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Carboxyl Terminus of Mitosin Is Sufficient to Confer Spindle Pole Localization

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Abstract Mitosin is a nuclear protein of 3,113 amino acids which has been shown to associate with the mitotic apparatus, especially the kinetochore, during mitosis. In this paper we further confirmed its association with the spindle poles in normal monkey kidney CV1 cells by indirect immunofluorescence microscopy. When the carboxyl portion of mitosin containing amino acids 2,094–3,113 (named mitosin-pTN) was stably expressed in rat fibroblast Rat2 cells using a tetracycline-inducible system, strong spindle pole association was observed in addition to expected centromere localization. The same results were achieved in Chinese hamster ovary (CHO) cells. On the other hand, mitosin-pTC containing amino acids 2,756–3,113 was not targeted to spindle poles. Use of the FLAG epitope [Hopp et al., 1988] genetically fused to each amino terminus of these mutants eliminated possible artifacts due to antibody cross-reaction, since the spindle pole localization of wild-type mitosin was confirmed with a FLAG-tagged mutant by an antibody (anti-FLAG M2 monoclonal antibody) irrelevant to antibodies to mitosin. Our data also suggested a possible interaction of mitosin with the spindle microtubules. Interaction of mitosin with the major parts of the mitotic apparatus further implies an important role in mitosis. J. Cell. Biochem. 66:441–449, 1997.

Key words: mitosin; CENP-F; spindle pole; kinetochore; centromere

The mitotic process is one of the most intriguing cellular events. In eucaryotes, mitosis is exerted through a highly conserved mitotic apparatus consisting of the spindle of microtubules, a pair of microtubule-organizing centers (MTOCs), and the kinetochores of chromosomes. In animals, MTOC takes the form of the centrosome, an organelle consisting of a pair of centrioles and a cloud of pericentriolar material (PCM). Microtubules are assembled from a ring-shaped template of γ-tubulin located in PCM [Zheng et al., 1995; Moritz et al., 1995]. The centrosome is a crucial organelle in many microtubule-based cellular events, including mitosis, cell locomotion, and cytoplasmic organization [for reviews see Mazia, 1987; Vorobjev and Nadezhdina, 1987; Kellogg et al., 1994]. Centrosomes duplicate during S phase and move in opposite directions to form spindle poles during mitosis. Numerous studies have been done to investigate the molecular organization of the centrosome so that the molecular mechanism of its function can be approached. Many proteins have been found to be localized to the centrosome either ectopically or transiently [for reviews see Kalt and Schliwa, 1993; Kellogg et al., 1995; Bouckson-Castaing et al., 1996; Mcnally et al., 1996]; some of them have been shown to be directly involved in centrosome functions [Doxsey et al., 1994; Tugendreich et al., 1995; Zheng et al., 1995; Moritz et al., 1995; Boleti et al., 1996; Walczak et al., 1996].

The kinetochore, located at the centromere of the chromosome, is a trilayer structure resolved by electron microscopy (EM). Under EM, spindle microtubules are found to be attached to the outer layer of the kinetochore. Disruption of the microtubule-kinetochore connection either biochemically, mechanically, or genetically impairs subsequent chromosome segregation. The kinetochore is therefore thought to be a critical cellular organelle for mitosis [reviewed

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by Rieder, 1982; Ault and Rieder, 1994; Pluta et al., 1995]. Several components of the mammalian kinetochore have been molecularly characterized [reviewed by Bloom, 1993; Pluta et al., 1995]. Some of them turn out to be associated with the kinetochore throughout the cell cycle—for instance, CENP-A [Palmer et al., 1991], CENP-B [Cooke et al., 1990] and CENP-C [Saitoh et al., 1992]. Others are associated with the kinetochore transiently. CENP-E [Yen et al., 1992], mitosin [Zhu et al., 1995a], CENP-F [Liao et al., 1995], and dynein [Steuer et al., 1990] fall into the latter category.

Mitosin was originally cloned by its interaction with retinoblastoma protein (Rb) in vitro [Shan et al., 1992; Zhu et al., 1995a]. It is basically identical to CENP-F [Liao et al., 1995], except that the existence of the first tandem repeat in CENP-F is not supported by our sequence for mitosin. We here refer to it as mitosin/CENP-F when shared properties are discussed. Mitosin/CENP-F is expressed in a cell cycle–dependent manner. In G1 phase, it is hardly detectable by both immunoblotting and indirect immunofluorescence (IIF) microscopy. It is detected in coincidence with S phase entry and is hyperphosphorylated after the G2/M transition [Zhu et al., 1995a]. At the end of M phase, it is quickly degraded. Mitosin/CENP-F is a nuclear protein during interphase. It is redistributed to prekinetochores from late G2 or early prophase and remains at the kinetochores until late anaphase. After this point, a portion of mitosin/CENP-F is located within the region resembling antiparallel spindle fibers. At the end of cytokinesis, it is concentrated at the midbody in trace amounts. Mitosin/CENP-F is a component of the outer kinetochore plate [Rattner et al., 1993; Zhu et al., 1995b]. Furthermore, the carboxyl-terminal portion of mitosin from amino acids 2,094–3,113 contains domains required for its in vitro Rb binding, nuclear localization, centromere localization, and dimerization [Zhu et al., 1995a,b].

In this paper, we further confirmed that mitosin was also associated with the spindle poles in M phase. Interestingly, the expressed deletion mutant "mitosin-pTN" showed strong spindle pole localization. Our results further imply the importance of this protein in mitosis.

METHODS

Maintenance of Mammalian Cell Lines

Normal monkey kidney CV1 and Chinese hamster ovary (CHO) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (GIBCO, Shanghai, China) supplemented with 10% calf serum (Sijiqin Company, Hangzhou, China) in an environment with 5% CO_2. Rat fibroblast Rat2 was maintained in DMEM containing 10% fetal calf serum (GIBCO). G418-resistant cell strains were maintained in appropriate media containing 0.2 mg/ml G418.

Antibody Production

Fusion proteins between glutathione S-transferase (GST) and mitosin, named GST-10Stu (containing amino acids 1,759–2,093 of mitosin) and GST-10Bgl (containing amino acids, 2,092–2,487) [Zhu et al., 1995a], were purified as described previously. Protein (100 μg) was used to immunize rabbits using standard procedures. The resulted antisera were designated as αRstu and αRbgl, respectively. The specificity of antisera was tested using both IIF staining and immunoblotting.

Indirect Immunofluorescence Studies

Cells for IIF staining were grown on coverslips for 20 h. All samples were fixed in methanol at −20°C for 15 min. To remove soluble fractions, we treated CV1 cells for 1 min at room temperature with the extraction buffer (10 mM MOPS, pH 6.8, 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 1 mM EGTA, 0.5% Triton X-100) before fixation in methanol. Procedures previously described for IIF staining [Zhu et al., 1995a] were followed. Among the primary antibodies used were anti-FLAG M2 mAb (IBI) (final concentration 3 μg/ml), rabbit antimitosin polyclonal antibody α10C (final concentration 2 μg/ml of purified IgG) [Zhu et al., 1995a,b], rabbit antimitosin polyclonal antisera αRstu (1:100) and αRbgl (1:500), and antitubulin mAb (1:20,000) (Sigma, St. Louis, MO). These antibodies were used either alone or in appropriate combination, according to the purpose of experiment. Fluorochrome-labeled secondary antibodies, Texas Red–conjugated donkey antirabbit IgG (Amersham, Arlington Heights, IL), and FITC-conjugated goat antimouse IgG (Biosource International, Inc., Camarillo, CA) were diluted 1:50 and used either separately or in combination when discrimination of immunostaining by different primary antibodies was required. In all cases, nuclear DNA and chromosomes were stained using the DNA-specific fluorescent dye 4′,6-diamidino-2-phenylindole (DAPI) (0.5 μg/ml) (Sigma). PermaFluor Aqueous Mountant (Lipshaw Immunon, Inc., Pitts-

burgh, PA) was applied to mount the coverslips on slides. Images were recorded using Kodak (Melbourne, Australia) Gold III (ASA 400) or Lucky Pan China (Lucky Film Corp., Baoding, China) SHD400 film under an Olympus (Tokyo, Japan) BX50 fluorescence microscope equipped with three excitation cubes (U-MWU, U-MWB, and U-MWG) and a PM-30 exposure control unit.

Plasmid Constructs

The vector pUHD10-3 for the tetracycline-inducible system [Gossen and Bujard, 1992] was modified to fit our experiment. pUHD10-3 was cleaved with Sma I, and the 160 bp fragment containing the minimal promoter was isolated. Another aliquot of pUHD10-3 was double-cleaved with Sac I and Sma I, followed by treatment with mung bean nuclease (New England Biolabs, Beverly, MA) to remove the single-strand terminus. The 3 Kb fragment was isolated and ligated to the 160 bp fragment. The resulted plasmid, termed pUHD20, was equivalent to pUHD10-3 but possessed unique Kpn I and Sma I sites.

Plasmid pTN was constructed by cloning the 3.3 Kb Kpn I–BamH I fragment from EΔN [Zhu et al., 1995b] into pUHD20 linearized with the same restriction enzymes. pTN thus contained sequences coding for the C-terminal portion of mitosin from amino acids 2,094–3,113 which was fused to the C-terminus of the FLAG epitope [Hopp et al., 1988]. The expressed FLAG-mitosin fusion protein from this construct was designated as mitosin-pTN to simplify description in text. The predicted molecular mass of mitosin-pTN is approximately 120 kDa.

Plasmid pTC was constructed as follows. First, the cohesive ends generated by Sac I cleavage of pTN were removed by treatment with T4 DNA polymerase (Promega, Madison, WI). After further digestion with BamH I, the 1.3 Kb fragment encoding amino acids 2,756–3,113 of mitosin was isolated. Second, the vector was prepared by cleavage of pTN with Nhe I. The cohesive ends were filled-in before cleavage with BamH I. The 3.1 Kb vector fragment was isolated, which contained sequences coding for the FLAG epitope immediately upstream of the Nhe I end. Both the 1.3 and 3.1 Kb fragments were ligated together so that the mitosin cDNA was fused in frame with the sequence coding for the FLAG tag. The Nhe I site was restored in the resulting plasmid pTC. The expressed fusion protein was termed mitosin-pTC (Mr ~43 kDa).

Both pTN and pTC were sequenced to ensure correct ligation between the FLAG-coding sequence and mitosin cDNA.

Transfection and Selection of Stably Transfected Cells

Rat fibroblast Rat2 or CHO cells were transfected with 20 μg of pTN or pTC in combination with 2 μg of p15-1neo (modified by P.L. Chen and W.-H. Lee by inserting a G418-resistant gene into p15-1, one of the two plasmids required for the tetracycline system) using the calcium phosphate method. Cells were subsequently cultured in presence of G418 (GIBCO) (0.8 mg/ml for Rat2 and 1 mg/ml for CHO) for 3 weeks. G418-resistant colonies were either subcloned or cultured as a whole to test inducible expression of the FLAG-tagged mitosin by both IIF microscopy and immunoblotting. Tetracycline (1 μg/ml) was always included in the culture medium until expression of exogenous mitosin was required [Gossen and Bujard, 1992]. G418-resistant cells were maintained in presence of G418 (0.2 mg/ml).

Immunoblotting

Cell samples were lysed directly in SDS-loading buffer and subjected to 10% SDS-PAGE. Proteins were electrophoretically transferred to Immobilon-P membrane (Millipore, Bedford, MA), and immunoblotting was performed as described [Zhu et al., 1995a] using anti-FLAG M2 antibody (3 μg/ml). Results were visualized either using NBT/BCIP or by enhanced chemiluminescence (ECL) (Amersham), depending on the use of alkaline phosphatase–conjugated IgG (Promega) or horseradish peroxidase–conjugated IgG (Amersham) as secondary antibody.

RESULTS

Immunofluorescence Study of Endogenous Mitosin in CV1 Cells

Mitosin/CENP-F has been shown to be a component of the kinetochore during early stages of mitosis [Zhu et al., 1995a; Liao et al., 1995]. Detailed examination of CV1 cells also suggests its distribution at the spindle poles, since two distinct foci were noticed symmetrically on both sides of the metaphase plate (Fig. 1A, panels 3,4) [Zhu et al., 1995a]. The intensities of these two foci varied among different mitotic cells examined (data not shown), probably due to background fluorescence of cytoplasmic mitosin

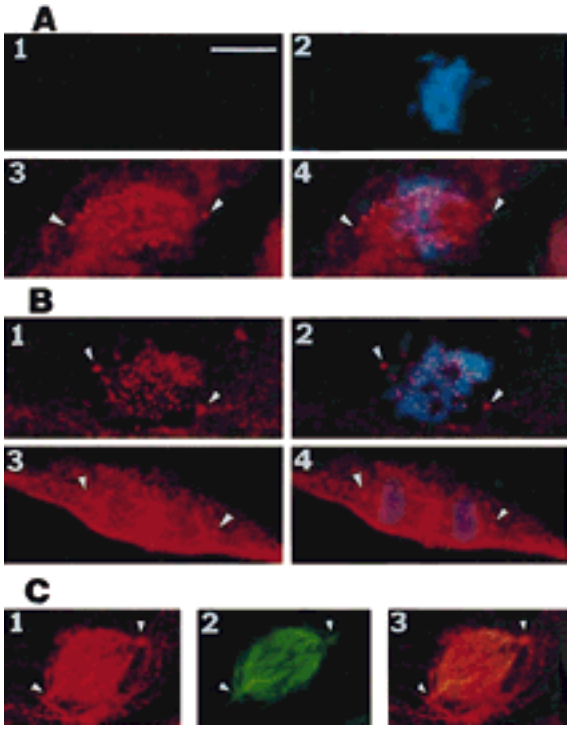

Fig. 1. IIF staining of both centromeres and spindle poles by antimitosin antibody. Monkey kidney CV1 cells growing on coverslips were directly fixed in cold methanol (**A**) or detergent-extracted to remove soluble fractions before methanol fixation (**B,C**). Fixed cells were subjected to IIF microscopy. Chromosomes were stained with DNA-specific fluorescent dye DAPI (blue color). Spindle poles were indicated by arrow heads. **A:** Immunostaining of intact mitotic CV1 cells using a preimmune serum (**panel 1**) and rabbit antimitosin antibody αRbgl (**panel 3**). Superimposed images of immunostaining (red) and chromosomes (blue) were shown in **panels 2** and **4,** respectively. Images in panels 1, 2 were overexposed one stop to show background staining. **B:** IIF staining of CV1 cells extracted with detergent. **Panels 1,2:** A cell at early metaphase. **Panels 3,4:** A cell at late anaphase. Also note the staining at the region of the metaphase plate in panels 3,4. **C:** Colocalization of mitosin with the spindle of microtubules. Mitosin (red) was stained using αRbgl and Texas Red–conjugated donkey antirabbit IgG (**panel 1**). Please note that some microtubule staining (which resembled the spindle) leaked into this channel, probably due to filter combination and strong microtubule fluorescence. Microtubules (green) were simultaneously stained using antitubulin mAb and FITC-conjugated goat antimouse IgG (**panel 2**). Spindle poles revealed in panel 1 and panel 2 were superimposed with each other (**panel 3**). Scale bar, 10 μm.

and/or epitope masking. These two foci were distinguished from the centromere fluorescence of mitosin by their failure to colocalize with chromosomes (Fig. 1A, panels 3,4). On the other hand, preimmune serum did not show such a staining pattern (Fig. 1A, panels 1,2), even when the images were overexposed.

To verify this notion, CV1 cells were extracted with salt and detergent to remove soluble fractions prior to IIF staining. As shown in Figure 1B, two bright foci distinct from the centromere staining were readily visible in all mitotic cells, especially in those at stages of the cell cycle from early metaphase (Fig. 1B, panels 1,2) to early anaphase (data not shown). It appeared that each focus consisted of a pair of closely adjacent dots. After anaphase, both foci appeared to be gradually overwhelmed by cytoplasmic staining (Fig. 1B, panels 3,4). The punctate staining in areas other than the spindle apparatus might reveal mitosin binding to other cellular organelles after removal of the soluble fraction or simply reflect a nonspecific background since a similar pattern was also observed in the cytoplasm of interphase cells subjected to the same extraction (data not shown). Consistent with previous results [Liao et al., 1995; Zhu et al., 1995a], immunofluorescence at the central region of spindle appeared at late anaphase, resembling the region of antiparallel spindle microtubules nucleated from both asters (Fig. 1B, panels 3,4).

Although both foci indicated in Figure 1A (panels 3,4) and 1B resembled spindle poles, a further experiment was performed to colocalize them with the spindle formed by the microtubule (Fig. 1C). Extracted CV1 cells were immunostained with both the rabbit αRbgl and a mouse antitubulin mAb. Texas Red–coupled antirabbit IgG was used to indicate the cellular distribution patterns of mitosin (Fig. 1C, panel 1), whereas FITC-coupled antimouse IgG was applied simultaneously to reveal distribution patterns of tubulin (Fig. 1C, panel 2). When both images were superimposed together, the indicated foci revealed by antibodies to mitosin indeed fell into the poles of microtubule spindle (Fig. 1C, panel 3). Though the excitation cubes in the fluorescence microscope were not optimal for such a dual staining (which resulted in slight leakage of microtubule images into the channel for Texas Red), the result was still conclusive.

In prophase, the spindle pole staining mingled with the centromere fluorescence. Both were basically indistinguishable. Colocalization with microtubules at prophase, however, confirmed that the spindle poles were also decorated by antibodies to mitosin (data not shown).

Behaviors of the Carboxyl Portion of Mitosin in Rat2 Cells

To further confirm that the spindle pole staining described above was indeed due to mitosin, we examined the behaviors of exogenously expressed mitosin in rat fibroblast Rat2 cells. The following concerns were considered in experiment design. 1) The FLAG epitope [Hopp et al., 1988] was genetically fused to the amino-terminus of each mitosin mutant. Since anti-FLAG M2 mAb (IBI) does not cross-react with mitosin [Zhu et al., 1995a,b], results obtained with this irrelevant antibody would answer whether antimitosin antibodies had somehow cross-reacted with other centrosomal protein(s). 2) The tetracycline-inducible system [Gossen and Bujard, 1992] was used to reduce the possibility of cell-cycle arrest caused by overexpression of mitosin [Zhu et al., 1995a]. 3) The promoter in the tetracycline system is weaker than the cytomegalovirus (CMV) early gene promotor used in previous study [Zhu et al., 1995a,b]. Less amount of expression may enable us to study high affinity targets of mitosin in cells.

A stable cell line, TNb22, was selected from G418-resistant Rat2 colonies cotransfected with pTN and p15-1neo. Immunoblotting showed that it expressed a protein of ~140 kDa upon induction of expression by removing tetracycline from the culture medium for 12 h (Fig. 2A, lanes 1,2). In fact, expression was detected in as early as 4 h after removal of tetracycline (Fig. 2B, lane 3). The expression level was stable after 8–12 h (Fig. 2B, lanes 4–6). There was virtually no leakage of expression even when results by ECL were overexposed (Fig. 2B, lanes 1,2). Although the induced protein migrated at approximately 140 kDa while the calculated molecular mass of mitosin-pTN was 120 kDa, we believed that TNb22 was a correct cell line for the following reasons. First, there are cases in which apparent molecular weights of proteins are slightly different from their calculated ones. Second, as confirmed by sequencing, the reading frame of FLAG-coding sequence was indeed followed by that of mitosin cDNA. Third, restriction analysis ruled out possible insertion of a foreign DNA into mitosin sequences. Fourth, the behaviors of this protein resembled those of wild-type mitosin (Fig. 3).

Indirect immunofluorescence staining further proved that TNb22 expressed correct FLAG-mitosin fusion protein (Fig. 3). In presence of tetracycline, no immunostaining was detectable using both anti-FLAG M2 (Fig. 3, panel 1) and antimitosin α10C [Zhu et al., 1995a] (Fig. 3, panel 2) antibodies. This result also indicated that α10C did not recognize the rat mitosin homologue to a significant extent. After removal of tetracycline for 12 h, strong nuclear fluorescence was observed (Fig. 3, panels 4,5). Since the nuclear targeting signal of mitosin has been included in construct pTN [Zhu et al., 1995b], the consistency of the experimental result with the predicted one made it convincing that the desired cell strain was obtained.

To our surprise, strong and typical spindle pole localization was observed in mitotic cells of TNb22 after induction (Fig. 3, panels 7–12). Both the antimitosin and anti-FLAG antibody gave the same results whether they were used simultaneously (Fig. 3, panels 7–12) or separately (data not shown). Although only results from metophase and anaphase are shown, the spindle pole staining was actually observed from

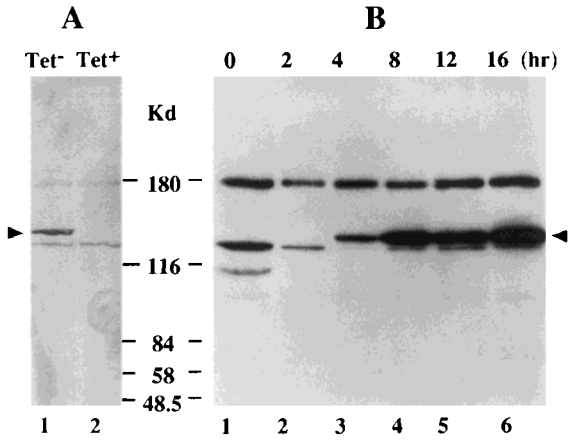

Fig. 2. Stable expression of mitosin-pTN in Rat2 cells. The rat fibroblast Rat2 cells were cotransfected with pTN and p15-1neo to express the C-terminal one-third of mitosin under control of the tetracycline-inducible system. After G418 selection for 3 weeks, drug-resistant colonies were tested for expression. One such clone, TNb22, was analysed. Cells maintained in DMEM containing 1 μg/ml of tetracycline overnight were fed with fresh medium with or without tetracycline for indicated periods of time. Cells were then lysed in SDS-loading buffer. The cellular proteins were subjected to 10% SDS-PAGE and immunoblotting using anti-FLAG M2 monoclonal antibody. **A:** *Lane 1:* A protein of ~140 kDa (indicated by an *arrowhead*) was inducibly expressed after removal of tetracycline for 12 h. *Lane 2:* No such protein was detectable in control cells without induction. **B:** The levels of mitosin-pTN after induction for indicated periods of time. This blot was visualized by ECL and overexposed to check background synthesis (leakage). Nonspecific immunostaining of other proteins also appeared, especially when overexposed.

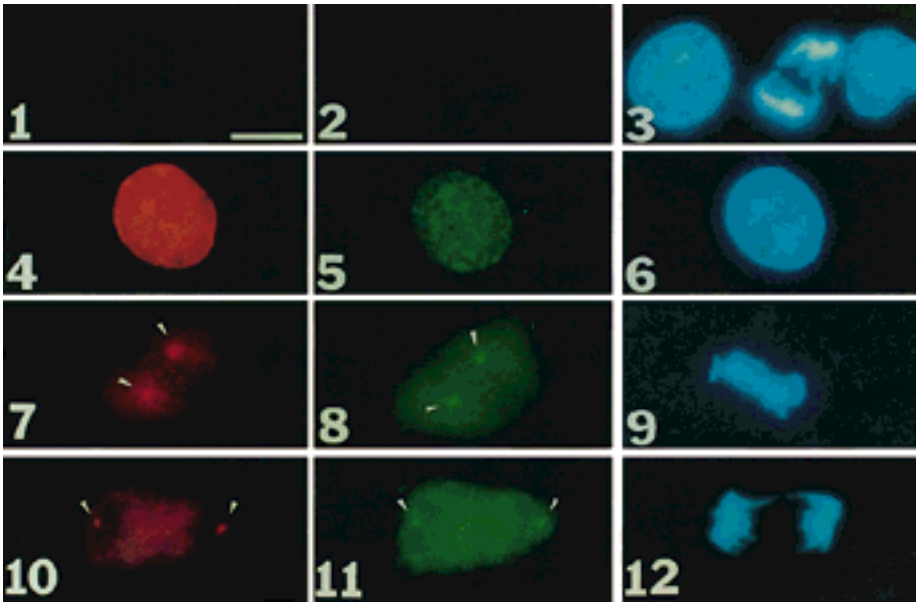

Fig. 3. Mitosin-pTN is targeted to the spindle poles in Rat2 cells. TNb22 cells were grown on coverslips in DMEM with (**panels 1–3**) or without tetracycline (**panels 4–12**) for 12 h before fixation using cold methanol. Indirect immunofluorescence staining was performed using a combination of anti-FLAG M2 antibody and rabbit antimitosin α10C. The FLAG epitope was visualized as red fluorescence by donkey anti-mouse IgG–Texas Red conjugate (panels 1,4,7,10) and human mitosin-pTN as green fluorescence by sheep antirabbit IgG-FITC conjugate (panels 2,5,8,11). Nuclear and chromosomal DNA were stained with DAPI (panels 3,6,9,12). Panels 1–3: Uninduced cells immunostained as described. Panels 4–6: Nuclear staining of an interphase cell expressing mitosin-pTN. Panels 7–9: Strong spindle pole staining (indicated by *arrowheads*) of a metaphase cell expressing mitosin-pTN. Centromere staining is also visible at the metaphase plate. Panels 10–12: Spindle pole staining (arrowheads) of an anaphase cell. Scale bar, 20 μm.

prometaphase to cytokinesis (data not shown). In addition to the spindle pole localization, mitosin-pTN was also targeted to the centromere as expected [Zhu et al., 1995b], appearing as dot speckles at the metaphase plate (Fig. 3, panels 7,8). The intensity of spindle pole fluorescence was much stronger than that of centromere fluorescence. In primate cells, however, wild-type mitosin exhibited stronger centromeric fluorescence (Fig. 1) [Zhu et al., 1995a].

Expression of Exogenous Mitosin in CHO Cells

To demonstrate that the spindle pole localization of exogenous mitosin was not restricted to Rat2 cells only, we tested Chinese hamster ovary (CHO) cells. G418-resistant colonies were cultured as a whole population. As a result, the percentage of positive cells increased about tenfold comparing to those obtained from transiently transfected populations. Consistent with previous results, expressed mitosin-pTN migrated as a 140 kDa protein by immunoprecipitation using the anti-FLAG M2 antibody (data not shown). It was localized to the nucleus in CHO cells, while untransfected cells failed to be immunologically stained (Fig. 4, panels 1,2). Both centromere and strong spindle pole staining were readily observed in metaphase cells (Fig. 4, panels 3,4). Fluorescence at spindle poles persisted through the following stages of M phase (Fig. 4, panels 5–8). Furthermore, the staining appeared as a pair of closely spaced bright dots (Fig. 4, panels 3,5,7), a morphology also observed for wild-type mitosin in CV1 cells (Fig. 1). At the end of cytokinesis, mitosin-pTN appeared both at the spindle pole regions and in nuclei of daughter cells (Fig. 4, panels 7,8). This is astonishing because the wild-type mitosin/CENP-F is degraded at this point and is not recruited into daughter nuclei [Zhu et al., 1995a; Liao et al., 1995]. A possible explanation is that a degradation signal may exist in mitosin/CENP-F and is deleted in mitosin-pTN. In interphase, however, mitosin-pTN was not detected at the position of microtubule-organizing centers when costained using antitubulin antibody (data not shown).

We then conducted a further experiment to exclude the possibility that any FLAG-fusion protein might be targeted to the spindle pole. In

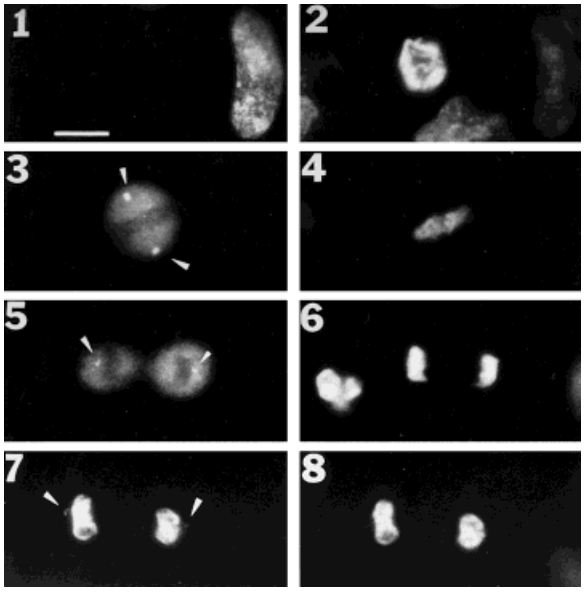

Fig. 4. Mitosin-pTN is also localized to spindle poles in CHO cells. CHO cells stably cotransfected with pTN and p15-1neo were induced for 16 h to express mitosin-pTN before fixation. IIF staining was performed using anti-FLAG M2 mAb and goat antimouse IgG-FITC conjugate (**panels 1,3,5,7**). Chromasomal and nuclear DNA was stained with DAPI (**panels 2,4,6,8**). Immunostaining foci at spindle poles are indicated by *arrowheads.* Subcellular localization of mitosin-pTN in cells at interphase (panel 1), metaphase (panel 3), telophase (panel 5), and late cytokinesis (panel 7) is shown. Centromere staining can also be noticed at the metaphase plate in panel 3. Cells not expressing mitosin-pTN (including a mitotic cell) are also included in panels 1,2 as negative controls. Scale bar, 10 μm.

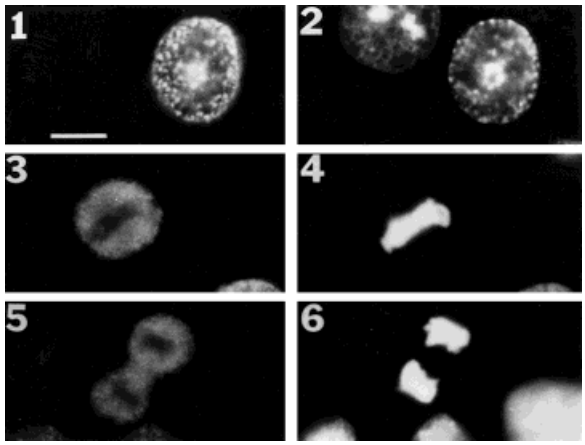

Fig. 5. Mitosin-pTC is not targeted to the spindle poles. CHO cells were cotransfected with pTC and p15-1neo. Transfected cells were selected and treated as described in Fig. 4. The subcellular localization of mitosin-pTC in cells at different stages of the cell cycle is illustrated in panels 1, 3, and 5, while corresponding cells stained with DAPI are shown in panels 2, 4, and 6, respectively. **Panels 1,2:** Nuclear staining of mitosin-pTC. **Panels 3–6:** Absence of spindle pole staining in a metaphase cell (panels 3,4) and an anaphase cell (panels 5,6) expressing mitosin-pTC. All possible planes of focus were examined. Cells not expressing mitosin-pTC were also included in different panels as negative controls. Scale bar, 10 μm.

a series of constructs, plasmid pTC was designed to express the portion of mitosin only from amino acids 2,756–3,113 (mitosin-pTC), which was approximately one-third the size of mitosin-pTN. It was also a nuclear protein, as expected (Fig. 5, panels 1,2). When mitotic cells expressing mitosin-pTC were examined, however, no fluorescence characteristic of the spindle pole staining was visible (Fig. 5, panels 3–6). According to this result, the spindle pole staining revealed previously was indeed due to certain region(s) of mitosin, not the FLAG peptide.

DISCUSSION

As a protein transiently associated with centromeres during mitosis, mitosin/CENP-F is probably implicated in chromosome segregation [Zhu et al., 1995a; Liao et al., 1995]. Here we provide further evidence to show that mitosin is also a spindle pole–associated protein. First, CV1 cells exhibited clear spindle pole fluorescence by IIF microscopy using antimitosin antibodies. Second, a deleted mutant (mitosin-pTN) expressed in both Rat2 and CHO cells conferred spindle pole localization. Why mitosin-pTN accumulates more at spindle poles compared to centromeres than its wild type, however, is presently not known. Both the wild-type mitosin and the mutant appeared as a closely spaced doublet at each spindle pole. Although this typical morphology resembles the pair of centrioles, further experiments by immunogold electron microscopy are required to understand the precise position of mitosin in spindle poles.

The properties of mitosin/CENP-F imply it is a microtubule-associated protein. Mitosin/CENP-F displays dynamic distribution at the centromere, spindle pole, and spindle midzone in M phase. Distribution along the polar spindle fibers was also observed (data not shown). Its localization at the outer kinetochore plate [Rattner et al., 1993; Zhu et al., 1995b] also favors this speculation. Moreover, the C-terminal 200 residues of mitosin/CENP-F are basic and proline-rich [Zhu et al., 1995a; Liao et al., 1995]. This is characteristic of the microtubule-binding domains of a variety of proteins, including CENP-E [Liao et al., 1994], MAP2, and tau [Lewis et al., 1988].

Probably due to its size, mitosin appears as a protein of multidomains. So far, the C-terminal portion of mitosin from amino acids 2,094–3,113 has been shown to contain four active regions responsible for in vitro Rb binding and nuclear, centromere, and spindle pole localization, respectively. Its Rb binding region (from amino acid residues 2,961–3,001) shares homology with that of E2F-1 [Zhu et al., 1995a]. Its nuclear localization signal (NLS) (from residues 2,930–2,958) falls into the category of the bipartite NLS [Dingwall and Laskey, 1991; Zhu et al., 1995b]. The region capable of centromere localization (amino acids 2,488–3,113) [Zhu et al., 1995b], however, did not show significant homology with centromere targeting regions defined for either CENP-B [Pluta et al., 1992] or CENP-C [Lanini and McKeon, 1995]. We also compared centrosome binding sequences (124 amino acids) of a Drosophila protein, CP190 [Oegema et al., 1995] with mitosin. Interestingly, this portion of CP190 exhibited an identity of 25% over 56 residues with a region of mitosin between residues 2,920 and 2,974 (data not shown). This homologous region, however, is unlikely to be responsible for the spindle pole association of mitosin, since mitosin-pTC, which covers this region, does not bind to spindle poles (Fig. 5). Whether it represents a portion of the centrosome localization signal of mitosin remains to be investigated.

As a nuclear protein in interphase, mitosin may therefore be sequestered to prevent it from perturbation of normal roles of the centrosome until M phase, when it is required at the mitotic spindle poles. In fact, similar regulatory mechanisms may also be implicated for several other proteins, including NuMA [reviewed by Cleveland, 1995], CP60 [Kellogg et al., 1995], and CP190 [Oegema et al., 1995]. On the other hand, the nuclear localization of mitosin may also be necessary for recruiting mitosin in time during kinetochore assembly at early M phase, so that other kinetochore proteins (e.g., CENP-E [Yen et al., 1992]) can be assembled subsequently after mitosin. After all, localization to certain structure(s) could be due to nonspecific interaction. Specific localization should thus associate with certain physiological functions which we have been trying to elucidate in the case of mitosin.

It will be interesting to further define domains of mitosin/CENP-F required for the centromere and spindle pole association. The experimental system provided here is more efficient for this purpose than the previously used transient system [Zhu et al., 1995b] mainly due to a higher percentage of positive mitotic cells. Furthermore, the moderate expression level would result in less disturbance on the physiological states of cells to be studied so that artifacts caused by overexpression could be reduced. In fact, detailed study by flow cytometry revealed that inducible expression virtually did not alter the cell-cycle progression in TNb22 (data not shown). Quality chromosome spread containing positive cells is also easily obtained (data not shown), which enables us to examine the precise location of mitosin mutants in mitotic cells. Detailed characterization of these domains will facilitate our studies on the physiological roles of mitosin and interaction between mitosin and other centrosomal or centromere proteins. Study of the relationship among these proteins may finally lead to the understanding of the molecular organization of the spindle pole and kinetochore.

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