum and 5 μCi/ml of 3[H] thymidine. After 24 h, cells were washed with 5% trichloro acetic acid (TCA) twice and solubilized into 0.5 ml of 10 N sodium hydroxide and 400 μl of extract was used for counting the thymidine incorporation. To measure the proliferation in terms of metabolic activity alamarBlue Assay (Biosource; Camarillo, CA) was used. In this assay, cell growth rate was measured by oxidation–reduction indicator that both fluoresces and changes color in response to chemical reduction of growth medium resulting from cell growth [Ahmed et al., 1994]. Changes in color were measured by spectrophotometer. Empty vector- and MLCK mutant-transfected cells were washed with PBS 24 h after serum stimulation and incubated at 37°C with 1 ml alamarBlue reagent for 2 h. Medium was collected from the plates and optical density (OD) was measured at 540 and 620 nm wavelength on spectrophotometer as a change in color. The ratio of 540/620 OD was considered as a relative metabolic activity in growing cells.

## RESULTS

### Expression and Biochemical Characterization of EC MLCK Mutants in CV1 Fibroblasts

As detailed in Methods, two EC MLCK deletion mutant constructs were generated. EC MLCK1745 fused with a V5 epitope tag at the C-terminus lacks the calmodulin-binding site, the auto-inhibitory domain, and the myosin-binding domain present within the 1,745–1,914 amino acid C-terminal segment (Fig. 1A). A second construct was characterized by the deletion of the ATP-binding site (1,580–1,617 amino acids, EC MLCKATPdel), which was also fused with the V5 epitope. To study the expression levels of these proteins, MLCK constructs under a CMV promoter were transiently expressed in CV1 cells (Fig. 1B) and Western blotted for V5 antibody. The C-terminal truncated EC MLCK1745 protein band was observed at ~190 kDa molecular weight as compared to the endogenous MLCK ~210 kDa protein from CV1 cells (data not shown). The EC MLCKATPdel band was observed at ~210 kDa molecular weight.

To define the physiological effects of mutant MLCK over-expression in CV1 fibroblasts, we next compared the effect of enzymatic activity in EC MLCK1745- and empty vector-transfected cells and observed an increase in MLC phosphorylation measured by diphosphorylated-MLC antibody (*MLCpp*) staining in EC MLCK1745 expressing cells. Thrombin, a well studied contractile agent both in fibroblasts and endothelium and an activator of EC MLCK [Garcia et al., 1995], significantly increased the levels of phosphorylated MLC in empty vector-transfected cells, which was significantly augmented in EC MLCK1745-transfected cells (Fig. 1B, lanes 1 and 3), representing ~40% increase in phosphorylated MLC over thrombin-stimulated controls. Transfection with the EC MLCKATPdel mutant did not significantly alter basal levels of MLC phosphorylation. However, EC MLCKATPdel transfection produced ~80% inhibition of the thrombin-stimulated MLC phosphorylation response at $Ser^{19}$/$Thr^{18}$ suggesting this construct functions as a dominant negative MLCK mutant (Fig. 1C).

### Effect of the EC MLCK1745 Mutant on Stress Fiber Formation

We next examined the effect of EC MLCK mutants on fibroblast stress fiber organization by transiently transfecting EC MLCK mutants into CV1 cells, and examining the levels of polymerized actin and MLC phosphorylation. Compared to the neighboring untransfected cells or empty vector transfected cells (Fig. 2A), cells expressing EC MLCK1745 identified by V5 immunostaining showed enhanced stress fiber formation in a highly organized pattern (Fig. 2B). EC MLCK1745 localization was predominantly confined to thick stress fibers with marginal staining in the cell periphery with cortical actin filaments and microspikes. To evaluate the effect of mutant expression on MLC phosphorylation, transfected cells were co-stained with diphospho MLC antibody and with rhodamine phalloidin to stain F-actin stress fiber pattern (Fig. 2C,F). EC MLCK1745 transfection produced a substantial increase in basal level MLC phosphorylation as compared to empty vector transfected cells (Fig. 2C compared to H), which correlated with enhanced organized stress fibers observed by actin staining (Fig. 2I). This suggests that EC MLCK1745 functions as a putative constitutive active EC MLCK mutant to induce organized stress fiber assembly in fibroblasts.

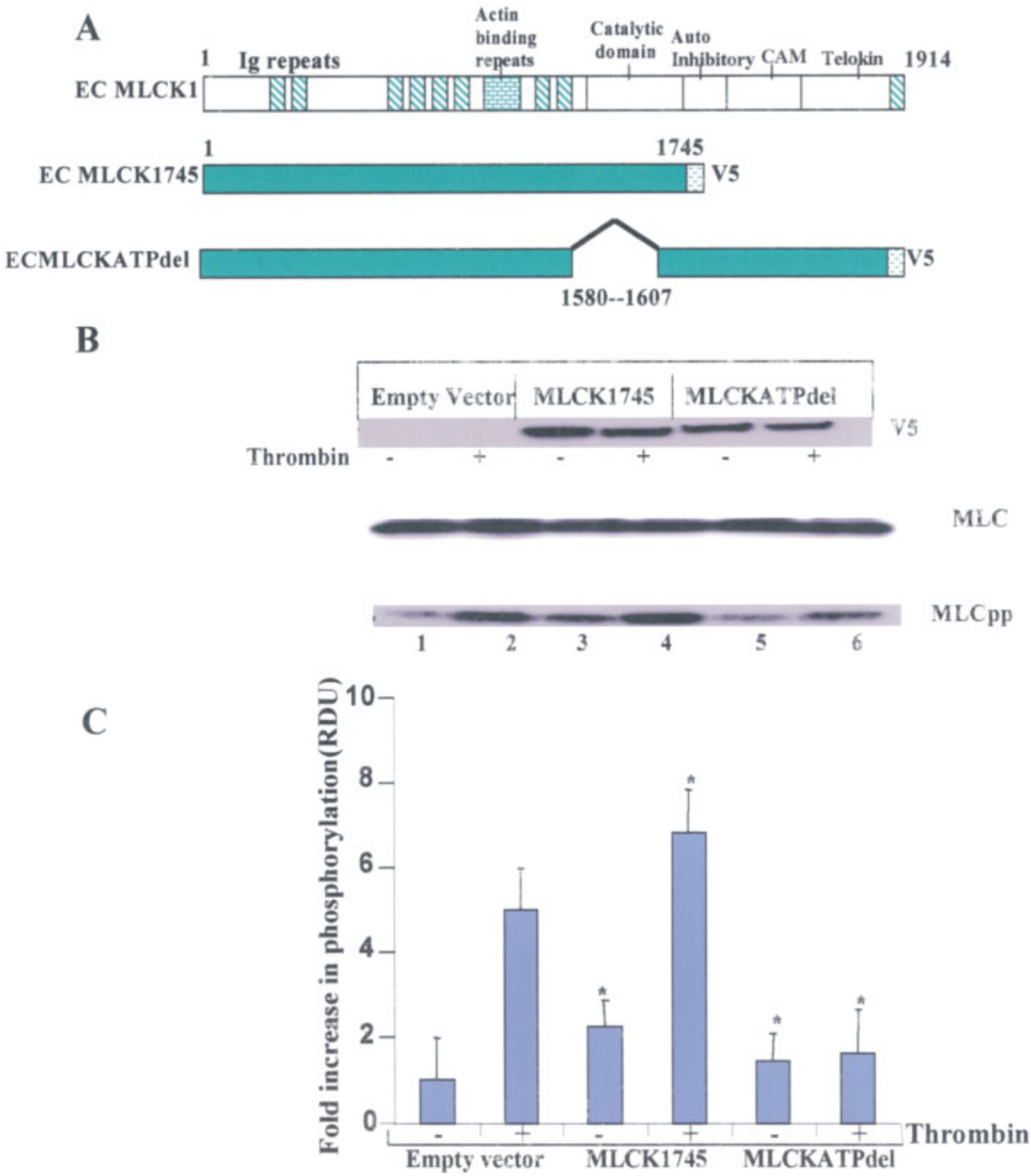


**Fig. 1.** Effect of EC MLCK mutants on CV1 fibroblast MLC phosphorylation. **A**: Schematic map showing the constructions of EC MLCK1745 and EC MLCKATPdel mutants. Using EC MLCK full-length cDNA as a template for PCR analysis, MLCK1745 and MLCKATPdel deletion mutants were synthesized and cloned into TOPO expression vector with CMV promoter and transfected into CV1 fibroblasts using FuGene method. **B**: MLC phosphorylation in mutant overexpressing cells. Total extracts from transfected cells were treated with and without human thrombin (100 ng/ml), separated on SDS–PAGE, Western blotted, and immuno-stained using V5, MLC-specific monoclonal, and diphosphorylated MLC-specific antibodies. **C**: Fold change in MLC phosphorylation in mutant overexpressing cells. MLCK mutant transfected cells were Western blotted and immunostained in more than three different experiments and quantitated by densitometric scanning. For statistical analysis, least significant difference multiple-range test was applied and randomized one way analysis of variants were determined, $P < 0.05$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

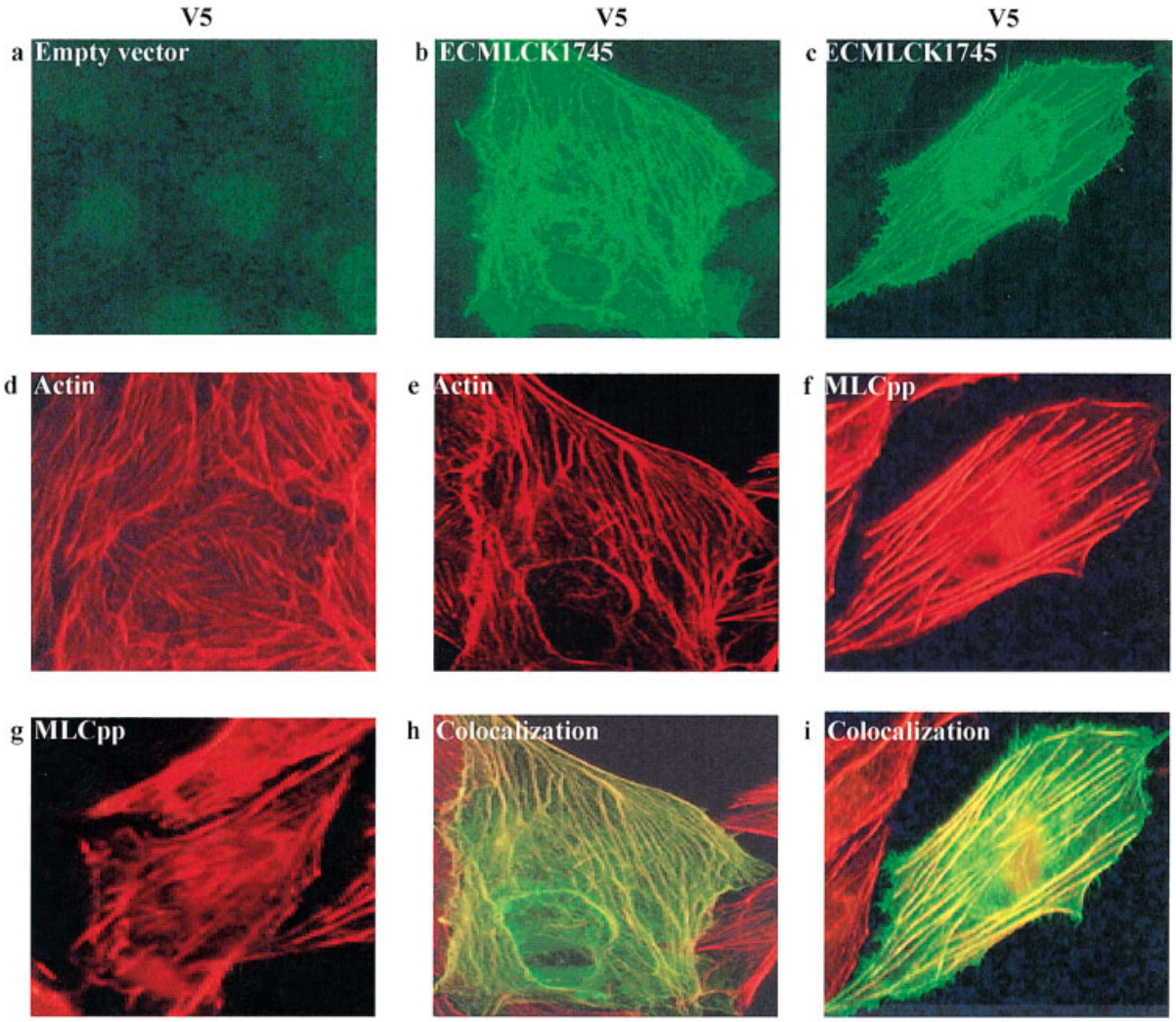


**Fig. 2.** Effect of EC MLCK1745 on stress fiber organization in CV1 cells. CV1 cells 48 h after transfection were fixed and permeabilized for immunostaining. Transfected cells with empty vector and MLCK1745 were identified using V5 epitope immunostaining (**A**, **B**, and **C**) and with MLC di-phospho antibody (**F** and **G**). The morphology of stress fibers was determined by phalloidin staining (**D** and **E**). Association of MLCK1745 with stress fibers was compared in (**B** and **C**) with MLC phosphorylation in (**F** and **I**) respectively. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

### Effect of Calmodulin Antagonist on Mut1745 Induced Stress Fiber Organization

To confirm the predicted calmodulin independence of MLCK1745, we initially compared the expression pattern of the constitutively active EC MLCK1745 kinase with a GFP-tagged full length MLCK protein. In unstimulated fibroblasts, GFPMLCK2 over-expression alone induced stress fiber formation (Dudek and Garcia, unpublished data), which was abolished after treatment with the CaM antagonist, trifluoperazine (TFP), which we have shown to inhibit endogenous MLCK activity (10). Empty vector-transfected CV1 cells (Fig. 3A) stimulated with thrombin demonstrated organized actin filaments, which were also inhibited by TFP pretreatment (Fig. 3A). Thrombin treatment increased organized stress fibers in both empty vector- and GFPMLCK2-transfected cells (Fig. 3A,B), which was completely attenuated by TFP (compare Fig. 3A(c) to 3B(f and g)). These results confirm that native and GFP-MLCK2-induced stress fiber assembly occurs in a calmodulin-dependent manner.

We next defined the calmodulin independent function of the MLCK1745 mutant compared to the endogenous MLCK kinase. Similar

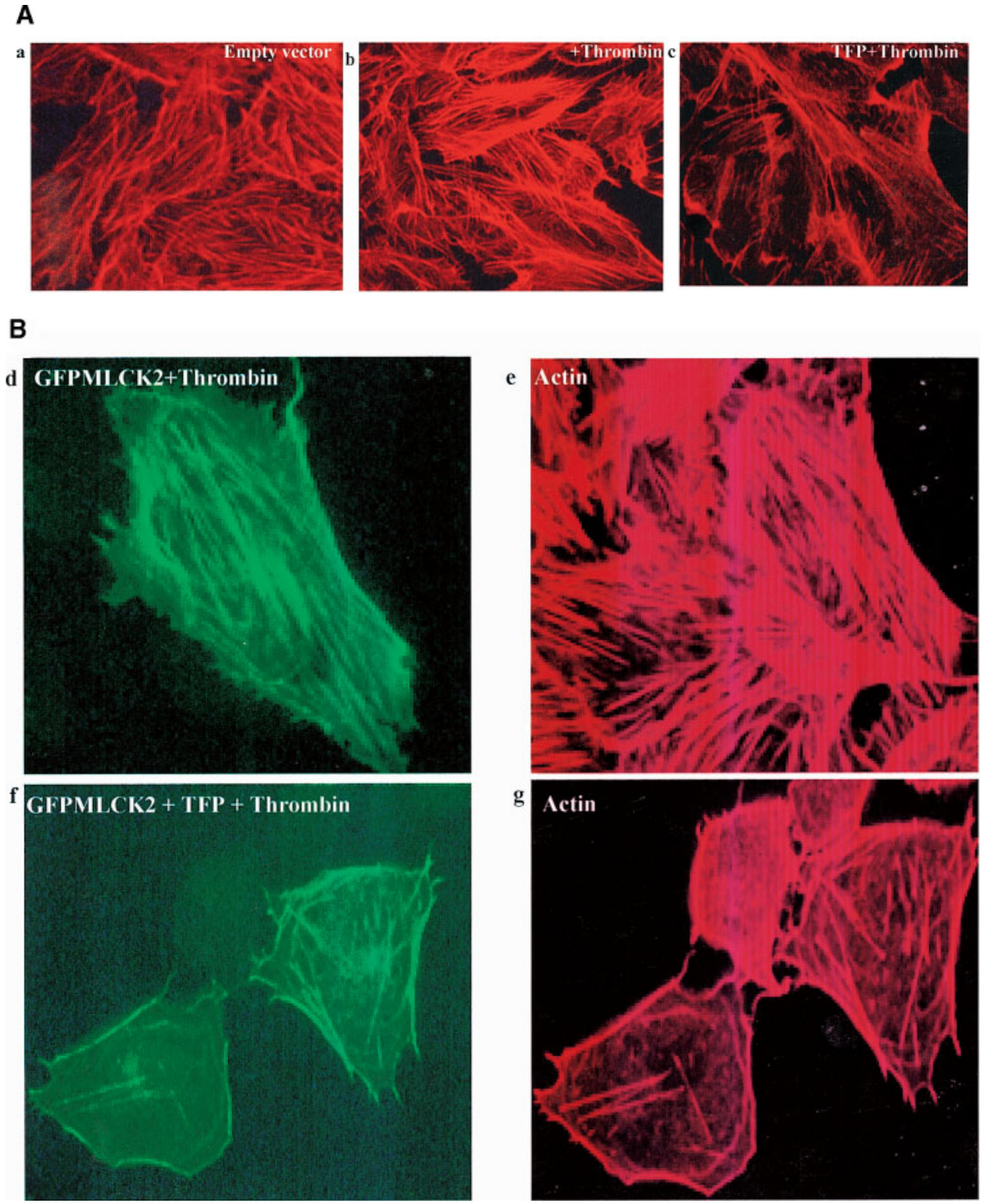


**Fig. 3.** Effect of $Ca^{2+}$/calmodulin antagonism on MLCK1745-induced stress fibers. **A**: Empty vector transfected cells treated with trifluoperazine (TFP, 25 μM) for 1 h and subjected to thrombin treatment, morphology of stress fibers was determined by phalloidin staining as shown in (**a**, **b**, **c**). **B**: Effect of TFP on GFPMLCK2 induced stress fiber organization. GFPMLCK2 transfected cells 48 h after transfection were treated with TFP and thrombin. GFPMLCK2 induced stress fibers were inhibited by TFP (compare **e** and **g**), GFPMLCK2 expressing cells incubated with TFP and further treated with thrombin did not show significant increase in stress fibers. **C**: Effect of TFP treatment on EC MLCK1745 induced stress fibers. EC MLCK1745 transfected cells treated with TFP and stained for V5 antibody compared with actin (**h** and **i**). TFP treatment does not attenuate the stress fiber assembly in transfected cells. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

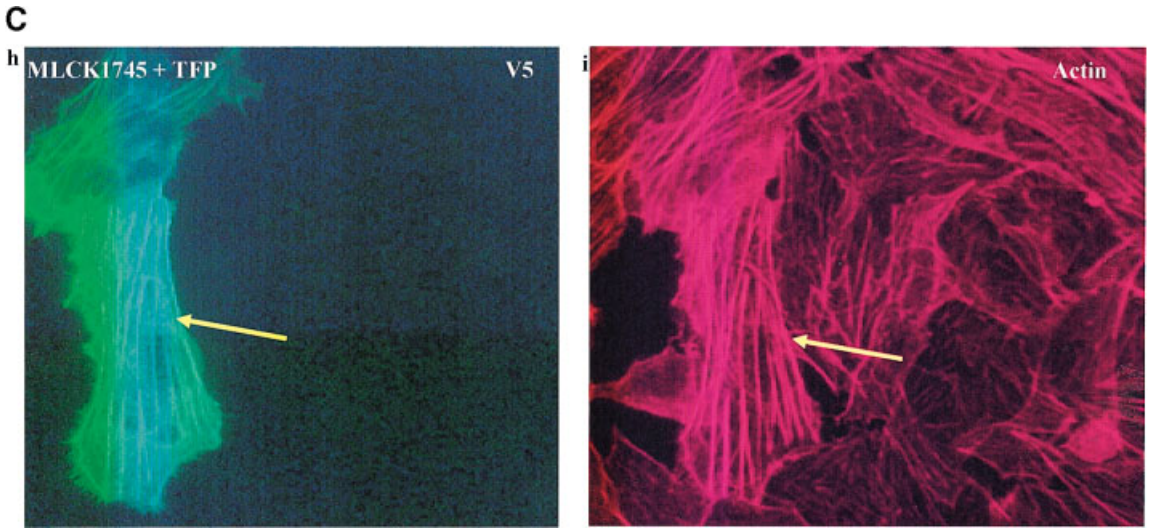


**Fig. 3.** *(Continued)*

TFP treatment (25 μM) in MLCK1745 overexpressing cells did not alter stress fiber formation, indicating that TFP inhibits endogenous MLCK without altering the stress fiber assembly induced by MLCK1745 (Fig. 3C). Furthermore, TFP treatment inhibited the thrombin-induced stress fibers in untransfected but not in EC MLCK1745 transfected cells (data not shown). These data confirm that EC MLCK1745-induced stress fiber assembly occurs independently of calmodulin binding to MLCK.

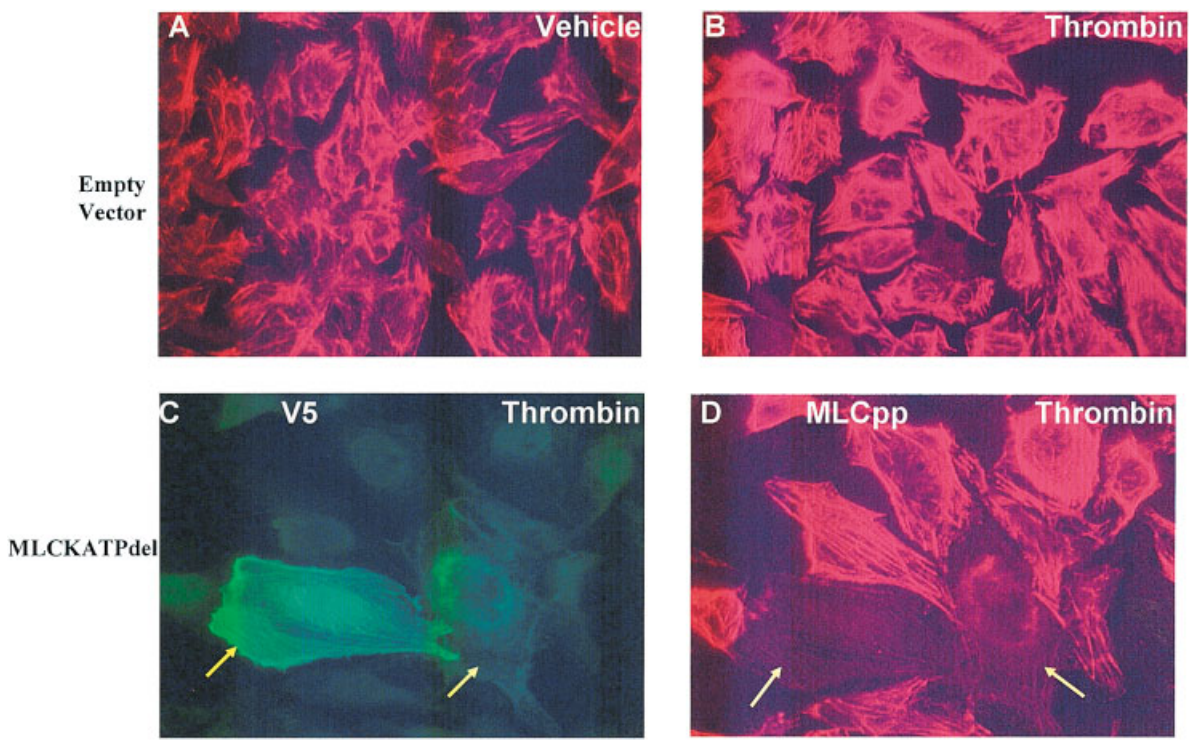


**Fig. 4.** Inhibition of MLC phosphorylation after EC MLCKATPdel overexpression. CV1 cells transfected with EC MLCKATPdel were treated with thrombin, fixed and permeabilized for immunostaining using V5 and MLC-di-phospho antibody. MLCKATPdel transfected cells are shown by arrow.

### Spatial Localization of EC MLCKATPdel After Agonist Treatment and MLC Phosphorylation

To evaluate the effect of EC MLCKATPdel mutant expression on MLC phosphorylation, empty vector and EC MLCKATPdel mutant transfected CV1 cells were co-stained with diphospho-MLC and V5 antibodies. The organized pattern of stress fibers and V5 staining noted with EC MLCK1745 was not observed in MLCKATPdel-transfected cells but rather diffuse epitope staining was observed throughout the cytoplasm (Fig. 4). CV1 cells transfected with these mutants showed significant differences in their morphology with MLCKATPdel mutant cells appearing rounded particularly when compared to MLCK1745 transfected cells, which showed spread morphology (Figs. 2D and 4C). Compared to empty vector controls, MLCKATPdel transfected cells stimulated with thrombin demonstrated complete inhibition of MLC phosphorylation and absence of actin stress fibers indicating that MLCK activity and MLC phosphorylation are necessary for stress fiber organization (Fig. 4) and both processes are abolished by the ATPdel dominant-negative MLCK mutant.

### Effect of EC MLCK Mutant Overexpression on CV1 Fibroblast Proliferation

The effects of changes in MLC phosphorylation by EC MLCK1745 and EC MLCKATPdel on cell growth and division were studied by measuring thymidine incorporation assay (Fig. 5) and by metabolic activity (Fig. 6). Quiescent CV1 fibroblasts transfected with EC MLCK1745 and exposed to 10% serum showed significant augmentation (~20% increase) in thymidine incorporation when compared to either empty vector-transfected or non-transfected control cells. In contrast, EC MLCKATPdel transfected cells produced ~33% inhibition in thymidine incorporation compared to serum stimulated empty vector and control cells. Finally, studies examining cell growth reflected by metabolic activity of growing cells were measured by alamarBlue assay and revealed an increased growth rate in EC MLCK1745 transfected cells (Fig. 6). These results suggest that MLCK-induced MLC phosphorylation participates in regulation of CV1 fibroblast metabolic activity and cell growth with this regulation modulated by targeted deletion, within the functional domains of the enzyme.

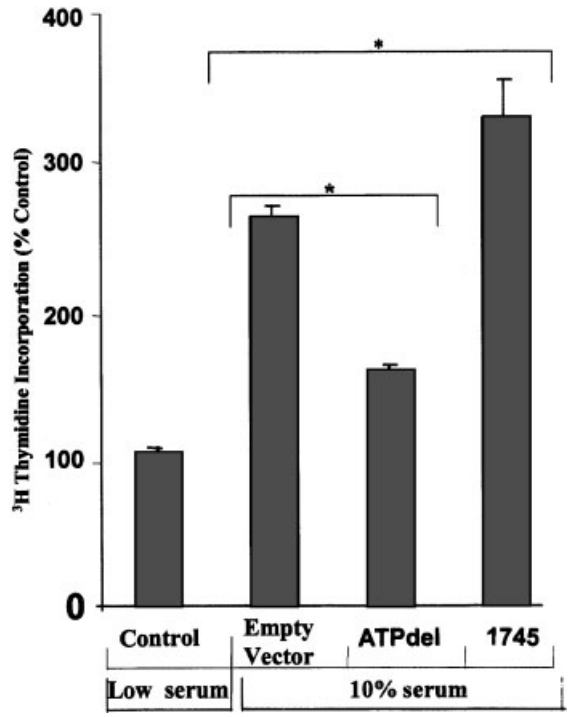


**Fig. 5.** Effect of MLCK mutants on CV1 growth and metabolic activities. CV1 cells transfected with empty vector, EC MLCK1745, EC MLCKATPdel, after 48 h, serum starved for 24 h, and induced with high serum containing $^3$[H] thymidine for 16 h. Cell extracts washed with TCA counted for thymidine incorporation. Transfection studies and thymidine incorporation were repeated atleast four times. Error bars are derived from three different experiments. For statistical analysis, least significant difference multiple-range test was applied and randomized one way analysis of variants were determined, $P < 0.05\%$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com]

## DISCUSSION

In non-muscle cells, phosphorylation of MLC at Ser$^{19}$ on myosin II correlates with activation of myosin ATPase, stress fiber formation, capping of cell surface receptors, and cytokinesis [Majercik and Bourguignon, 1988; Yamakita et al., 1994; Goeckeler and Wysolmerski, 1995; DeBiasio et al., 1996]. However, the role of MLCK in these processes remains largely uncharacterized. In fibroblasts, inhibition of MLCK by microinjection of MLCK specific antibodies induces stress fiber disassembly and MLC dephosphorylation [Lamb et al., 1988]. Reduction in MLCK expression via antisense techniques produces fibroblast cell rounding and decreased proliferation [Shoemaker et al., 1990] and attenuates chemoattractant-stimulated cell locomotion [Walker et al., 1998; Kishi et al., 2000]. These observations directly

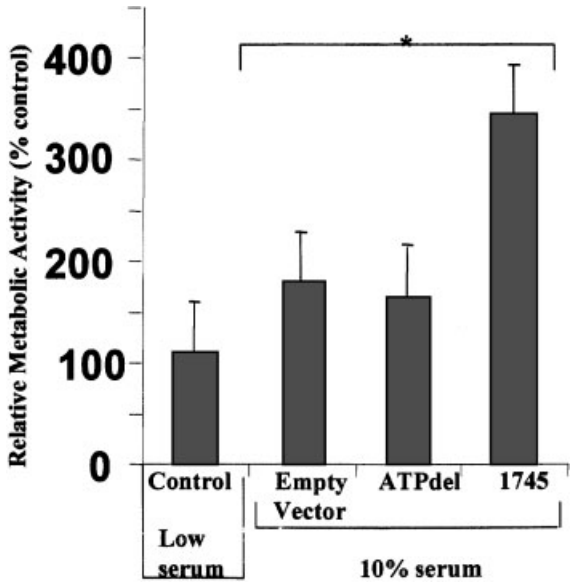


**Fig. 6.** Metabolic activity in MLCK mutant expressing cells. Empty vector, EC MLCK1745 and EC MLCKATPdel transfected cells serum starved for 16 h and induced with high serum complete CV1 medium for 4 h. Complete medium was replaced by alamarblue reagent and cells were incubated at 37°C for 2 h. Total change in optical density (OD) of oxidized and reduced form of the reagents was measured at 540 and 620 OD. The 540/620 OD ratio was considered as a metabolic activity in growing cells. Error bars are derived from three different experiments. For statistical analysis, least significant difference multiple-range test was applied and randomized one way analysis of variants were determined, $P < 0.05\%$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com]

implicate MLCK in the signaling pathways that control the non-muscle cell motility and cell morphology.

Studies of MLCK activation have been largely restricted to evaluation of the smooth muscle MLCK isoform one of the three gene products of MYLCK [Shoemaker et al., 1990]. In the presence of $Ca^{2+}$, calmodulin binds to the calmodulin-binding sequence in the regulatory subunit region of MLCK and plays an important role in $Ca^{2+}$-dependent MLCK activation. In the stationary state, the regulatory segment of MLCK folds back on the catalytic core to render the kinase inactive. However, following $Ca^{2+}$ influx, the regulatory segment is subsequently displaced from the catalytic site and calmodulin collapses at a position near the end but adjacent to the catalytic core [Kamm and Stull, 1985]. The exposed catalytic site of the kinase allows the N-terminus of MLC to bind with closure of the cleft for phosphorylation [Kamm and Stull, 2001]. The purpose of this study was to further understand the regulation of the novel high molecular weight EC MLCK isoform, vis-a-vis the $Ca^{2+}$/CaM regulatory segment, as we have shown EC MLCK to be homologous to smooth muscle MLCK in amino acids #922–1,914 but to exhibit altered CaM binding affinity [Birukov et al., 2001]. Non-stimulated cells transfected with a mutant kinase (EC MLCK1745) without the $Ca^{2+}$/CaM regulatory segment, myosin binding region, or the entire C-terminal domain demonstrated organized stress fibers and increased kinase activity, which were insensitive to calmodulin antagonist. These observations, combined with our in vitro phosphorylation studies, strongly suggest that MLCK1745 is a CaM-binding, independent, constitutively active kinase.

EC MLCK1745 was generated from the high molecular weight form of MLCK present in non-muscle and endothelial cells that contains five actin binding DXRXXL motifs which are required to localize the kinase to thin filaments, restricting compartmental movement [Poperechnaya et al., 2000]. For MLC phosphorylation, proximity of the kinase to the myosin filaments is required. A C-terminal low affinity myosin interacting segment in MLCK directs the kinase for myosin phosphorylation. We have shown that association of MLCK1745 was sufficient to induce MLC phosphorylation and stress fiber formation indicating, that the extreme C-terminal myosin binding is not an absolute requirement for kinase activity, MLC phosphorylation, or stress fiber formation. $Ca^{2+}$/calmodulin binding activates the kinase but does not dissociate MLCK from actin suggesting that association of MLCK with actin monomers could be an earlier step in force generation. Without disturbing the anchoring of MLCK to thin filaments, we deleted the calmodulin-binding site, with the resulting kinase acting independently of an increase in $Ca^{2+}$ signaling.

The role of MLCK in cell growth has been recently addressed [Fishkind et al., 1991; Poperechnaya et al., 2000]. Previous work on smooth muscle MLCK has shown that stop codon insertion between catalytic and regulatory domains generates a truncated, constitutively active kinase [Obara et al., 1995]. Expression of the smooth muscle truncated catalytic domain of MLCK (tMK) in NIH-3T3 cells increased MLC phosphorylation [Hecht et al., 1996; Cai et al., 1998], but inhibited NIH 3T3 cell growth and increased the doubling

time, results in conflict with our current report which showed an increased growth rate measured by thymidine incorporation as well as metabolic activity assay. A notable difference in these studies is the molecular structure of the EC MLCK1745, which compared to smooth muscle tMK, contains an additional N-terminal 922 amino acids sequence. Furthermore, the prior studies utilizing the smooth muscle tMK constructs were evaluated in stably transfected cells whereas our studies represent transient transfections and could complicate interpretation and comparison with our observations. The novel N-terminus in the EC MLCK1745 may have an important function in signaling pathways [Birukov et al., 2001], which does not inhibit the kinase activity but may be involved in binding, inducing the growth rate in fibroblast. Our future work in progress will focus on comparison of the tMK-like smooth muscle kinase mutant with EC MLCK1745 activity in cytoskeletal rearrangement and signaling events.

Finally, our results confirm the role of MLCK in non-muscle stress fiber formation utilizing a novel dominant-negative MLCK mutant. Transfection with the EC MLCKATPdel mutant did not significantly alter basal levels of MLC phosphorylation, however, EC MLCKATPdel transfection produced an ~80% inhibition of thrombin-stimulated MLC phosphorylation at $Ser^{19}/Thr^{18}$ suggesting that this construct inhibits agonist-induced MLCK activity. Furthermore, the EC MLCKATPdel reduced serum-induced fibroblast growth. Given the multifunctional role of EC MLCK in non-muscle cells such as endothelium and fibroblasts, this construct may prove to be of particular utility in exploring the role of EC MLCK in vascular pathobiology.

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