Activation of m-Calpain Is Required for Chromosome Alignment on the Metaphase Plate during Mitosis*

Received for publication, August 11, 2003, and in revised form, October 30, 2003
Published, JBC Papers in Press, December 19, 2003, DOI 10.1074/jbc.M308841200

Shinobu Honda‡§, Tomotoshi Marumoto‡, Toru Hirot‡, Masayuki Nitta‡, Yoshimi Arima‡, Michio Ogawa§, and Hideyuki Saya‡¶

From the Departments of ‡Tumor Genetics and Biology and the §Departments of Tumor Genetics and Biology and Gastroenterological Surgery, Graduate School of Medical Sciences, Kumamoto University, 1-1-1 Honjo, Kumamoto 860-8556, Japan

Calpains form a superfamily of Ca2+-dependent intracellular cysteine proteases with various isoforms. Two isoforms, μ- and m-calpains, are ubiquitously expressed and known as conventional calpains. It has been previously shown that the mammalian calpains are activated during mitosis by transient increases in cytosolic Ca2+ concentration. However, it is still unknown whether the activation of calpains contributes to particular events in mitosis. With the use of RNA interference (RNAi), we investigated the roles of calpains in mitosis. Cells reduced the levels of m-calpain, but not μ-calpain, arrested at prometaphase and failed to align their chromosomes at the spindle equator. Specific peptidyl calpain inhibitors also induced aberrant mitosis with chromosome misalignment. Although both m-calpain RNAi and calpain inhibitors affected neither the separation of centrosomes nor the assembly of bipolar spindles, Mad2 was detected on the kinetochores of the misaligned chromosomes, indicating that the prometaphase arrest induced by calpain inhibition is due to activation of the spindle assembly checkpoint. Furthermore, when calpain activity was inhibited in cells having monopolar spindles, chromosomes were clustered adjacent to the centrosome, suggesting that calpain activity is involved in a polar ejection force for metaphase alignment of chromosomes. Based on these findings, we propose that activation of m-calpain during mitosis is required for cells to establish chromosome alignment by regulating some molecules that generate polar ejection force.

Chromosome alignment at the spindle equator, or congression, is a remarkably conspicuous event during mitosis that defines the metaphase stage of the cell cycle. This movement of chromosomes to the spindle equator is necessary for accurate segregation of replicated DNA in organisms as diverse as plants, insects, and mammals (1). Stable metaphase chromosome positioning and the preceding prometaphase chromosome movements are a consequence of the balance or imbalance of poleward and antipoleward movements. For some chromosomes that are closer to one pole, the polar ejection force that pushes chromosomes away from the pole is necessary to move toward the spindle equator to establish metaphase alignment. Recently, a kinesin-like DNA-binding protein (Kid),1 a chromokinesin, has been identified as taking a leading role in producing the polar ejection force for metaphase alignment of chromosomes (2–7). However, the underlying mechanisms that regulate the polar ejection force to establish initial congression of chromosomes and maintenance of alignment prior to anaphase are largely unknown.

Cell cycle progression is regulated by a complex process, which involves kinase/phosphatase cascades, protease action, signaling by second messengers, and other operations. These mechanisms cooperatively function to execute various events during the cell cycle. Recently, it has become well known that changes in the concentration of intracellular Ca2+, a second messenger of intracellular signaling, plays a crucial role in cell cycle progression (8, 9). In particular, Ca2+ oscillations occur at the G1-S boundary and near the G2-M transition when the downstream molecules of Ca2+ signaling are considered to be activated. However, the target molecules involved in cell cycle progression through Ca2+ signaling are obscure.

Calpains are a superfamily of Ca2+-dependent intracellular cysteine proteases whose members are expressed ubiquitously or in a tissue-specific manner. Numerous isoforms of calpains were identified in organisms ranging from mammals to invertebrates, and homologues have been found even in yeast and bacteria. Among them, the mammalian μ- and m-calpains (called conventional calpains) are the most extensively analyzed and have been shown to play a role in various physiological functions, including cell motility (10, 11), apoptosis (12), cell growth (13, 14), and cell cycle progression (8, 9, 15, 16). Several lines of evidence suggest that calpains are critically involved in mitotic progression. First, paxillin, a protein involved in focal adhesion, is cleaved specifically by activated m-calpain during mitosis when focal adhesions dissociate (17). Second, calpains and their endogenous inhibitor, calpastatin, alter their distributions during mitosis (18). Third, injection of calpains into the nuclei of stage-arrested starfish oocytes results in the resumption of meiosis (8). These findings allow us to speculate that calpain activation contributes to the mitotic progression through proteolysis of their specific substrates.

In this study, we demonstrate that reduction of m-calpain expression by RNA interference induced chromosome misalignment in mitotic cells and atypical nuclear morphology in interphase cells. The similar phenotypes were observed when mi-

---

*This work was supported by a grant for cancer research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to H. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†To whom correspondence should be addressed: Dept. of Tumor Genetics and Biology, Graduate School of Medical Sciences, Kumamoto University, 1-1-1, Honjo, Kumamoto 860-8556, Japan. Tel.: 81-96-373-5116; Fax: 81-96-373-5120; E-mail: hsaya@gpo.kumamoto-u.ac.jp.

↑The abbreviations used are: Kid, kinesin-like DNA-binding protein; PBS, phosphate-buffered saline; PIPES, 1,4-piperazinediethanesulfonic acid; DMSO, dimethyl sulfoxide; siRNA, small interfering RNA; DIC, differential interference contrast; GFP, green fluorescent protein; FACS, fluorescence-activated cell sorting; APC, anaphase-promoting complex.

This paper is available on line at http://www.jbc.org 10615
totic cells were treated with specific calpain inhibitors. In those cells, prometaphase duration was significantly prolonged due to the activation of spindle assembly checkpoint. Furthermore, we demonstrate that inhibition of calpain activity impairs the polar ejection force, which is required for chromosomes to achieve bipolar microtubule attachment and to align on metaphase plate. Our findings suggest that calpain is required for chromosome alignment in mitosis by regulating the machinery of polar ejection force through proteolysis of target proteins.

MATERIALS AND METHODS

Cell Culture and Synchronization—HeLa cells were cultured in a mixture of Dulbecco’s modified Eagle’s medium and Ham’s F12 medium supplemented with 10% fetal bovine serum. Synchronization was performed using double thymidine block protocol (first 22 h of incubation with 2 mM thymidine, an interval of normal medium incubation for 8 h, and second incubation with 2 mM thymidine for 18 h), a sequential thymidine/nocodazole protocol (first 22 h of incubation with 2 mM thymidine, an interval of normal medium incubation for 6–7 h, and 100 ng/ml nocodazole incubation for 8–12 h) or thymidine/butyloractone I protocol (first 22 h incubation with 2 mM thymidine, and following 50 μM butyloractone 1 incubation for 8 h). Mitotic cells were collected by mechanical shake-off from the culture plate after a sequential thymidine/nocodazole protocol as above. Connected mitotic cells were washed with PBS, released into medium containing indicated reagents, and harvested at indicated times.

RNA Interference—The sequences of the small interfering RNAs (siRNAs) specific for human large subunits of m-calpain and μ-calpain were 5’-CCAGGACUAGGAGGGCGACdTdT-3’ and 5’-GCUAGUUGUCGUGACUCGACdTdT-3’, respectively. The sequences of the siRNA specific for human Mad2 was 5’-ACCUUUAUCUGAGGUGAGdTdT-3’. A siRNA-targeting luciferase (GL-2: 5’-GCUAGGCCGAUAUCUCGAdTdT-3’) was used as a control (19). The 21-nucleotide RNA-ΔRNA chimera duplexes were obtained from Japan BioService (Asaka, Japan). Both annealing of the component strands of each siRNA and transfection was done using a transfection reagent (Invitrogen) as previously described (19).

Flow Cytometry and Mitotic Index—For cell cycle analysis and DNA content evaluation, cells were fixed in 70% methanol at −20 °C for several hours (20). Cells were centrifuged at 2000 rpm and resuspended in PBS containing RNase A (Sigma) at 0.1 mg/ml. Samples were incubated at 37 °C for 15 min, and propidium iodine was added to a final concentration of 50 μg/ml. Samples were analyzed by a FACScellibur flow cytometer using CellQuest software (BD Biosciences).

Mitotic index was determined as a percentage of mitotic cells in the total population. Cells with mitotic condensed chromatin were visualized by aceto-orcein (Merck) in 60% acetic acid and analyzed microscopically as previously described (21).

Antibodies—Antibodies used in these experiments were as follows: rabbit polyclonal antibody against domain III of m-calpain was obtained from Sigma; mouse monoclonal antibody against domain III of μ-calpain was obtained from RBI; mouse monoclonal antibody against cyclin B were obtained from Transduction Lab; goat polyclonal antibody against the N-terminal peptide of m-calpain (N-19), goat polyclonal antibody against securin and mouse monoclonal antibody against p53 (DO-1) was obtained from Santa Cruz Biotechnology; rat monoclonal antibody against α-tubulin was obtained from Harlan Sera Lab; rabbit antisera against Mad2 was generated by injecting rabbits with recombinant GST-hsMad2 fusion protein.

Inhibitors—The membrane-permeable inhibitors of calpain used in this study were calbenozyx-leucyl-leucyl-aldehyde (Z-LLal: Peptide Institute, Osaka), calbenozyx-valyl-phenylalanal (MDL28170: Calbiochem), calbenozyx-leucyl-leucyl-thr-CHF (Z-LLT-FMK: Calbiochem), 4-fluorophenylisulfonfyl-Val-Leu-CHO (SJA6017: Calbiochem). The Amount of m-Calpain Is Regulated in a Cell Cycle-dependent Manner and Elevated in G2-M Phase—To investigate the involvement of conventional calpains in the progression of mitosis, we first assessed the changes of protein levels of conventional calpains during cell cycle. HeLa cells were synchronized at the beginning of S phase by double thymidine block method. At different times after release from block, cells were harvested and analyzed by immunoblotting (Fig. 1). Both the full-length and autolysed fragments of m-calpain increased in G2-M phase, similar to cyclin B. In contrast, the levels of full-length and proteolytic fragments of μ-calpain were not.

RESULTS

The Amount of m-Calpain Is Regulated in a Cell Cycle-dependent Manner and Elevated in G2-M Phase—To investigate the involvement of conventional calpains in the progression of mitosis, we first assessed the changes of protein levels of conventional calpains during cell cycle. HeLa cells were synchronized at the beginning of S phase by double thymidine block method. At different times after release from block, cells were harvested and analyzed by immunoblotting (Fig. 1). Both the full-length and autolysed fragments of m-calpain increased in G2-M phase, similar to cyclin B. In contrast, the levels of full-length and proteolytic fragments of μ-calpain were not.
dramatically changed throughout the cell cycle. These findings raised the possibility that m-calpain, rather than μ-calpain, is involved in events during G2-M phases.

Reduction of m-Calpain by RNA Interference Induces Aberrant Mitosis with Chromosome Misalignment—To assess the functions of conventional calpains in cell cycle progression, we used siRNAs targeting mRNAs of μ-calpain and m-calpain. The protein levels of μ- and m-calpains were effectively reduced by siRNA transfection (Fig. 2A). Reduction of m-calpain expression led to a significant increase in the mitotic index (Fig. 2B) and induced aberrant mitosis with chromosome misalignment, although bipolar spindles were normally formed (Fig. 2C). However, reduction of μ-calpain did not affect mitotic index (Fig. 2B) and chromosome alignment (Fig. 2C). HeLa cells, which stably express a GFP-tagged histone H2B (H2B-GFP) fusion protein, were transfected with m-calpain siRNA and investigated the nuclear dynamics by using time-lapse fluorescence and DIC video microscopy (Fig. 2D). Cells with reduced m-calpain expression showed mitotic delay with misalignment of chromosomes followed by aberrant cell division. Moreover, the depletion of m-calpain significantly induced abnormal nuclear morphology, such as large and multiple lobed nuclei (Fig. 2F), in a time-dependent manner (Fig. 2F), suggesting that they had become aneuploid after aberrant mitosis. In contrast, the abnormal nuclei were not observed in cells treated with control siRNA (GL2 oligo) and μ-calpain siRNA (Fig. 2, E and F). These results suggest that m-calpain plays a significant role in chromosome alignment and proper segregation of chromosomes.

Calpain Inhibitor Induces Mitotic Delay with Misalignment of Chromosomes Despite Normal Bipolar Spindle—To further confirm the involvement of calpains in mitotic event, we treated HeLa cells with Z-LLal, a specific peptidyl calpain inhibitor. Fluorescence-activated cell sorting (FACS) analysis of HeLa cells treated with Z-LLal or MDL28170 for 8 h showed an accumulation of cells with 4N DNA in a dose-dependent manner (Fig. 3A). We also microscopically investigated chromosome configuration by aceto-orcein staining and found that the proportion of mitotic cells (mitotic index) were increased after Z-LLal treatment (Fig. 3B). Synchronized HeLa cells stably expressing histone H2B-GFP fusion protein were incubated with Z-LLal from G2 phase, and the nuclear dynamics were investigated by time-lapse microscopy (Fig. 3C). The duration of mitosis appeared to be prolonged from 6 h to over 10 h, and the cells eventually divided abnormally or underwent cell death (Fig. 3C). In those Z-LLal-treated cells, alignment of chromosomes on the metaphase plate was heavily impaired while chromosome condensation and nuclear envelope breakdown occurred normally. In most of those mitotic cells, some chromosomes did not congress near the spindle equator and remained near or behind the poles (Fig. 3D) although the bipolar spindles were normally produced. This finding was consistent with that we found in cells treated with m-calpain siRNA (Fig. 2C). Treatment with other calpain inhibitors, MDL28170, Z-LLY-CH2F, and SJA6017, showed similar effects on mitosis of HeLa cells (Fig. 3D), HCT116 cells, NIH/3T3 cells, and HEK293T cells (data not shown).

Calpain Inhibitor Activates Mad2-dependent Spindle Assembly Checkpoint—Metaphase-anaphase transition is driven by activation of ubiquitin-proteasome-mediated proteolysis, which is promoted by anaphase-promoting complex (APC). The spindle assembly checkpoint blocks the transition from metaphase to anaphase by inhibiting APC activity until all chromosomes are aligned at the metaphase plate and achieve bipolar attachment to the mitotic spindle. To investigate whether mitotic arrest induced by calpain inhibition is due to the activation of spindle assembly checkpoint, we first examined the APC activity in cells treated with calpain inhibitors. HeLa cells were synchronized at prometaphase by using the thymidine/nocodazole protocol, and released into medium containing Me2SO, Z-LLal, or MG132, a general proteasome inhibitor. In the cells released into normal medium (Me2SO) from nocodazole, both cyclin B and securin that are known substrates of APC were rapidly degraded. In contrast, in cells released into Z-LLal or MG132, levels of cyclin B and securin did not change at 3 h after the release from nocodazole (Fig. 4A), suggesting that APC is inactivated by calpain inhibition. To rule out the possibility that Z-LLal directly inhibits proteasome function, we examined the effect of Z-LLal on the stability of p53 protein, which is known to be degraded by an Mdm2-mediated ubiquitin/proteasome mechanism but not by the APC-dependent pathway. The p53 was stabilized by the treatment with MG132 but not by Z-LLal treatment (Fig. 4B).

APC activity is suppressed by the activation of spindle assembly checkpoint (22). To explore whether the checkpoint activation is involved in prometaphase arrest of the calpain-inhibited cells, we examined the recruitment of Mad2 protein to kinetochores; such recruitment is considered to occur at unattached kinetochores and is necessary for maintenance of spindle assembly checkpoint signaling. In cells arrested at prometaphase by Z-LLal-treatment, Mad2 was stained as paired dots at kinetochores of most of the misaligned chromosome, whereas aligned chromosomes were negative for Mad2 staining (Fig. 4C). The misaligned chromosomes contained Mad2-positive kinetochores, suggesting that these kinetochores were not properly captured by bipolar spindles. Furthermore, transfection of the cells with siRNA specific for Mad2 resulted in abrogation of Z-LLal-induced prometaphase arrest (Fig. 4D); the cells entered into mitosis and then quickly exited. In contrast, cells transfected with a control siRNA were arrested at prometaphase after exposure to Z-LLal. These findings suggest that the activation of calpain is required for proper microtubule attachment at kinetochores of misaligned chromosomes and, thereby, calpain inhibition results in the activation of the Mad2-dependent spindle assembly checkpoint, which leads to prometaphase arrest.

Calpain Inhibitor Has No Effect on Bipolar Spindle Formation—Spindle assembly checkpoint is activated by reagents such as nocodazole and taxol, which affect the stability of mitotic spindles. Therefore, we examined the status of mitotic spindles in cells arrested at prometaphase by calpain inhibi-
tors. Images obtained by fluorescence confocal microscopy showed that two spindle poles were positioned contralaterally and bipolar spindles formed normally when HeLa cells were treated with various calpain inhibitors including Z-LLal (Fig. 3D). These findings indicate that calpain inhibitors affected neither the separation of centrosomes nor the assembly of bipolar spindles.

To test whether calpain inhibitors alter the stability of mitotic spindle in the same manner as taxol, we added 5 μl/ml of Me₂SO (control), 50 μM of Z-LLal, 200 ng/ml of nocodazole, or 100 nM of taxol to HeLa cells pretreated with nocodazole or Z-LLal. In the presence of preceding nocodazole, additional Z-LLal treatment did not affect nocodazole-induced depolymerization of mitotic spindles while taxol markedly stabilized them (Fig. 4E, upper panels). Conversely, in the presence of preceding Z-LLal, additional nocodazole treatment resulted in complete depolymerization of mitotic spindles (Fig. 4E, lower panels), suggesting that Z-LLal did not stabilize mitotic spindles. These findings provide evidence that prometaphase arrest induced by calpain inhibitors is not caused by abnormalities in bipolar spindle formation and stability.

Chromosomes on Monopolar Spindles Were Clustered Adjacent to the Centrosome by Inhibition of Calpain—Proper chromosome alignment during mitosis is achieved via a complex
Role of m-Calpain in Chromosome Alignment

and variable series of movements that include the rapid poleward translocation of monoorienting chromosomes, oscillation of monooriented chromosomes toward and away from their attached spindle pole by poleward and polar ejection forces, and bipolar attachment followed by congression to the spindle equator (1, 23, 24). Microscopic analysis of cells arrested at prometaphase by treatments with m-calpain siRNA (Fig. 2C) and calpain inhibitors (Fig. 3D) showed that some chromosomes interact with microtubules derived from one of the two spindle poles and are closely localized to the pole. These findings prompted us to examine whether calpain activity is required to generate a polar ejection force during mitosis.

It has been shown previously that the polar ejection force can be monitored by examining chromosome position on monopolar spindles (5). Cells treated with monastrol, a specific Eg5 inhibitor, formed monopolar spindles due to failure of centrosome separation (25). Chromosomes formed a ring around the single pole, oriented in a “V” shape formed by poleward force at kinetochores and polar ejection force at their arms, and a chromosome-clear zone was seen widely at the center of the ring. The balance between poleward and polar ejection forces determines this chromosome position. Given that the polar ejection force is normally stronger than the poleward force in normal prometaphase cells, a chromosome-clear zone is evident (Fig. 5, A, upper panels, and B, left panel). However, cells treated with Z-LLal showed that chromosomes on monopolar spindles tended to cluster adjacent to the centrosome and did not orient in the “V” shape (Fig. 5, A, lower panels and B, right panel). Furthermore, we calculated the areas occupied by cytoplasm and chromosomes using images obtained by fluorescence confocal microscopy (Fig. 5E). The areas of chromosomes in cells treated with Z-LLal (average: 455.7 μm²) were significantly smaller than that in cells treated with Me₂SO (average: 619.8 μm²) (p < 0.0001, Student’s t test), although the areas of cytoplasm did not differ between cells treated with Z-LLal and Me₂SO (average: 1041.3 μm² and 1018.6 μm², p = 0.28) (Fig. 5F). Therefore, the ratio of chromosome area to cytoplasm was significantly smaller in Z-LLal-treated cells than control cells (average: 0.45 in Z-LLal-treated cells and 0.60 in control cells, p < 0.0001). The same effect was observed when HeLa cells were transfected with m-calpain siRNA and treated with monastrol (Fig. 5D). The ratio of chromosomal area to cytoplasm in each cell was significantly smaller in m-calpain-depleted cells than cells treated with control siRNA (average: 0.51 in m-calpain-depleted cells and 0.59 in control cells, p < 0.0001). These findings suggest that m-calpain inhibition suppresses the polar ejection force on the chromosome arms. We thus speculated that m-calpain is activated during mitosis and proteolyses a target molecule(s) that regulates the polar ejection force of mitotic spindles.

Calpain Activity Is Required to Maintain Chromosome Alignment at the Spindle Equator—In Xenopus egg extracts, the polar ejection force produced by chromokinesin Kid (Xkid) is required for maintenance of chromosome alignment on the metaphase plate (4). To investigate whether calpain activity is required, not only for chromosome congression on the metaphase plate but also for maintenance of chromosome align-
ment during metaphase, we treated HeLa cells with MG132 to induce metaphase arrest (Fig. 6A) (26) and subsequently treated with Z-LLal in the presence of MG132, followed by time-lapse fluorescence and DIC video microscopy. In cells treated with Me2SO in the presence of MG132, all chromosomes were aligned at the equator and arrested at metaphase for up to 14 h (data not shown). By contrast, the addition of Z-LLal into the medium containing MG132 rapidly induced impairment of chromosome alignment (Fig. 6B) and which never aligned again until the end of our observations (Fig. 6, C

**FIG. 4. Membrane-permeable calpain inhibitor induces prometaphase delay through activation of Mad2-dependent spindle assembly checkpoint.** A, HeLa cells were synchronized at prometaphase by the sequential thymidine/nocodazole protocol, then released into the media of the indicated reagents and lysed at the indicated time point. Samples were subjected to immunoblot analysis using antibodies against cyclin B, securin, or α-tubulin (loading control). B, HCT116 cells expressing wild-type p53 were treated with indicated reagents for 6 h and then subjected to immunoblot analysis using antibodies against p53, an APC-independent substrate of proteasome. α-Tubulin was analyzed as loading control. C, HeLa cells arrested at prometaphase by treatment with Z-LLal were fixed and stained with an antibody against Mad2 (green) and TOTO-3 (red) for DNA. Scale bar, 5 μm. D, HeLa cells transfected with siRNA targeting GL2 (control) or Mad2 were synchronized at G1-S boundary and released into medium containing Z-LLal. Phase contrast images of cells at 0 and 13 h after release are shown in left panels. The percentage of mitotic cells was determined (right panel). E, HeLa cells were preincubated with nocodazole for 8 h and treated with Me2SO (DMSO), taxol or Z-LLal for an additional 3 h in the presence of nocodazole (upper panels). Similarly, cells were incubated with Z-LLal for 8 h and treated with Me2SO (DMSO), nocodazole, or taxol (lower panels) in the presence of Z-LLal. Cells were stained for α-tubulin (green) and DNA (red). Scale bar, 5 μm.
Fig. 5. Treatment of calpain inhibitors perturbs polar ejection force on chromosomes. A, HeLa cells were treated with Me₂SO (DMSO) or Z-LLal in the presence of monastrol (MA) for 8 h. Cells were fixed and stained for α-tubulin (green) and DNA (red). Scale bar, 5 μm. B, cells treated with Me₂SO or Z-LLal in the presence of MA were fixed and stained with serum of CREST syndrome patient for kinetochores (green) and with propidium iodide for DNA (red). Scale bar, 5 μm. C, areas of cytoplasm (left) and chromosomes (center) in cells treated with MA+DMSO or MA+Z-LLal were calculated and plotted. In addition, the ratios of chromosome area to cytoplasm area in each cell were plotted (right). Statistical analysis (Student’s t test) was performed. D, areas of cytoplasm (left) and chromosomes (center) and the ratios of chromosome area/cytoplasm area (right) in cells treated with MA and indicated siRNA were calculated and plotted. Statistical analysis (Student’s t test) was performed. E, illustration indicates how the areas of chromosome and cytoplasm were calculated.
nuclear envelope breakdown, and oscillate about the spindle equator. Other chromosomes initially interact with microtubules at only one kinetochore, resulting in rapid chromosome movement toward one of the poles. Near the spindle pole, both sister kinetochores capture spindles from the same pole. This monopolar attachment is corrected by activating the Aurora B kinase-INCENP complex until one of the kinetochores is captured by microtubules from opposite pole to form bipolar attachment (27). Polar ejection force is required for the monoriented chromosomes to move away from their attached spindle pole to facilitate bipolar attachment, followed by congression to the spindle equator. Impairment of polar ejection forces may induce frequent detachment of kinetochores from microtubules until bipolar attachment is formed. This might trigger the Mad2-dependent spindle assembly checkpoint, which causes prometaphase arrest in cells disrupted m-calpain function.

We propose that m-calpain plays a role in generating polar ejection forces to promote chromosome alignment for the following reasons. First, both cells depleted m-calpain by RNAi and treated with calpain inhibitor failed to congress their chromosomes to the metaphase plate. Second, cells treated with calpain inhibitor in the presence of monastrol demonstrated that the spreading area of chromosomes on monopole was reduced and chromosomes did not orient in a typical "V" shape. This phenotype is similar to those observed in cells microinjected with antibodies against Kid (5), which has been lately demonstrated to be a major player for generating polar ejection force (3–5). We have recently observed that m-calpain proteolyses recombinant Kid protein in vitro (data not shown). This finding together with the reported finding that overexpression of Kid disrupts chromosome alignment (7) supports the hypothesis that m-calpain may regulate turnover of Kid protein, which is required for proper chromosome congression.

Arthur et al. (28) proposed that conventional calpains are potentially essential for embryonic development, but not for cell growth and division, by analyzing mouse embryonic fibroblast cells (MEFs) derived from mice lacking the Capn4 gene (28). Since Capn4 is a common small regulatory subunit for both m- and v-calpains, Capn4−/− MEFs potentially lack the activities of conventional calpains. Discrepancy between our findings and their results obtained using Capn4−/− MEFs may be explained by the recent experimental observation that the conventional calpain can be activated without binding of Capn4 (29). This hypothesis is supported by our findings that neither Capn4 RNAi nor the treatment of PD150606, an inhibitor of Capn4, shows significant effect on mitotic progression of HeLa cells (data not shown). It is thus conceivable that inhibition of calpain 2, a large subunit of m-calpain, may lead to biological responses different from those induced by inhibition or depletion of Capn4 in mammalian cells.

Proteolysis is a key event in control of the cell cycle. Increasing evidence has revealed that changes in intracellular Ca^{2+} concentrations also have a crucial role in the progression of the cell cycle, particularly mitosis. Therefore, it has been suggested that the activation of Ca^{2+}-dependent protease m-calpain is essential to mitotic progression, although the substrates of m-calpain during mitosis have been unknown (9, 15, 30). Our data demonstrated that m-calpain is required to establish bioriented chromosomes during prometaphase by regulating polar ejection forces. To identify the specific substrates of m-calpain in mitosis provides molecular insight as to how calcium signaling regulates chromosome stability during mitosis.

Acknowledgments—We thank Drs. M. Nakao, T. Mimori, H. Kuwahara, T. Hara, K. Matsuhashi, S. Iida, T. Ozawa, D. Zhang, Y. Ota, and I. Sakaguchi (Kumamoto University) for helpful discussion and technical advice; Dr. T. C. Saido (Brain Science Institute, RIKEN, Japan) for critical reading of the manuscript; Drs. G. M. Wahl (The Salk

**FIG. 6. Requirement of calpain activity for maintenance of chromosome alignment at the spindle equator.** HeLa cells stably expressing the H2B-GFP were pretreated with MG132 until all chromosomes aligned at the spindle equator (A), and then Z-LLal (50 μM) was added to the medium at 1.5 h after the start of observation by time-lapse fluorescence and DIC video microscopy (A–D). Numerals on the figure indicate time (hours:minutes) from the start of observation. and D). The final phenotype appeared to be very similar to that observed in cells treated with the calpain inhibitor alone (Fig. 3D). These results indicated that chromosome alignment at metaphase is not static and requires m-calpain activity to be maintained.

**DISCUSSION**

We have shown that the amount of m-calpain increases in G_{2–M} phase in HeLa cells, while μ-calpain is almost static throughout the cell cycle. Cells treated with siRNA oligos against m-calpain, not μ-calpain, accumulated in mitosis with chromosomes failed to align at the spindle equator. Atypical larger lobulated nuclei, possibly resulted from aberrant mitotic spindles to kinetochores of several chromosomes near spindle poles. Some chromosomes biorient immediately upon congress to the spindle equator a chromosome must biorient, i.e. attach to spindle microtubules with each kinetochore interacting with microtubules derived from one of the two spindle poles. Some chromosomes biorient immediately upon...
Institute, La Jolla) and T. Kanda (Hokkaido University, Japan) for providing the H2B-GFP expression vector; Y. Fukushima and T. Arino for secretarial assistance. We are also grateful to members of the Gene Technology Center in Kumamoto University for their important experimental contributions.

REFERENCES
1. Kapoor, T. M., and Compton, D. A. (2002) J. Cell Biol. 157, 551–556
2. Tokai, N., Fujimoto-Nishiyama, A., Toyoshima, Y., Yonemura, S., Tsukita, S., Inoue, J., and Yamamota, T. (1996) EMBO J. 15, 457–467
3. Funahiki, H., and Murray, A. W. (2000) J. Cell Biol. 150, 975–988
4. Kapoor, T. M., Mayer, T. U., Coughlin, M. L., and Carafoli, E. (1998) Cell Calcium 23, 123–130
5. Cox, E. A., and Huttenlocher, A. (1998) Microsc. Res. Tech. 43, 412–419
6. Bhatt, A., Kaverina, I., Otey, C., and Huttenlocher, A. (2002) J. Cell Sci. 115, 3415–3425
7. Wang, K. K., Posmantur, R., Nadimpalli, R., Nath, R., Mohan, P., Nixon, R. A., Talanian, R. V., Reece, B., and Saltsman, J. A. (1998) Arch. Biochem. Biophys. 356, 187–196
8. Kimura, Y., Koga, H., Araki, N., Mugita, N., Fujita, N., Takehisa, H., Nishi, T., Yamashima, T., Saito, T. C., Yamasaki, T., Moritake, K., Saya, H., and Nakas, M. (1998) Nat. Med. 4, 915–922
9. Xu, Y., and Mellgren, R. L. (2002) J. Biol. Chem. 277, 21474–21479
10. Santarella, L. (1998) Biochem. Biophys. Res. Commun. 244, 317–324
11. Carafoli, E., and Molinari, M. (1998) Biochem. Biophys. Res. Commun. 247, 193–203
12. Yamaguchi, R., Maki, M., Hatanaka, M., and Sabe, H. (1994) FEBS Lett. 356, 114–116
13. Lane, R. D., Allan, D. M., and Mellgren, R. L. (1992) Exp. Cell Res. 203, 5–16
14. Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T. (2001) Nature 411, 494–498
15. Nitta, M., Tsuiki, H., Arima, Y., Harada, K., Nishizaki, T., Sasaki, K., Mimori, T., Ushio, Y., and Saya, H. (2002) Genes Cells 7, 151–162
16. Hirota, T., Morisaki, T., Nishiyama, Y., Marumoto, T., Tada, K., Hara, T., Masuko, N., Inagaki, M., Hatakeyama, K., and Saya, H. (2000) J. Cell Biol. 149, 1073–1086
17. Bhatt, A., Kaverina, I., Otey, C., and Huttenlocher, A. (2002) J. Cell Biol. 154, 707–717
18. Arthur, J. S., Elce, J. S., Hegadorn, C., Williams, K., and Greer, P. A. (2000) Mol. Cell. Biol. 20, 4474–4481
19. Hata, S., Sorimachi, H., Nakagawa, K., Maeda, T., Abe, K., and Suzuki, K. (2001) FEBS Lett. 501, 111–114
20. Sherwood, S. W., Kung, A. L., Roitelman, J., Simon, R. D., and Schimke, R. T. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 3353–3357
Activation of m-Calpain Is Required for Chromosome Alignment on the Metaphase Plate during Mitosis
Shinobu Honda, Tomotoshi Marumoto, Toru Hirota, Masayuki Nitta, Yoshimi Arima, Michio Ogawa and Hideyuki Saya

J. Biol. Chem. 2004, 279:10615-10623.
doi: 10.1074/jbc.M308841200 originally published online December 19, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M308841200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 30 references, 14 of which can be accessed free at
http://www.jbc.org/content/279/11/10615.full.html#ref-list-1