Microinjection of the Catalytic Fragment of Myosin Light Chain Kinase into Dividing Cells: Effects on Mitosis and Cytokinesis

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Abstract. Myosin light chain kinase (MLCK) is thought to regulate the contractile activity in smooth and non-muscle cells, and may play an important role in controlling the reorganization of the actin–myosin cytoskeleton during cell division. To test this hypothesis we have microinjected the 61-kD catalytic fragment of MLCK into mitotic cells, and examined the effects of unregulated MLCK activity on cell division. The microinjection of active 61 kD causes both a significant delay in the transit time from nuclear envelope breakdown to anaphase onset, and an increase in motile surface activity during and after metaphase. Control experiments with intact MLCK or with inactive catalytic fragment suggest that these effects are specifically induced by the unregulated myosin light chain kinase activity. Immunofluorescence analysis suggests that delays in mitosis are coupled to disruptions of spindle structures, while increased surface motility may be related to changes in the organization of actin and myosin at the cell cortex. Most importantly, despite the expression of strong phenotypes, 61 kD-injected cells still form functional cleavage furrows that progress through cytokinesis at rates identical to those of control cells. Together, these results suggest that the activity of MLCK can affect mitosis and cortical activities, however additional control mechanisms are likely involved in the regulation of cytokinesis.

1. Abbreviations used in this paper: MLCK, myosin light chain kinase; NEB, nuclear envelope breakdown; NRK, normal rat kidney.
absence of the Ca²⁺/calmodulin complex (Ikebe et al., 1987a, 1989). Microinjection of this fragment into smooth muscle cells induces contraction without the requirement for the release of Ca²⁺ (Itoh et al., 1989). In this study, we have prepared this catalytic fragment and microinjected it into mitotic normal rat kidney (NRK) cells soon after nuclear envelope breakdown (NEB). Interestingly, the microinjection caused a significant delay in mitosis during prometaphase/metaphase and an increase in surface activities during metaphase, anaphase, and telophase. However, after the onset of anaphase, the cleavage furrow formed at the proper time and location in the cell, at a rate identical to that of controls. We conclude that while the regulation of MLCK activity can affect mitosis and cortical activities, the control of cytokinesis probably involves additional factors.

**Materials and Methods**

**Preparation of MLCK and its Catalytic Fragment**

MLCK was prepared from turkey gizzards by the method of Walsh et al. (1983), as modified by Ikebe et al. (1987a). After phoshocellulose chromatography, MLCK was further purified by calmodulin-affinity chromatography as described by Adelstein and Klee (1982) and dialyzed into a storage buffer of 50 mM KCl, 30 mM Tris-HCl, 0.5 mM DTT, pH 7.5.

The catalytic fragment of MLCK was prepared by limited proteolysis of MLCK in the presence of Ca²⁺ and calmodulin as detailed by Ikebe et al. (1987a, 1989). Briefly, MLCK (0.5 mg/ml) in the storage buffer containing 0.2 mM CaCl₂ was proteolysed with trypsin (1:100, wt/wt) in the presence of 0.2 mg/ml calmodulin for 7.5 min at 25°C. The reaction was terminated by the addition of a twofold (wt/vt) excess of soybean trypsin inhibitor over trypsin, and the solution was subjected to anion-exchange chromatography on either a DEAE-Sepharose Fast Flow exchanger (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ) or a TSK DEAE-SPW HPLC column (Pharmacia LKB Biotechnology, Inc.). DEAE columns were eluted with a 100-500 mM NaCl gradient in a buffer of 30 mM Tris-HCl, 0.5 mM DTT, pH 7.5, and peak fractions containing the 61-kD catalytic fragment of MLCK were pooled and further purified by hydroxylapatite chromatography (Ikebe et al., 1987a). The purified 61-kD fragment was concentrated to ~1 mg/ml, dialyzed against storage buffer, and stored at 4°C.

The extent and rate of MLCK phosphorylation was assayed by incubating MLCK (10 μg/ml) or the 61-kD fragment (5 μg/ml) with 0.75 mg/ml gizzard myosin in a buffer of 150 mM NaCl, 25 mM Tris-HCl, 5 mM MgCl₂, 0.5 mM DTT, pH 7.5, containing either 0.2 mM CaCl₂/5 μg/ml calmodulin or 1 mM EGTA (and 5 pg/ml calmodulin in assays of MLCK). Reactions were initiated by the addition of ATP to 1 mM. 50-μl aliquots of reaction mixtures were removed at various times, precipitated with 3-5% cold TCA, and washed with cold acetone prior to resuspension in urea-glycerol PAG (Perrie and Perry, 1970) on 10% mini slab gels stained with Coomassie blue, and quantified using an Ultrascan XL laser densitometer (Pharmacia LKB Biotechnology, Inc.).

Before injection into cells, MLCK and the 61-kD fragment were dialyzed into injection buffer (40 mM KCl, 20 mM Tris-HCl, 0.1 mM DTT, pH 7.0). The kinase activities were routinely assayed before and after microinjections. Inactive preparations of the 61-kD fragment were obtained by either dialyzing the active fragment for 24 h into a buffer of 10 mM Tris-acetate, 0.2 mM EDTA, 0.01% bromophenol blue, pH 8.6. Samples were analyzed by urea-glycerol PAGE (Perrie and Perry, 1970) on 10% mini slab gels stained with Coomassie blue, and quantified using an UltraScan XL laser densitometer (Pharmacia LKB Biotechnology, Inc.).

**Cell Culture, Microinjection, and Video Microscopy**

NRK cells (NRK-52E; American Type Culture Collection, Rockville, MD) were cultured and plated for microinjection as previously described (Cao and Wang, 1990a). Microinjection dishes were cultured at 33°C on a microscope body (Sigma Chemical Corp., St Louis, MO). Images were stored as digital files in an image-processing computer, which was assembled by G. W. Hannaway and Associates (Boulder, CO) and consisted of a Silicon Graphics 4D/20 workstation (Mountain View, CA) and image-processing boards from Imaging Technology (150 series; Woburn, MA). Sequences of phase images were used to determine the timing of key stages of mitosis, and motile activities were analyzed using a motion display program as described previously (Wang, 1989).
Figure 2. Phosphorylation activity of MLCK and the 61-kD catalytic fragment prepared for microinjection studies. MLC2o is phosphorylated by MLCK in the presence of the Ca2+/calmodulin complex (open triangles), but not in the presence of EGTA and calmodulin (closed triangles). In contrast, the 61-kD catalytic fragment has a strong activity both in the presence of EGTA (closed circles) and in the presence of Ca2+ (data not shown). After dialysis into a low ionic strength buffer or extended periods of storage on ice, the 61-kD fragment shows a greatly reduced activity (open circles). Gel insets show the urea-glycerol PAGE analysis of MLC2o phosphorylation by an active 61-kD (a) and inactive 61-kD (b) preparation of the catalytic fragment of MLCK, at 0, 1, 5, and 10 min after the addition of ATP. LC2oD, dephosphorylated MLC2o; LC2oP, phosphorylated MLC2o; LC17, 17-kD myosin light chain. The band migrating slightly faster than LC17 is most likely a proteolytic fragment of myosin.

Other Procedures

Calmodulin was prepared from bovine brain according to Burgess et al. (1980) and smooth muscle myosin was prepared from turkey gizzard according to Ikebe and Hartshorne (1985). Protein concentrations were determined by the method of Lowry et al. (1951) using BSA as a standard. SDS-PAGE was performed according to Laemmli (1970) using 5–20% micro-slab gels (Matsudaira and Burgess, 1978), and Sigma SDS-6 and SDS-6H molecular weight standards.

Results

Biochemical Characterization of the 61-kD Catalytic Fragment of MLCK

MLCK is believed to be a highly specific enzyme that is stringently regulated by the binding interaction with the Ca2+/calmodulin complex. Using methods described by Ikebe et al. (1987a, 1989), we prepared a Ca2+/calmodulin-independent fragment of MLCK. As shown in Fig. 1, limited tryptic proteolysis of MLCK in the presence of Ca2+ and calmodulin resulted in the cleavage of the protein into several well-defined fragments, from which a 61-kD fragment was purified using anion exchange and hydroxylapatite chromatography (Fig. 1, lane 4).

In biochemical assays, the 61-kD fragment caused rapid phosphorylation of MLC2o independent of the presence of Ca2+ and calmodulin (Fig. 2, 61kDa/EGTA). However, because this activity was relatively short lived, showing a ~50% decrease within 7 d of the proteolytic cleavage of MLCK, microinjection experiments were performed within 5 d of preparation. Preparations of the 61-kD fragment that showed little or no catalytic activity in these assays (Fig. 2, 61kDa/inactive) were used as controls in microinjection experiments described below.
Figure 4. Time-lapse series of phase micrographs of a buffer-injected NRK cell during mitosis. After NEB (a, t = 0 min), the cell was microinjected at t = 5 min and observed through cell division. Images were recorded during prometaphase (b, t = 9.7 min), metaphase (c, t = 19.4 min), anaphase onset (d, t = 28.3 min), anaphase II (e, t = 29.8 min), and midcytokinesis (f, t = 32.2 min). Note that the plasma membrane appears smooth around the cell, except for slender projections anchoring the cell to the substrate (arrows). Bar, 10 μm.

**Microinjection of the 61-kD Catalytic Fragment of MLCK into Dividing Cells**

To define the timing of different stages of mitosis in NRK cells, we observed normal dividing cells by video microscopy and measured the period from NEB to midcytokinesis under conditions where the culture temperature was precisely maintained (see Materials and Methods). Uninjected cells showed a transit time from NEB to midcytokinesis averaging ~31 min, with the period from NEB to anaphase onset lasting ~26 min, and the period from anaphase onset to midcytokinesis lasting ~5 min (Table I).

In contrast, cells injected with the active 61-kD fragment showed a significant delay in the progression through prometaphase and metaphase, with transit times between NEB and anaphase onset ranging from 25 to 97 min (Fig. 3; Table I). In addition, ~10% of the injected cells failed to show normal karyokinesis or cytokinesis (see below). Surprisingly, all of the cells that completed karyokinesis showed no effect on the duration of anaphase or the rate of cytokinesis, as measured from the point of anaphase onset to midcytokinesis (Table I). Microinjection of cells with buffer alone, the inactive 61-kD fragment, or intact MLCK showed no effect on either the timing of mitosis or the rate of cytokinesis (Table I). Cells injected with control solutions exhibited typical morphological features of uninjected cells as they progressed through mitosis and cytokinesis, including chromosome condensation and NEB, chromosome congregation and alignment, chromosome separation, and formation of the cleavage furrow (Fig. 4).

In contrast, a large proportion (75%) of cells injected with the active 61-kD fragment showed striking surface blebbing activities during later stages of mitosis. As shown in Fig. 5, the injected cell progressed through prometaphase with a normal phase morphology, however during anaphase highly active blebs showing rapid expansion and retraction activities were observed on the cell surface. The blebbing activity was initiated during metaphase in ~40% of injected cells, and during anaphase in ~35% of injected cells. Once started, the blebbing continued through the end of cell division. Blebs were initially observed over the entire surface during metaphase (Fig. 6), but as cells entered cytokinesis the blebbing activity subsided in the equatorial region where apparently normal cleavage furrows formed (Figs. 5 and 6). Upon completion of cytokinesis, the blebbing activity gradually decreased while daughter cells spread out in a manner similar to control cells. When 61-kD was injected during late metaphase and early anaphase, active blebbing was similarly observed in regions outside the equator, and there were no apparent effects on karyokinesis or the rate of cytokinesis (data not shown).

In a small percentage of injected cells that failed to show normal karyokinesis (mentioned above), metaphase chromosomes formed dispersed aggregates, while erratic blebs and constrictions were observed on the cell surface (Fig. 7, a and b). The failure to undergo normal cytokinesis was likely related to the abnormal karyokinesis observed in these cells.

**Fluorescence Localization of F-actin, Myosin, and Tubulin after Microinjection of the 61-kD Fragment**

The distribution of F-actin and myosin in cells microinjected with the 61-kD fragment was observed after staining with fluorescent phalloidin or specific antibodies against myosin.
Figure 5. Time-lapse series of phase micrographs of a mitotic NRK cell injected with the 61-kD catalytic fragment of MLCK. After NEB (a, t = 0 min), the cell was microinjected at t = 5 min and followed through cell division. Although the phase morphology appears normal during prometaphase (b, t = 7.5 min) and metaphase (c, t = 20.0 min), blebbing activity initiates after the onset of anaphase (d, t = 26.9 min) and continues through anaphase II (e, t = 28.4 min) and cytokinesis (f, t = 29.4 min). Note that the formation of the cleavage furrow at the equator is unaffected by the adjacent blebbing activity, and cytokinesis occurs at a rate similar to that for controls. Arrows indicate rapid extension and retraction of surface blebs. Bar, 10 μm.

In cells exhibiting dynamic surface activity, blebs were delineated by intense cortical staining of actin (Figs. 8 a and 9 a). In some cases actin and myosin were clearly localized near the base of the blebs (Fig. 9, a and b, arrows). In dividing cells both actin filaments and myosin were present in the cortex of the cleavage furrow, although the degree of staining varied from cell to cell as previously shown by others (Nunnally et al., 1980; Cao and Wang, 1990a). Similar distributions of actin and myosin in the furrow were observed for control cells and cells injected with the 61-kD fragment. Note that the variability of myosin staining in the furrow of NRK cells was observed both with the monoclonal and polyclonal antibodies used in this study, however only the mAb appeared to show a distinct punctate staining pattern as previously observed in lymphoblasts (Bornens et al., 1989) and PtK2 cells (Klotz et al., 1986).

To determine whether the delay of prometaphase and metaphase was related to disruptions of the mitotic spindle, injected cells were processed for immunofluorescence with anti-tubulin antibodies. Disrupted spindle structures, lacking well-defined kinetochore fibers, were observed in injected cells exhibiting prolonged periods of prometaphase.

Figure 6. Time-lapse sequence of phase micrographs of a dividing NRK cell injected with the 61-kD catalytic fragment of MLCK. The cell was microinjected 5 min after NEB (t = 0 min), and exhibited a significant delay in mitosis with a transit time from NEB to anaphase onset of 61.9 min. After an extensive period of metaphase blebbing (a, t = 60.1 min), the surface activity subsided in the equatorial region during anaphase (b, t = 61.9 min) and cytokinesis (c, t = 66.4 min; and d, t = 67.6 min). The cleavage furrow appears similar to that in control cells. Bar, 10 μm.
and metaphase (Fig. 10, d and e). However, upon the onset of anaphase, the organization of spindle microtubules in 61-kD delayed cells (Fig. 10 f) appeared similar to that of control cells (Fig. 10, g–l).

**Figure 8.** Distribution of F-actin and myosin in a mitotic NRK cell microinjected with the 61-kD fragment of MLCK and in an uninjected control cell. After injection of the 61-kD fragment at 5 min after NEB, the treated cell (a and b) displayed rapidly expanding and retracting surface blebs during metaphase. Both the injected cell and the control cell (c and d) were fixed at ~15 min after NEB, which represents a typical time for metaphase in control cells, and stained for F-actin (a and c) and myosin (b and d). F-actin staining is localized on the cortex of the injected cell, while myosin staining is concentrated both on the cortex near the base of blebs (arrows) and as punctate foci in the cytoplasm. Similar results were obtained with polyclonal antibodies to myosin (see Materials and Methods), however only the mAb shows the distinct punctate staining pattern shown here (also see Fig. 9). Bar, 5 μm.

**Discussion**

Past biochemical studies have suggested a potentially important role for MLCK in regulating contractile activities in nonmuscle cells. The phosphorylation of the MLC20 at serine 19, after the activation of MLCK by Ca2+-calmodulin complexes, could control both the assembly of myosin filaments and the generation of forces by actin–myosin interactions (Sellers and Adelstein, 1987). Thus it is possible that, during cell division, transient changes in the intracellular concentration of Ca2+ and/or the Ca2+-calmodulin complex may regulate both spatially and temporally cortical contractile activities.

We sought to address this possibility by microinjecting the 61-kD constitutively active domain of MLCK. Such injections are expected to disturb the temporal and spatial regulations of the MLCK activity, and disrupt cellular functions that rely on the precise regulation of MLC phosphorylation. Two distinct effects, delays in the progression through prometaphase and metaphase, and surface blebbing activities during later stages of mitosis, were observed following the microinjections. Given the lack of effects after injections of buffer, intact MLCK, and inactive 61-kD fragment (Table I), the observed responses are likely due to the unregulated kinase activity of the 61-kD fragment, rather than nonspecific effects of the microinjection. The positive biological effects of the active catalytic fragment suggest that the activity of endogenous MLCK must be tightly regulated in dividing cells.

**Effects of the 61-kD Fragment on Cortical Activities**

One of the most dramatic effects of the microinjection of the 61-kD fragment is the induction of active surface blebbing during metaphase and anaphase (Figs. 5 and 6). Although the mechanism for blebbing is poorly understood, it is likely that the 61-kD fragment causes an increase in the phosphorylation of myosin and stimulates contractile activities, which in turn may generate surface blebs through deformation of the cortical cytoskeleton and extrusion of the inner cytoplasm. This explanation is supported by the concentration of actin filaments and myosin in the cortex and near the base of blebs (Figs. 8 and 9; also see Herman et al., 1981, and Zeligs and Wollman, 1977). In addition, a recent study has reported abnormal surface activities in Dictyostelium cells expressing a truncated myosin II, which lacks the ability to undergo disassembly and appears to show enhanced interactions with cortical actin (Egelhoff et al., 1991). Blebbing has also been observed in some transformed cell lines (Porter et al., 1973) and under conditions that presumably weaken the mechanical strength of the cortex, e.g., after the treatment of cells with cytochalasin B (Puck et al., 1972; Miranda et al., 1974; Perry and Snow, 1975; Godman and Miranda, 1978), or in melanoma cells deficient in the actin filament cross-linking protein filamin (Byers et al., 1991).

The apparent lack of blebbing during prometaphase in 61-kD-injected cells suggests that the activation of myosin-dependent motility may depend on multiple factors. For example, other kinases or phosphatases may affect the extent and/or sites of phosphorylation on MLC20 (Lamb et al., 1988; Fernandez et al., 1990). A particularly attractive possibility is that MLCK-dependent phosphorylation of MLC20 may be modulated by the activity of the p34<sup>cdc2</sup> kinase (Sat-
terwhite et al., 1990), which phosphorylates MLC$_{20}$ at serines 1, 2, and threonine 9, and inhibits MLCK-mediated phosphorylation of MLC$_{20}$ at serine 19 (Ikebe et al., 1987b; Sellers and Adelstein, 1987; Pollard et al. 1990). Thus, even though the 61-kD fragment is likely to be active during prometaphase, phosphorylation of MLC$_{20}$ at serine 19 may be inhibited until the activity of p34$^{c_{dc2}}$ declines later in mitosis. Future studies to test the level and sites of MLC$_{20}$ phosphorylation in dividing cells will be extremely valuable.

**Effects of the 61-kD Fragment on Cytokinesis**

Several mechanisms for the regulation of cytokinesis have been proposed (Rappaport, 1986; Salmon, 1989; Mabuchi, 1990; Pollard et al., 1990). One attractive hypothesis involves the preferential phosphorylation of myosin in the equatorial region by MLCK, presumably after the dephosphorylation of MLC$_{20}$ at the p34$^{c_{dc2}}$ directed sites and a localized activation of MLCK by the Ca$^{2+}$-calmodulin complexes (Pollard et al., 1990). The increase in the phosphory-
lation of MLC₂₀ at serine 19 then activates contraction of the cleavage furrow. Based on this model, one would predict that changes in the degree, timing, or location of myosin phosphorylation could disrupt the process of cytokinesis. Specifically, microinjection of the 61-kD fragment should cause an unregulated phosphorylation of myosin throughout the entire cortex of dividing cells, that in turn might stimulate a global contraction and potentially disrupt both the timing and pattern of cell cleavage.

To our surprise, cytokinesis proceeded normally in the majority of 61-kD injected cells with respect to both the site and rate of cell cleavage (Figs. 5 and 6; Table I). These results indicate that additional factors, independent of the phosphorylation of MLC₂₀, are likely involved in regulating cytokinesis. Based on previous studies, it is clear that cytokinesis involves not only acto-myosin interactions, but also formation of contractile structures in the cleavage furrow through the recruitment and organization of actin filaments and accessory proteins (Fujitaya et al., 1978; Aubin et al., 1979; Nunnally et al., 1980; Sanger et al., 1989; Cao and Wang, 1990a; b; Mabuchi, 1990; Pollard et al., 1990). Thus, the assembly of cross-linking proteins in the equatorial region may account for the suppression in blebbing activity concomitant with the organization of actin–myosin interactions into an effective cleavage force. In contrast, the lack of such organization in other regions may account for the sustained blebbing activities observed outside the furrow.

The regulation of cytokinesis may also involve the concerted activities of multiple kinases and phosphatases. While MLC₂₀ may be continually phosphorylated by the 61-kD catalytic fragment in injected cells, it is possible that only the equatorial region is able to maintain a specific pattern and level of phosphorylation during cytokinesis. The activity and localization of myosin in dividing cells may also be regulated by additional factors, such as phosphorylation of the heavy chains (Egelhoff et al., 1991), and/or association with myosin-binding proteins (Yabkowitz and Burgess, 1987). However, the physiological role of these factors in mammalian tissue culture cells remains to be elucidated.

**Effects of the 61-kD Fragment on Mitosis**

One of the unexpected results of this study is that injection of the 61-kD catalytic fragment affects not only cortical activities, but also the progression of prometaphase and metaphase. Given the variety of evidence indicating that myosin II heavy chains are not directly involved in chromosome congression or segregation (Mabuchi and Okuno, 1977; Kiehart et al., 1982; De Lozanne and Spudich, 1987; Knecht and Loomis, 1987; Zurek et al., 1990), we are left with the question of how an increase in MLCK activity and elevation of phosphorylated MLC₂₀ might cause delays in mitosis.

The most direct explanation for such delays is that the dephosphorylation of MLC₂₀ at serine 19 is somehow required for the progression from metaphase to anaphase. This implies that MLC₂₀, in addition to regulating myosin heavy chain activities, may also interact with other molecules involved in mitosis. One possibility is that, since MLC₂₀ can serve as a substrate for several kinases as well as phosphatases (Sellers and Adelstein), it is possible that increases in the phosphorylation state of MLC₂₀ may alter its interaction with these enzymes, resulting in a change in the balance of activities among different kinases and phosphatases involved in the regulation of mitosis. Alternatively, the catalytic domain of MLCK is known to share some homology with other members of the Ca²⁺-calmodulin-dependent kinase family (Hanks et al., 1988; Olson et al., 1990; Shoemaker et al., 1990). Thus, the removal of certain noncatalytic domains during the preparation of 61-kD could affect its subcellular localization and/or substrate specificity, and cause delays in mitosis by phosphorylating proteins involved in spindle function. Finally, immunofluorescence studies suggest that MLCK may undergo redistribution from stress fibers in interphase cells (De Lanerolle et al., 1981) to kinetochore spindle-fibers in mitotic cells (Guerrero et al., 1981). This raises the question of whether MLCK may normally have additional substrates in the mitotic spindle, in addition to MLC₂₀.

Future experiments to test these ideas should yield new and important insights regarding the mechanism of mitosis and cytokinesis.

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