The N-terminus of the long MLCK induces a disruption in normal spindle morphology and metaphase arrest

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Summary
We have shown previously that only the long myosin light chain kinase (MLCK), which is the predominant MLCK isoform expressed in nonmuscle cells, localizes to the cleavage furrow. To further examine the in vivo localization of the long MLCK in HeLa cells and the mechanisms responsible for kinase targeting during the cell cycle, we examined the distribution of the endogenous kinase and constructed green fluorescent protein (GFP) fusions of long HeLa MLCK truncations. A GFP fusion containing the N-terminal IgG domain and the five DXR motifs localized to stress fibers during interphase and the cleavage furrow during mitosis. Although individual fusions of the five DXRs and IgG domain both independently localized to stress fibers, only the five DXRs demonstrated a cortical localization in mitotic cells. Thus, robust targeting of the long MLCK to the cleavage furrow required the five DXRs and additional sequences from the IgG domain. Expression of the IgG domain alone or with five DXRs increased the number of multinucleate cells tenfold, whereas expression of the five DXRs or GFP had no effect. Furthermore, expression of the IgG domain alone or with five DXRs disrupted normal spindle morphology during mitosis. Extended astral microtubules and increased bundling of kinetochore microtubules, and spindle pole fragmentation were detected in mitotic cells. These microtubule defects were associated with abnormalities in metaphase chromosome alignment and a subsequent metaphase arrest caused by activation of the spindle assembly checkpoint at the kinetochores of mono-oriented chromosomes. Together, these results suggest that MLCK has an unexpected regulatory function during mitosis.

Key words: Myosin light chain kinase, Cell division, Mitosis, Metaphase arrest, Microtubules

Introduction
Biochemical studies have shown that phosphorylation on Ser-19 of the regulatory light chain (RLC) of myosin-II takes place late in mitosis (Satterwhite et al., 1992; Yamakita et al., 1994), and studies using a myosin-II biosensor that monitors Ser-19 phosphorylation (DeBiasio et al., 1996) and Ser-19 phosphoepitope antibodies (Matsumura et al., 1998) confirmed these early observations. More recently, the functional significance of this phosphorylation event has been addressed. In Drosophila, germ-line cells expressing an RLC with alanine substitutions at Ser-21 and Thr-20 (equivalent to Ser-19 and Thr-18) are deficient in cytokinesis (Jordan and Karess, 1997). In mammalian cells, expression of an RLC with alanine substitutions at Ser-19 and Thr-18 induces multiple defects during mitosis (Komatsu et al., 2000). These in vivo studies emphasize the importance of Ser-19 phosphorylation during mitosis and indicate that activation of the myosin-II motor is an important regulatory step in cell division.

Myosin light chain kinase (MLCK) is a leading candidate for mediating myosin-II phosphorylation during mitosis because it is a dedicated, Ca2+/calmodulin-regulated kinase that exclusively phosphorylates Thr-18 and Ser-19 on the regulatory light chain of myosin-II. Our previous biochemical studies demonstrated that the activity of the long MLCK is cell cycle regulated, with a twofold decrease in specific activity during metaphase (Popeneschnaya et al., 2000). In addition, inhibition of MLCK expression via antisense techniques causes cell rounding and decreased proliferation (Shoemaker et al., 1990), suggesting that MLCK participates in the signaling pathways that mediate cell division. Moreover, MLCK-mediated phosphorylation of Ser-19 has been implicated in regulating a wide array of cellular functions (Kamm and Stull, 2001).

In vertebrates, the smooth/nonmuscle MLCK gene locus produces two MLCK isoforms: a high-molecular-weight MLCK (long MLCK) and a low-molecular-weight MLCK (short MLCK), which have molecular masses of ~210 kDa and 108-125 kDa, respectively. The long MLCK is identical to the short MLCK except for a unique N-terminal extension of ~900 residues (Garcia et al., 1997; Watterson et al., 1995). Both the long and short MLCKs are expressed in several smooth and nonmuscle tissues but the long MLCK is the predominant isoform expressed in cultured cells (Blue et al., 2002). In vitro, the long and short MLCKs have comparable $K_m$ and $V_{max}$ values (Birukov et al., 2001), indicating that the N-terminal extension is not required for catalytic activity.

Sequence analysis of the long and short MLCKs indicates that they have several distinct structural motifs. The short MLCK contains three immunoglobulin (Ig) motifs, one Fn motif (a fibronectin-like module), a PEVK-rich repeat region and three repeats of DFRXXXL on the N-terminus. The N-terminal extension of the long MLCK contains six additional Ig motifs followed by two additional repeats of DVRXXXL and DFRXXL. The DFRXXXL and DVRXXXL sequences, which we
generally refer to as DXR motifs, have been shown to mediate the interaction of MLCK with F-actin (Smith et al., 2002; Smith and Stull, 2000; Smith et al., 1999), whereas the extreme C-terminal Ig motif in the long and short MLCKs serves as a myosin-II binding module that interacts with the head-tail junction of the myosin heavy chain (Shirinsky et al., 1993; Silver et al., 1997). However, the functions of the other structural modules, including the six Ig motifs in the N-terminal extension, remain to be elucidated.

To examine further the mechanisms responsible for kinase targeting during the cell cycle, we evaluated the distribution of the endogenous kinase and constructed green fluorescent protein (GFP) fusions of HeLa long MLCK truncations. Our observations suggest that targeting of the long MLCK to the cleavage furrow is mediated primarily by the five DXRs but additional sequences from the N-terminal Ig motifs increase the affinity of the kinase for the contractile ring. To address the contribution of MLCK to mitotic progression, we examined the effects of expression of N-terminal fragments of the kinase on cell division. Live cell imaging of transfected HeLa cells showed that expression of N-terminal fragments of the long MLCK induced a metaphase arrest, which was associated with abnormal spindle morphology as well as spindle pole fragmentation. These microtubule defects were coupled with abnormalities in metaphase chromosome alignment and activation of the spindle assembly checkpoint at the kinetochores of mono-oriented chromosomes. Altogether, our results suggest that MLCK has an unexpected regulatory function during mitosis.

Materials and Methods

Cell culture

HeLa cells were grown as monolayer cultures at 37°C in a humidified atmosphere of 5% CO2 in DMEM containing 10% fetal bovine serum (FBS).

GFP fusion constructs

The HeLa long MLCK (accession number AY339601) was amplified from a HeLa Marathon-Ready cDNA library (Clontech) using forward and reverse primers of 5'-GGGGATGTTAG-AGCTGTTGTGGCTCTGCA-3' and 5'-GACCTAAACCGATAATC-TATCACACTAGGTGCTT-3', respectively. High fidelity PCR (Invitrogen) produced a PCR product of the expected length (~5870 nucleotides), which was subcloned into pCR2.1-TOPO (Invitrogen) for sequencing and subsequent analysis.

To prepare GFP fusions of the HeLa IgG domain (residues 2-867) or the IgG domain containing all five DXR motifs (residues 2-1024), nucleotides 4-2601 or 4-3072 were amplified from the full-length long MLCK in pCR2.1-TOPO and subcloned into the EcoRI/KpnI sites of pEGFP-C2 (Clontech), respectively. Using the Quick Change XL site-directed mutagenesis kit (Stratagene), E1626 was substituted with K to create a HeLa kinase-dead MLCK (nucleotides 4-5714) with GFP at the N-terminus (Invitrogen) for centrifugation at 16,000 g for 15 minutes. The supernatant was applied to a glutathione-Sepharose column (Amersham Pharmacia Biotech) equilibrated in Cleavage Buffer (20 mM Tris-HCl pH 8.0, 200 mM KCl, 300 mM NaCl, 1% NP-40, 2 mM EDTA, 2 mM EGTA, 5 mM DTT, 10% sucrose, 0.02% NaN3) and loaded onto a MonoQ column equilibrated in Buffer A (20 mM Tris pH 7.5, 50 mM NaCl, 1 mM DTT, 10% sucrose, 0.02% NaN3). The column was washed with 20 column volumes of Buffer A and developed with a 20 column volume linear gradient of 50-600 mM NaCl in Buffer A. Purified IgG domain was dialysed and loaded onto a MonoQ column equilibrated in Buffer A (20 mM Tris pH 7.5, 50 mM NaCl, 1 mM DTT, 10% sucrose, 0.02% NaN3). The purified recombinant IgG domain contains seven additional amino acids at the N-terminus (Gly-Pro-Leu-Glu-Ser-Pro-Glu-Phe). The molecular weight of the recombinant IgG domain was confirmed by mass spectrometry.

Antibodies

Rabbit polyclonal antibodies to the HeLa MLCK IgG domain were prepared using the recombinant HeLa IgG domain and standard methods (Covance). The IgG domain antibodies were purified by affinity chromatography on an Aminolink column (Pierce) coupled with the HeLa IgG domain. The specificity of the IgG domain antibodies was assessed by immunoblot and immunoprecipitation analysis using HeLa cell lysates and the purified recombinant IgG domain as a positive control.

The MLCK monoclonal antibody (clone K36) and β-tubulin monoclonal antibody (clone tub 2.1) were purchased from Sigma. The full-length polyclonal antibody against GFP was from Clontech. The human anti-centromere-protein polyclonal antibody (autoimmune serum ACA) was from Antibodies Incorporated. The CENP-E rabbit polyclonal antibody was a kind gift from D. Cleveland (University of
immunofluorescence
For MLCK immunofluorescence, cells were fixed for 10 minutes in 3.7% formaldehyde in cytoskeleton stabilization buffer (137 mM NaCl, 5 mM KCl, 1.1 mM NaH2PO4, 0.4 mM KH2PO4, 2 mM MgCl2, 2 mM EGTA, 5 mM PIPES, and 5.5 mM glucose, pH 6.1) (Small, 1981) at room temperature, rinsed with stabilization buffer and permeabilized for 20 minutes at room temperature in 0.5% Triton X-100 in stabilization buffer. The cells were blocked for 20 minutes with 1% bovine serum albumin (BSA) in PBS and then incubated with affinity-purified MLCK IgG domain antibodies at 7.5 μg ml−1 in PBS containing 1% BSA for 30 minutes at room temperature. After washing with 1% BSA in PBS, the cells were incubated with Alexa-546-conjugated goat anti-rabbit antibodies (Molecular Probes) containing 1% BSA for 30 minutes at room temperature. The cells were rinsed with 1% BSA in PBS and mounted using Pro-Long Anti-Fade (Molecular Probes).

HeLa cells were transfected with the MLCK-GFP constructs using lipofectin (Invitrogen) or polyfect (Qiagen). Transfected cells were fixed 26-38 hours after transfection in freshly prepared 4% formaldehyde in PBS for 15 minutes at room temperature. After rinsing the cells in PBS containing 0.02% sodium azide, the coverslips were mounted in Pro-Long Antifade (Molecular Probes). Transfected HeLa cells were permeabilized as follows. Briefly, cells were cooled to 4°C, washed with ice-cold PBS and extracted for 30-90 seconds with ice-cold 10 mM Tris 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 pH 7.0, 30 mM KCl, 5 mM MgCl2, 1 μM CaCl2, and fixed and mounted as described above.

ECL chemiluminescence detection system (Amersham Pharmacia Biotech). To evaluate the expression levels of the MLCK-GFP fusions, the intensity of the GFP signal was compared with a standard curve of purified recombinant GFP run on the same gel. The level of GFP-fusion protein expression was quantified by densitometry using ImageQuant software. The MLCK-GFP fusions constituted 0.27-0.59% of the total cellular protein (kinase-dead MLCK, 0.37%; IgG domain + five DXRs, 0.59%; IgG domain, 0.36%; five DXRs, 0.45%; GFP, 0.27%, respectively) compared with myosin-II, which comprises approximately 1-1.5% of total cellular protein (Honer et al., 1988). Consistently, we found that expression of GFP and the MLCK-GFP fusions did not affect the expression of β-tubulin or GFP. Immunoreactive proteins were detected using the ECL chemiluminescent detection system (Amersham Pharmacia Biotech). To evaluate the expression levels of the MLCK-GFP fusions, the intensity of the GFP signal was compared with a standard curve of purified recombinant GFP run on the same gel. The level of GFP-fusion protein expression was quantified by densitometry using ImageQuant software. The MLCK-GFP fusions constituted 0.27-0.59% of the total cellular protein (kinase-dead MLCK, 0.37%; IgG domain + five DXRs, 0.59%; IgG domain, 0.36%; five DXRs, 0.45%; GFP, 0.27%, respectively) compared with myosin-II, which comprises approximately 1-1.5% of total cellular protein (Honer et al., 1988). Consistently, we found that expression of GFP and the MLCK-GFP fusions did not affect the expression of β-tubulin in these cells (Fig. 2B, bottom).

Microscopy and live cell imaging
Images were acquired using IPLab Spectrum software (Scanalytics) with a CoolSNAP HQ interline 12-bit, cooled CCD camera (Roper Scientific) mounted on an Olympus IX70 microscope with a PlanApo 60x, 1.4 N.A. objective (Olympus) and HQ bandpass filters (Chroma Technology Corp). IPLAB images were deconvolved with Vastek Microtome software. Images were processed using Photoshop (Adobe Systems).

For live cell studies, transfected HeLa cells were imaged beginning at 26-38 hours after transfection. Mitotic, transfected cells in metaphase were identified by their GFP fluorescence and the alignment of the chromosomes at the cell equator, and were followed from metaphase until late cytokinesis. Anaphase onset was usually detectable within 5-30 minutes. For clarity, the time was set to zero in the frame 1 minute before anaphase onset was observed. For arrested cells, each cell was imaged for approximately 3 hours.

During video microscopy, the cells were maintained at 37°C in phenol-red-free DMEM containing 10% FBS and penicillin/streptomycin with a PDM-2 stage micro-incubator (Harvard Apparatus) and the pH maintained by gassing with 5% CO2. Evaporation was prevented by overlaying the medium with mineral oil. Under these conditions, we observed normal cytokinetic events in control cells for up to 8 hours. Phase images were acquired every 30 seconds using IPLAB software (Scanalytics).

Immunoblot, immunoprecipitation and kinase activity assays
For immunoblots, approximately 30 hours after transfection, cells were washed twice with chilled PBS and lysed in RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS). Whole cell lysates expressing the GFP-kinase-dead MLCK, truncated MLCK-GFP fusions or GFP alone were clarified by centrifugation at 4°C for 10 minutes at 14,000 g and protein concentrations determined using the Bio-Rad DC protein assay. 25 μg of each lysate was separated on 8% or 12% SDS-PAGE (Laemmli, 1970). Proteins were transferred to nitrocellulose membranes and reacted with antibodies to MLCK, β-tubulin or GFP. Immunoreactive proteins were detected using the ECL chemiluminescent detection system (Amersham Pharmacia Biotech). To evaluate the expression levels of the MLCK-GFP fusions, the intensity of the GFP signal was compared with a standard curve of purified recombinant GFP run on the same gel. The level of GFP-fusion protein expression was quantified by densitometry using ImageQuant software. The MLCK-GFP fusions constituted 0.27-0.59% of the total cellular protein (kinase-dead MLCK, 0.37%; IgG domain + five DXRs, 0.59%; IgG domain, 0.36%; five DXRs, 0.45%; GFP, 0.27%, respectively) compared with myosin-II, which comprises approximately 1-1.5% of total cellular protein (Honer et al., 1988). Consistently, we found that expression of GFP and the MLCK-GFP fusions did not affect the expression of β-tubulin in these cells (Fig. 2B, bottom).

For immunoprecipitation of the wild-type MLCK or kinase-dead MLCK-GFP fusions, transiently transfected cells were washed twice with PBS and resuspended in a lysis buffer composed of 50 mM Tris-HCl pH 8.2, 400 mM NaCl, 0.5% Triton X-100, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM DTT, 1 mM PMSF and 50 μg ml−1 each of chymostatin, leupeptin and pepstatin A. After a 20 minute incubation on ice, the lysate was clarified by centrifugation at 4°C for 15 minutes at 16,000 g. The supernatant was diluted 1:3 in 20 mM Tris-HCl pH 7.5, 0.5 mM Trition X-100, 0.5 mM EDTA, 1 mM DTT and 1 mM PMSF with protease inhibitors as described above. Before the immunoprecipitation, GFP polyclonal antibodies were bound to protein-A/Sepharose in PBS containing 1 mg ml−1 BSA. The diluted supernatant was incubated with the antibody/protein-A/Sepharose for 2 hours at 4°C. Immune complexes were collected by centrifugation and washed five times with 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mM magnesium acetate and 0.5 mM DTT. The Ca2+/calmodulin-dependent kinase activity of the wild-type and kinase-dead MLCK GFP fusions was evaluated by measuring 32P incorporation into...
nonmuscle RLCs as described in Poperechnaya et al. (Poperechnaya et al., 2000).

Results
Distribution of the endogenous HeLa long MLCK
To examine the distribution of the endogenous long MLCK, we fixed and stained untransfected HeLa cells with antibodies against the IgG domain of the HeLa long MLCK. The antibody recognizes a single polypeptide of ~210 kDa on immunoblots of HeLa cell lysates (Fig. 1A) and immunoprecipitates a single 210 kDa band from whole cell homogenates of HeLa cells (data not shown). In conventional fluorescence micrographs, the endogenous MLCK had a punctate appearance, localized to the cell cortex and cell equator during anaphase, and concentrated in the cleavage furrow through telophase (Fig. 1B, left). This distribution of the endogenous MLCK was even more apparent in deconvolved images of conventional fluorescence micrographs (Fig. 1B, right). Enrichment of the endogenous kinase in the cell cortex during metaphase could be detected only in deconvolved images, possibly because of the limited amount of MLCK in the cortex of metaphase cells.

Fig. 1. Distribution of endogenous long MLCK during cell division.
(A) Immunoblots of HeLa whole cell lysates. Lane 1, incubation with an MLCK monoclonal antibody and lane 2, incubation with a HeLa MLCK IgG domain polyclonal antibody. Molecular weight standards are indicated in kDa. (B) Conventional fluorescence micrographs (left) and deconvolved images (right) of dividing HeLa cells stained with the HeLa MLCK IgG domain antibody. Scale bar, 10 μm. The endogenous long MLCK localizes to the cell cortex (arrows).

Targeting of the long MLCK to actin-containing structures
The N-terminal half of the long MLCK contains six C2-type Ig motifs (termed the IgG domain) followed by five DXR motifs (Fig. 2A). Our previous studies with the avian long MLCK demonstrated that a GFP fusion composed of the IgG domain and five DXRs localizes to stress fibers and the cleavage furrow, and displays a localization that is indistinguishable from the full-length long MLCK (Poperechnaya et al., 2000). To extend these studies and to examine the determinants responsible for targeting the long MLCK to the cleavage furrow, we cloned the long MLCK from HeLa cells. The unique portions of the chicken and HeLa long MLCKs (922 and 934 amino acids long, respectively), which are composed of six Ig and two DXR motifs, share only 57% identity. The HeLa long MLCK cDNA, which was amplified from a HeLa library, is 99.93% identical to the HUVEC MLCK (Garcia et al., 1997), with only four nucleotide changes. Three of these alterations are conservative, with no change in the amino acid sequence. The fourth nucleotide substitution results in the loss of a BamHI site (beginning at nucleotide 2042) and the conversion of Trp681 to a Cys.

We transiently expressed GFP-tagged human long MLCK truncations in HeLa cells to analyze their distribution in fixed cells. Immunoblot analysis with antibodies to GFP and MLCK demonstrated that the full-length GFP truncations as well as the full-length kinase-dead MLCK are expressed in HeLa cells (Fig. 2C). As anticipated, substitution of glutamic acid 1626 with a lysine resulted in a catalytically inactive kinase, because immunoprecipitates of the kinase-dead MLCK did not display any detectable kinase activity in the presence of Ca2+/calmodulin (Fig. 2B).

The kinase-dead MLCK (data not shown) and the HeLa IgG domain with five DXRs primarily concentrated on stress fibers (Fig. 3A) and exhibited a localization that is identical to a GFP fusion of the full-length avian MLCK (Poperechnaya et al., 2000). Similarly, individual GFP fusions of the IgG domain and the five DXRs independently localize to stress fibers (Fig. 3A), confirming previous observations on the localization of GFP fusions of the avian domains (Smith et al., 2002). We also observed independent localization of the IgG domain and five DXRs to stress fibers when these domains were expressed in NIH 3T3 cells (data not shown). Following Triton permeabilization, which releases unbound, soluble protein, both the IgG domain and five DXRs remained associated with stress fibers, indicating relatively high-affinity binding to these structures. However, we observed significantly greater stress fiber binding for the IgG domain with five DXRs in permeabilized cells than for either domain alone, which is consistent with both domains mediating the targeting of MLCK to stress fibers. Interestingly, in an in vitro cosedimentation assay, we did not detect binding of the IgG domain to either actin or myosin-II (data not shown), suggesting that targeting of this domain involves at least partial association with a novel protein.

In mitotic cells, the HeLa IgG domain with five DXRs was enriched in the cell cortex during metaphase and concentrated in the cleavage furrow throughout anaphase and telophase (Fig. 3B), confirming our previous observations that the catalytic core is not required for kinase targeting during mitosis (Poperechnaya et al., 2000). By contrast, individual GFP fusions of the IgG domain and the five DXRs distributed
uniformly throughout the cytoplasm and were excluded from the cell cortex. The localization of these truncations was also assessed in Triton-permeabilized mitotic cells (Fig. 3C). Permeabilization of mitotic cells reduced the diffuse fluorescence around the chromosomes and revealed strong cortical and cleavage furrow localization only for the IgG domain plus five DXRs (Fig. 3C), whereas the IgG domain had a diffuse distribution that was similar to the distribution of GFP alone (Fig. 3C). Approximately half of the cells expressing the five DXRs displayed some fluorescence at the equatorial cortex (Fig. 3C, arrows). These observations suggest that targeting of the long MLCK to the cleavage furrow is primarily mediated by the five DXRs but that additional sequences from the IgG domain increase the affinity of the kinase for the contractile ring. Interestingly, only the IgG domain localized to the central region of the midbody (Fig. 3C, arrowhead), which is known to contain spindle and chromosome-derived proteins (Zeitlin and Sullivan, 2001), suggesting that the IgG domain might interact with one or more of the proteins found in the midbody.

Expression of the N-terminus of the long MLCK induces a mitotic arrest

Consistent with a role in targeting the long MLCK to the contractile ring, expression of GFP fusions of the HeLa IgG domain alone or with five DXRs caused an approximately tenfold increase in the number of multinucleate cells, whereas expression of the five DXRs or GFP had no effect (Fig. 4). Expression of MyBP-C or MyBP-H, which contain seven and two Ig-C2 motifs, respectively (Welikson and Fischman, 2002), also had no influence, indicating that the observed effects on cytokinesis are not due to some non-specific consequence of overexpressing a C2-type Ig motif. We observed that cells transfected with the HeLa IgG domain alone or with five DXRs had to be analysed within 34 hours of transfection (i.e. one division cycle), because expression of these MLCK domains for longer times resulted in extensive cell death (N. G. Dulyaninova et al., unpublished). Expression of MyBP-C or MyBP-H was not deleterious because cells expressing these constructs could still be analysed 48 hours after transfection or even later.

To determine which aspects of mitotic progression were affected by expression of the IgG domain or IgG domain plus five DXRs, we examined transiently transfected HeLa cells by video microscopy 26-38 hours after transfection. Transfected cells in metaphase were identified by their GFP fluorescence and the alignment of the chromosomes at the cell equator and were followed from metaphase until late cytokinesis. For untransfected cells or cells transfected with GFP, anaphase onset was usually detectable within 30 minutes, and we typically observed a transit time of 4-5 minutes from late metaphase to the onset of furrowing (n=22 cells) (Fig. 5A). In the case of a mitotic arrest, cells were imaged for approximately 3 hours, until a loss of chromosome alignment

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was observed or visible cell flattening occurred (Fig. 5C,E). 53% of the cells expressing the IgG domain plus five DXRs (19 of 36 cells) and 58% of the cells expressing the IgG domain (seven of 12 cells) displayed a similar transit time to control cells (Table 1, Fig. 5A,B,D). However, 47% of the cells expressing the IgG domain plus five DXRs (17 of 36), 42% of the cells expressing the IgG domain (five of 12) and 100% of the cells expressing the kinase-dead MLCK failed to divide (Table 1, Fig. 5B,D). Of particular note, in cells expressing the IgG domain, IgG domain plus five DXRs or the kinase-dead MLCK, the chromosomes congressed to the metaphase plate but chromosome separation was unsuccessful, resulting in metaphase arrest. In addition, the chromosomes often failed to maintain a stable equatorial alignment. In cells expressing the IgG domain or IgG domain plus five DXRs, the chromosomes usually began to lose their metaphase alignment within 1 hour of mitotic arrest, even though the chromatin remained condensed (Fig. 5E), whereas the loss of metaphase chromosome alignment in cells expressing the kinase-dead MLCK occurred within several minutes, and was often followed by extensive cell blebbing (Fig. 5F). In addition, we rarely observed cells expressing the kinase-dead MLCK in anaphase and telophase. For cells expressing the IgG domain or IgG domain plus five DXRs, we observed arrested cells for up to 4 hours and, within this time frame, the cells remained arrested in metaphase without normal progression through mitosis and cytokinesis. In some cases, after prolonged metaphase arrest, we observed cell flattening and mitotic exit without any evidence of anaphase chromosome movement or cytokinesis (Fig. 5E). A similar mitotic arrest was observed in cells expressing the avian IgG domain plus five DXRs (data not shown). Last, the more dramatic phenotype observed with the kinase-dead MLCK is not surprising, because the catalytically inactive kinase will both displace the endogenous wild-type MLCK and sequester potential substrate.

To examine the eventual fate of cells that displayed this mitotic arrest, we identified metaphase arrested cells at approximately 23 hours after transfection and periodically observed them for an additional 8-25 hours (31-48 hours after transfection) (Table 2). Sixteen percent of the cells expressing the IgG domain plus five DXRs (n=7 cells) eventually divided and another 20% (n=9 cells) had an interphase morphology with a binucleate nucleus. The remaining 64% of the cells (n=29 cells) appeared to undergo apoptosis, based on their shrunk appearance, the condensation of the

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**Fig. 3.** Distribution of MLCK-GFP truncations in interphase and mitotic cells. Fluorescence micrographs of HeLa cells transfected with the HeLa IgG domain plus five DXRs, the HeLa IgG domain, the rabbit five DXRs or GFP. Scale bars, 10 μm. (A) Representative images of intact (top) and Triton-permeabilized (bottom) interphase cells. (B) Representative images of cells in metaphase, early anaphase, late anaphase and telophase. (C) Representative images of Triton-permeabilized cells in anaphase and late telophase.
chromatin and nuclear fragmentation, which was readily apparent with the cell permeant DNA-staining fluorescent dye Hoechst 33342 (data not shown). A similar proportion of cells expressing the IgG domain divided, became multinucleate or underwent apoptosis: 15% \((n=4)\), 19% \((n=5)\) and 66% \((n=18)\), respectively (Table 2). The observation that approximately 20% of the arrested cells become multinucleated is consistent with our earlier findings that approximately 10% of the cells expressing the IgG domain or IgG domain plus five DXRs are multinucleate (Fig. 4). Interestingly, most (~80%) binucleate cells could not progress through the next division cycle and appeared to undergo apoptosis when observed at 48 hours after transfection.

Expression of the N-terminus of the long MLCK disrupts normal spindle morphology and affects chromosome alignment

To examine whether the structure of the mitotic spindle is affected in cells expressing the IgG domain or IgG domain plus five DXRs, we identified metaphase-arrested cells, followed them for 3 hours and then fixed and stained for microtubules (Fig. 6A). In contrast to GFP controls and cells expressing the five DXRs, in which normal chromosome alignment and well-organized bipolar spindles were observed (Fig. 6Ag,h), cells expressing the kinase-dead MLCK (data not shown), IgG domain plus five DXRs or IgG domain frequently displayed abnormal spindle morphology (Fig. 6Aa-f). Typically, we observed extension of the astral microtubules, which extended and curved into the cytosol beyond the chromosomes (Fig. 6Aa,b,d,f, arrows). In some cases, spindle pole fragmentation was also detected or cells appeared to contain an additional aster together with a bipolar spindle (Fig. 6Aa,c), but \(\gamma\)-tubulin staining indicated the presence of two morphologically normal centrosomes in all mitotic arrested cells (Fig. 6B). Thus, the additional asters observed in cells expressing the IgG domain or IgG domain plus five DXRs are probably caused by microtubule bundling, because cross-linking of microtubules has been shown to induce aster formation (Verde et al., 1991).

Previous studies have shown that inhibition of CENP-E via antibody microinjection or expression of dominant negatives leads to spindle pole fragmentation and the appearance of minor acentriole poles similar to those observed in our arrested cells (McEwen et al., 2001). In addition, inhibition of CENP-E induces a mitotic arrest with a mixture of aligned and unaligned chromosomes (McEwen et al., 2001). To determine whether these same phenotypes are due to mislocalization of CENP-E in cells expressing the IgG domain or IgG domain plus five DXRs, we examined the distribution of CENP-E in transfected cells. In all cells examined, CENP-E localized to kinetochores (Fig. 6Ca-d). When the chromosomes aligned along the length of the spindle, the kinetochores were oriented towards the spindle, suggesting that kinetochore-microtubule

Table 1. Cells expressing the IgG domain or IgG domain+5 DXRs display a mitotic arrest

| Construct            | Number of cells observed | Percent divided cells | Percent arrested cells |
|----------------------|--------------------------|-----------------------|------------------------|
| Kinase-dead MLCK     | 6                        | –                     | 100% (6)               |
| IgG domain+5 DXRs    | 36                       | 53% (19)              | 47% (17)               |
| IgG domain           | 12                       | 58% (7)               | 42% (5)                |
| GFP                  | 8                        | 100% (8)              | –                      |
| Untransfected control| 14                       | 100% (14)             | –                      |

Table 2. The majority of arrested cells expressing the IgG domain or IgG domain+5 DXRs undergo apoptosis

| Construct            | Number of apoptotic cells observed | Percent apoptotic cells | Percent divided cells | Percent binucleate cells |
|----------------------|-----------------------------------|------------------------|-----------------------|-------------------------|
| IgG domain+5 DXRs    | 45                                 | 64% (29)               | 16% (7)               | 20% (9)                 |
| IgG domain           | 27                                 | 66% (18)               | 15% (4)               | 19% (5)                 |
interactions were taking place (Fig. 6Ca,b). Moreover, immunoblot analysis demonstrated that the level of CENP-E expression was the same in cells expressing GFP or the MLCK truncations (data not shown).

In addition to alterations in the astral microtubules, there appeared to be defects in the spindle microtubules as well. To examine the stable kinetochore microtubule population in more detail, labile, non-kinetochore-associated microtubules were depolymerized by incubation on ice and the morphology of the remaining spindle microtubules evaluated by β-tubulin staining. In cells expressing the IgG domain or IgG domain plus five DXRs, the spindle microtubules were bundled, instead of containing well-organized kinetochore fibers (Fig. 6Ca,b). This phenotype is not likely to be due to direct interaction of the IgG domain with the microtubules because in vitro cosedimentation assays indicate that the IgG domain does not bind to Taxol-stabilized microtubules (data not shown). Microtubules in interphase transfected cells were not effected (data not shown).

We also detected abnormalities in chromosome alignment in metaphase arrested cells (Fig. 6Aa-f). In particular, despite bipolar spindle formation and a robust metaphase plate, at least one chromosome typically remained off the equator. Using staining with ACA serum to monitor the position of the

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**Fig. 5.** Time-lapse series of phase and fluorescence micrographs of mitotic HeLa cells expressing GFP or GFP fusions of the IgG domain, IgG domain plus five DXRs or kinase-dead MLCK. Scale bar, 10 μm. (A) A cell expressing GFP. (B,C) Cells expressing the IgG domain plus five DXRs. (D,E) Cells expressing the IgG domain. Arrowheads indicate an actively growing lamellipodia of a cell that exits M phase. (F) Cell expressing the kinase-dead MLCK. Arrows indicate blebs that form in arrested cells.
Fig. 6. Expression of the N-terminus of the long MLCK disrupts normal spindle morphology during mitosis and inhibits chromosome alignment. Scale bars, 10 μm. (A) Metaphase arrested cells were identified, followed for approximately 3 hours, fixed and stained for microtubules with antibodies against β-tubulin (top) and for DNA with DAPI (middle). Merged images of microtubules (red) and DNA (blue) (bottom). In GFP controls and cells expressing the five DXRs, alignment of the chromosomes and well-organized bipolar spindles were observed (g,h). Cells expressing the IgG domain or IgG domain plus five DXRs frequently showed abnormal spindle morphology (a-f). The arrows indicate extended, curving astral microtubules (a,b,d,f). In some cases, spindle pole fragmentation was observed (a,c). Chromosome alignment was also abnormal in metaphase-arrested cells. Several mono-oriented chromosomes were observed close to the spindle pole (b,d) and sometimes the chromosomes aligned along the length of the spindle (a,e).

(B) Transfected cells were fixed and stained for pericentrosomal material with antibodies against γ-tubulin and DNA with DAPI. γ-Tubulin staining showed that cells expressing N-terminal fragments of MLCK possessed two morphologically normal centrosomes. (C) Transfected cells were chilled to depolymerize the labile non-kinetochore-associated microtubules and the morphology of the remaining spindle microtubules evaluated by β-tubulin staining (bottom). The cells were also stained with antibodies against CENP-E, which localized to kinetochores (middle). In cells expressing the IgG domain or IgG domain plus five DXRs (a,b), the spindle microtubules were bundled, instead of containing well-organized kinetochore fibers (c,d). (D) Cells expressing N-terminal fragments of MLCK display abnormalities in chromosome alignment. Indirect immunofluorescence with the centromere marker ACA was used to monitor the position of the kinetochores (top) and DAPI to visualize the chromosomes (middle) in metaphase transfected cells. Merged images of centromeres (red) and DNA (blue) (bottom). In 60% of the cells expressing the IgG domain or IgG domain plus five DXRs, there were uncongressed chromosomes close to one of the spindle poles or between the equator and one spindle pole (a,b). In cells expressing GFP or the five DXRs, less than 10% of mitotic cells with visible metaphase chromosome alignment had uncongressed chromosomes.
kinetochores and DAPI to visualize the chromosomes, approximately 60% of the cells expressing the IgG domain or IgG domain + five DXRs always showed a few uncongressed chromosomes located close to one of the spindle poles or between the equator and one spindle pole (Fig. 6Da,b). In cells expressing GFP or the five DXRs, less than 10% of mitotic cells with visible metaphase chromosome alignment had uncongressed chromosomes, which were usually close to spindle equator. Following prolonged mitotic arrest in cells expressing the IgG domain or IgG domain plus five DXRs, the chromosomes displayed a twisted alignment (Fig. 6Ad,e) or contained several mono-oriented chromosomes that were unusually close to the spindle pole (Fig. 6Ab,d,e). Sometimes, the chromosomes aligned along the length of the spindle rather than at the spindle equator (Fig. 6Aa,Ca,b).

Spindle assembly checkpoint is activated in metaphase cells expressing the N-terminus of the long MLCK
In the absence of tension, the 3F3/2 phosphoepitope is expressed at kinetochores, resulting in an anaphase delay (Nicklas et al., 1995; Daum et al., 2000), so we used immunostaining with the 3F3/2 antibody to examine the presence of unattached or mono-oriented chromosomes (Fig. 7A). In untransfected metaphase cells or cells expressing GFP or the five DXRs, we typically did not detect any 3F3/2 fluorescence, indicating proper chromosome congression and attachment. In cells expressing the IgG domain or IgG domain plus five DXRs, 3F3/2 staining was observed in more than 50% of cells with a visible metaphase plate, typically on uncongressed chromosomes.

Recent studies suggest that dephosphorylation of the 3F3/2 phosphoepitope at kinetochores is not required for inactivation of the spindle assembly checkpoint (Canman et al., 2002), so we examined the localization of the Mad1 and Bub3 checkpoint proteins to kinetochores (Taylor et al., 1998; Chen et al., 1998). In cells expressing the five DXRs, IgG domain or IgG domain plus five DXRs, both proteins localized to unattached kinetochores during prophase and prometaphase (Fig. 7B,Ca,c,e). In addition, bright Mad1 and Bub3 staining was observed on the kinetochores of uncongressed chromosomes in metaphase cells (Fig. 7B,C, arrows), whereas only residual staining was detected on the kinetochores of aligned chromosomes. Both checkpoint proteins were diffusely

![Fig. 7. Localization of the 3F3/2 phosphoepitope and checkpoint proteins in cells expressing N-terminal fragments of MLCK. Scale bars, 10 μm. (A) Immunostaining with the 3F3/2 antibody was used to examine the presence of unattached or mono-oriented chromosomes in transfected cells (left). DNA was visualized with DAPI (right). In cells expressing the IgG domain or IgG domain plus five DXRs, 3F3/2 staining was observed in more than 50% of cells with a visible metaphase plate, typically on misaligned/uncongressed chromosomes. In untransfected metaphase cells or cells expressing GFP or the five DXRs, 3F3/2 fluorescence was not detected. In transfected cells, the Bub3 (B) and Mad1 (C) checkpoint proteins (top) localized to unattached kinetochores during prophase and prometaphase (a,c,e). In cells expressing the IgG domain or IgG domain plus five DXRs, bright Bub3 and Mad1 staining was observed on the kinetochores of misaligned chromosomes near spindle poles or uncongressed chromosomes in metaphase cells (arrows), whereas only residual staining was detected on the kinetochores of aligned chromosomes. Chromosomes and nuclei were stained with DAPI (middle). Merged images of checkpoint proteins (red) and DNA (blue) (bottom).]
distributed throughout the cell during metaphase (Fig. 7B,Cb,d,f) and anaphase, which is similar to the localization pattern observed in other cell lines (Taylor et al., 1998; Chen et al., 1998). These data indicate that expression of the IgG domain or IgG domain plus five DXRs in HeLa cells does not affect the recruitment of Bub3 and Mad1 to unattached kinetochores or the release of these components once the chromosomes have congressed to the cell equator.

Discussion

The N-terminal half of the long MLCK contains six Ig-type motifs (termed IgG domain) followed by five DXR motifs, which contain five repeated DXRXXL sequences. We showed previously that the IgG domain plus five DXRs displays a localization that is identical to the full-length long MLCK in interphase and mitotic cells (P popech nay et al., 2000); however, the relative contributions of the IgG domain and the five DXRs to kinase targeting in mitotic cells has not been examined. Our results indicate that the IgG domain and the five DXRs independently localize to stress fibers, but only the five-DXR fragment displays a modest cortical localization to the cleavage furrow. In addition, our fluorescence studies indicate that the IgG domain plus five DXRs demonstrates more prominent stress fiber staining than the IgG domain or five-DXR fragment alone, suggesting that the IgG domain plus five DXRs has a higher affinity for stress fiber binding than either individual domain. This is supported by the observation that the long MLCK binds to Triton-insoluble cytoskeletons with a higher affinity than the short MLCK (Kudryashov et al., 1999; Smith et al., 2002). This difference in affinity can be attributed to the IgG domain and not to the two additional DXR motifs present in the long MLCK because biochemical studies demonstrate that the five DXR motifs from the long MLCK and the three DXR motifs from the short MLCK have comparable binding affinities for F-actin (Smith et al., 2002).

The six immunoglobulin motifs composing MLCK’s IgG domain belong to the C2-type superfamily. The Ig-C2 motif was originally identified in the extracellular domain of adhesion molecules (Williams and Barclay, 1988) but this motif is now recognized in a number of intracellular cytoskeletal proteins including titin, MyBP-C, MyBP-M, MyBP-H, myotilin and palladin (Einheber and Fischman, 1990; Labeit et al., 1990; Noguchi et al., 1992; Parast and Otey, 2000; Salmikangas et al., 1999; Vaughan et al., 1993). It is notable that most of these molecules are specific to skeletal muscle, suggesting that the C2 motifs contribute to the maintenance of cytoskeletal architecture and organization. In general, Ig motifs are believed to have structural as well as biological functions, because the Ig domain can serve as a modular spacer (Williams and Barclay, 1988) and bind to a diversity of ligands via the variable polypeptide loops, which are attached to the core β-sheet structure.

The C2 motif is commonly present in multiple copies with functional specialization of individual repeats within a single protein. For example, titin contains 112 copies of the C2 motif (Labeit and Kolmerer, 1995) but only two C2 motifs at the N-terminus (Z1, Z2) bind to the Z-line protein T-cap (Gregorio et al., 1998), whereas C2 motifs Z9 and Z10 are responsible for the binding interaction between titin and obscurin (Young et al., 2001). C2 motifs have also been implicated in myosin-II binding. The C-terminal C2 repeats in MyBP-C and MyBP-H (Alyonycheva et al., 1997; Okagaki et al., 1993), which have a total of seven and two Ig-C2 motifs, respectively, confer high-affinity binding to light meromyosin, whereas the remaining C2 motifs mediate other protein-protein interactions within the sarcomere. Similarly, the extreme C-terminal C2 motif in the long and short MLCKs has been shown to be a myosin-II binding module (Shirinsky et al., 1993; Silver et al., 1997); however, in vitro cosedimentation studies performed in our laboratory indicate that the N-terminal six Ig-C2 motifs present in the long MLCK do not bind myosin-II or F-actin (data not shown). This is consistent with previous observations that the Ig-C2 motif represents a diverse protein interaction module with a wide array of binding partners. Additional experiments will be needed to identify the protein or proteins that interact with the C2 motifs in the IgG domain. However, sequence analysis has shown that the six Ig-C2 motifs in the N-terminus of the long MLCK are most homologous to C2 motifs present in titin and palladin (N. G. Dulyaninova et al., unpublished).

Although the detailed mechanistic features remain to be elucidated, our findings demonstrate a clear link between MLCK and cell cycle progression. In particular, our in vivo time-lapse studies indicate that expression of the kinase-dead MLCK, IgG domain plus five DXRs or IgG domain can induce a metaphase arrest with defects in karyokinesis. These defects are clearly attributable to just the IgG domain as cells expressing the five DXRs do not have any detectable defects in mitosis.

Cells expressing the N-terminal domain of the long MLCK, which lacks the catalytic core, progress through interphase and enter mitosis with the assembly of bipolar spindles. However, chromosome alignment, anaphase onset and chromosome segregation, all fail owing to the disruption of normal spindle assembly. Our studies demonstrate that the observed microtubule defects induce abnormalities in chromosome alignment, the appearance of mono-oriented chromosomes and the subsequent activation of the spindle assembly checkpoint at the kinetochores of uncongressed chromosomes. Prolonged mitotic arrest results in the instability of chromosome equatorial alignment and a failure of cytokinesis.

Although initial kinetochore malorientations occur with some frequency, they are typically corrected in prometaphase by multiple detachments and reattachments of the kinetochore microtubules until the correct orientation is achieved (Nicklas, 1997). However, we suggest that, in our studies, activation of the spindle assembly checkpoint, which usually provides sufficient time for these errors to be corrected, aggravates these mistakes rather than promotes their correction. This suggestion is based on the observation that the chromosomes often fail to maintain a stable equatorial alignment and usually within 1 hour of mitotic arrest begin to lose their metaphase alignment (Nicklas, 1997). However, we suggest that, in our studies, activation of the spindle assembly checkpoint, which usually provides sufficient time for these errors to be corrected, aggravates these mistakes rather than promotes their correction. This suggestion is based on the observation that the chromosomes often fail to maintain a stable equatorial alignment and usually within 1 hour of mitotic arrest begin to lose their metaphase alignment even though the chromatin remains condensed. We propose that this failure to achieve correct chromosome orientation arises from improper chromosome attachment, aggravated by spindle defects. However, our studies cannot discount the possibility that the defects in spindle assembly are the primary cause of chromosome misalignment in arrested cells.

Our previous studies have shown that the long MLCK displays both cortical and cytoplasmic distributions during metaphase and that the IgG domain plus five DXRs is sufficient to target the kinase to the cortex in metaphase cells.
(Poperechnaya et al., 2000). The current studies indicate that both the IgG domain and the five DXRs are required for the observed cortical localization in metaphase cells. These observations suggest that the apparent abnormalities detected in mitotic cells expressing the IgG domain or IgG domain plus five DXRs are probably caused by mistargeting of the endogenous MLCK present in the cytoplasmic kinase pool. If one considers that there are two pools of MLCK in the cell, cortical and cytoplasmic with discreet regulatory functions during mitosis, it is possible that the cortex-associated MLCK mediates cortical actomyosin interactions, whereas the cytoplasmic pool regulates the mitotic apparatus and karyokinesis. A role for MLCK in modulating karyokinesis is consistent with previous studies demonstrating that expression of a T18A-S19A regulatory light chain, which cannot be phosphorylated by MLCK, induces a delay in the progression from metaphase to telophase with some cells displaying defects in chromosome segregation (Komatsu et al., 2000). In addition, studies in crane-fly primary spermatocytes have shown that the small molecule MLCK inhibitor ML-7 prevents chromosome attachment to the spindle (Silverman-Gavrila and Forer, 2001).

The regulatory function of the IgG domain is further supported by phosphopeptide mapping studies of in vivo derived MLCK, which indicate that the long MLCK is highly phosphorylated on serine residues during metaphase with most of the phosphorylation sites located within the IgG domain (N. G. Dulyaninova et al., unpublished). These observations also suggest that, in cells expressing N-terminal fragments of the long MLCK, inhibition of the mitotic kinase that phosphorylates the IgG domain could have deleterious effects on kinetochore-microtubule interactions or chromosome alignment and the maintenance of bipolar kinetochore attachments. Interestingly, inhibition of Aurora B produces similar defects in the microtubule spindle and in chromosome alignment (Kallio et al., 2002) as observed in cells expressing the IgG domain and IgG domain plus five DXRs. Last, we cannot rule out that the IgG domain has a structural function and that expression of this domain not only prevents localization of the endogenous kinase but recruits and interferes with the proper localization of other proteins that are required for multiple aspects of mitosis. Taken together, our findings point to an unexpected regulatory function for MLCK during mitosis.

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