Mitosis/CENP-F as a Negative Regulator of Activating Transcription Factor-4

Received for publication, December 20, 2004, and in revised form, January 24, 2005
Published, JBC Papers in Press, January 26, 2005, DOI 10.1074/jbc.M414310200

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Mitosis/CENP-F is a human nuclear matrix protein with multiple leucine zipper motifs. Its accumulation in S-G2 phases and transient kinetochore localization in mitosis suggest a multifunctional protein for cell proliferation. Moreover, its murine and avian orthologs are implicated in myocyte differentiation. Here we report its interaction with activating transcription factor-4 (ATF4), a ubiquitous basic leucine zipper transcription factor important for proliferation, differentiation, and stress response. The C-terminal portion of mitosin between residues 2488 and 3113 bound to ATF4 through two distinct domains, one of which was a leucine zipper motif. Mitosin mutants containing these domains were able to either supershift or disrupt the ATF4-DNA complex. On the other hand, ATF4, but not ATF1–3 or ATF6, interacted with mitosin through a region containing the basic leucine zipper motif. Moreover, overexpression of full-length mitosin repressed the transactivation activity of ATF4 in dual luciferase-based reporter assays, while knocking down mitosin expression manifested the opposite effects. These findings suggest mitosin to be a negative regulator of ATF4 in interphase through direct interaction.

Mitosis/CENP-F is a large protein of 3113 residues possibly with multiple functions during the cell cycle. In M phase, it is a protein located at the outer kinetochore plate (1–4) and is dynamically transported from kinetochores to spindle poles along microtubules by cytoplasmic dynein (5). At the end of mitosis, it is rapidly degraded (3). It is both hyperphosphorylated and farnesylated in M phase (3, 6). Farnesylation of mitosin/CENP-F is critical for G2/M progression and its postmitotic degradation (7). In interphase, mitosin/CENP-F is ubiquitously expressed mainly from S to G2 phases as a nuclear matrix protein (1–3). Such a property has been utilized as a proliferation marker for a variety of human malignancies (8–10). The physiological significance of nuclear mitosin, however, is not clear. Studies on its chicken and murine orthologs, CMF1 and LEK1, respectively, have provided clues for its roles in interphase. CMF1 is highly expressed in differentiating chicken heart (11, 12), with subcellular localization shifting from the nucleus to cytoplasm following skeletal myoblast differentiation (13). Its expression repression by antisense oligos diminished myosin expression in differentiating myoblasts (11, 13). CMF1 is thus implicated in early events of cardiac and skeletal muscle differentiation. LEK1 is also implicated in muscle differentiation (12, 14).

ATF4, also named CREB2, is a member of the ATF1/CREB family (15). Despite this, ATF4 lacks typical sites for protein kinase A critical for cAMP-dependent signaling and is thus unlikely a substrate of this kinase (16). However, human ATF4 is phosphorylated at Ser-245 by a growth factor-regulated kinase, RSK2, and the phosphorylation is implicated in skeletal development (17). In addition, phosphorylation of ATF4 on the DSGXXXS motif by an unknown kinase provokes its interaction with the SCFTrCP ubiquitin ligase, leading to attenuated stability (16). ATF4 is important for several stress responses, in which the translation of ATF4 mRNA is specifically promoted, despite a general translational inhibition caused by phosphorylation of the translation initiation factor eIF2α (18–21). Consistently, ATF4-deficient cells show defects in expressing genes involved in amino acid import, glutathione biosynthesis, and oxidative stress resistance (20). ATF4 also functions in long term memory and synaptic plasticity (22–24). In addition, ATF4-deficient mice show severe anemia, microphthalmia, and bone defects (17, 25–27), suggesting its critical roles in cell proliferation and differentiation.

ATF4 has been shown to interact with a variety of proteins through its C-terminal bZip region, implying its functional diversity and complex regulation. Through leucine zippers, it dimerizes with members either in the ATF/CREB family (28, 29) or in other bZip transcription factor families, for instance c-Fos (29, 30) and c-Jun (30) in AP-1 family and C/EBP (31) and IGEBP1 (32) in C/EBP family. Moreover, heterodimers with other bZip family members often retain the CRE binding specificity (29, 31). In addition, the Tax protein of the human T-cell leukemia virus and the γ-aminobutyric acid type B receptor also bind ATF4 to potentially modulate its activities (33, 34).

One of the striking structural characteristics of mitosin/CENP-F family proteins is the richness in leucine zipper motifs (2, 3, 14). These motifs may mediate protein-protein interactions with other proteins. In an attempt to explore the role of mitosin in interphase, we found evidence for its functional relationship with ATF4.

* This work was supported by Grants 30025021, 30330330, and 30421005 from the Natural Science Foundation of China. 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 1754 solely to indicate this fact.
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**EXPERIMENTAL PROCEDURES**

**Plasmid Constructs**—To express mitosin mutants fused to Gal4 BD in yeast, cDNA fragments encoding mitosinmt10 from amino acids 2488–3113 and mitosinTG from 2792 to 2887 (40) were cloned in-frame into pAS2-1 (Clontech) (Fig. 1A). A cDNA coding for the full-length mitosin was derived from cDNA fragments (3) and cloned into pUHD00P (35) to express FLAG fusion in mammalian cell culture for vector-based RNA interference assay, a synthetic DNA fragment containing a 22-bp inverted repeat corresponding to nucleotides 200–221 of human mitosin cDNA (GenBank™ accession NM-016343) was ligated into pBS/U6 vector (36) to create pBS/U6/Mi-1.

The full-length ATF4 cDNA was obtained by PCR from a placental cDNA library (Clontech) and cloned into pEGFP-N1 (Clontech) to express fusion or into pCEP4F (3) to express FLAG fusion. pACT-ATF4245/295 was obtained from the two-hybrid screen, which expressed a Gal4-AD fusion (Fig. 2A). pACT-ATF4185/351 was constructed from pACT-ATF4245/295, whereas pACT-ATF4245/129 was obtained by PCR.

To express other family members of ATF4 in yeast, cDNAs containing the entire open reading frames from pATF1 (kindly provided by Dr. Y. Zhang), RSV-ATF2 (from Dr. M. Castellazzi), pGCG-ATF3 (from Dr. T. Hai), and pCGN-ATF6 (from Dr. R. Frywiel) were cleaved with appropriate restriction enzymes and cloned into pACT2.

For protein expression in *Escherichia coli*, the full-length ATF4 cDNA was cloned into pGEX-2T to express GST fusion. A mitosin cDNA fragment coding for amino acids 2484–3113 was isolated from pMAL-mitosinmt10 (3) and inserted into pFLAG1 (IBI) to express FLAG-mitosinmt10. The construction of pMAL-mitosin10/NB and pMAL-mitosin10/KN and expression of MBP fusions have been previously described (3). pMAL-mitosin10/KN was constructed to mutate codons for Leu-2571 and Leu-2578 in the leucine zipper motif within mitosin10/NB into Ala by PCR-based mutagenesis. All the PCR-amplified sequences were confirmed by sequencing.

**Yeast Two-hybrid Screen**—The screen was performed following manufacturer's protocols for the Matchmaker 2 system (Clontech). Briefly, pAS-mitosinmt10 was cotransformed with a human placental cDNA library (Clontech) into yeast strain Y190 using the lithium acetate method. Yeast colonies grown on S.D./-Leu/-Trp/-His/-AT agar medium were assayed for β-galactosidase activity. Plasmid DNA was prepared from candidate positive clones and transformed into the *E. coli* strain T701 to propagate. A second round of cotransformation with bait plasmids was then performed to confirm the interaction. Plasmids having passed the confirmation were subjected to sequencing and further analysis.

**In Vitro Binding Assay**—Bacterial lysates containing GST or MBP fusion proteins in lysis buffer A (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.25% Nonidet P-40, 10 mM EDTA, 1 mM dithiothreitol, and protease inhibitors) were incubated at 4 °C for 30 min with 40 μl 50% slurry of glutathione-agarose beads (Sigma) or amyllose resin (New England BioLabs), respectively. Each aliquot of the glutathione beads was then incubated with 50 μl of bacterial lysate containing FLAG-mitosinmt10 at 4 °C for 2 h, followed by three times of wash with 500 μl of lysis buffer B. The bound proteins were then analyzed by immunoblotting or Coomassie Blue staining after SDS-PAGE. Similarly, each aliquot of the amyllose beads was incubated with 50 μl of HEK293T lysates containing GFP-ATF4 in lysis buffer B (50 mM HEPES-KOH, pH 7.8, 500 mM NaCl, 1% Nonidet P-40, 5 mM EDTA, 3 mM dithiothreitol, and protease inhibitors) (37) at 4 °C for 2 h, followed by five rounds of wash using phosphate-buffered saline (PBS). The bead-bound MBP fusion proteins were eluted using 50 μl of 10 mM maltose in PBS and analyzed by Western blotting or Coomassie Blue staining after 8% SDS-PAGE.

**Dual Luciferase Reporter Assay**—The firefly luciferase reporter construct pCRE-ATF4K2 contains two artificial CRE sites upstream of a minimal promoter and was a gift from Dr. T. Hai (Department of Molecular and Cellular Biochemistry, Ohio State University). The reporter construct pBSccyAluc (provided by Dr. K. Oda, Department of Biological Science and Technology, Science University of Tokyo, Japan) contained the firefly luciferase gene driven by rat cyclin A1 promoter (38).

HEK293T cells in 6-well plate were cotransfected with 6 μg of plasmid mixture per well, including reporter construct (1 μg) and pRL-TK (0.1 μg for each transfection in Fig. 5C and 0.2 μg in Fig. 5D) for constitutive expression of *Renilla* luciferase (Promega) as an internal control. p15–1 (2 μg), which is essential for activation of the tetracycline-responsive promoter in the pUHD vector (39), was included in the cotransfection mixture. Other cotransfected plasmids are indicated in Fig. 5. Luciferase assays were performed using a dual-luciferase reporter assay system (Promega) 48 h after transfection. The ratio of firefly luciferase activity to *Renilla* luciferase activity was presented in arbitrary units as the relative luciferase activities.

**RESULTS**

**Interaction of ATF4 with Mitosin**—We have previously shown that mitosincore containing amino acids 2488–3113, was capable of kinetochore localization (40). This mutant contains three leucine zipper motifs potential for protein-protein interactions (3). To screen for its interacting proteins, yeast two-hybrid screen was performed using mitosinTG as bait. A positive clone was identified, coding for amino acids 185–351 of the transcription factor ATF4. Although ATF4 was not a kinetochore protein (data not shown), it might link mitosin to transcription regulation in interphase since mitosin orthologs in mice and chicken appear to be involved in differentiation (11–14). Further assays were therefore performed.

The interaction between mitosincore and ATF4185/351 was specific, since mitosincore containing two leucine zipper motifs between residues 2792 and 2887 failed to bind ATF4 in yeast (Fig. 1A and B). Moreover, full-length GST-ATF4 immobilized on glutathione beads pulled down FLAG-mitosinmt10, a mutant expressed in *E. coli* and containing residues 2484–3113 (Fig. 1C, lane 2), while GST alone did not (Fig. 1C, lane 1), further suggesting a direct interaction between mitosin and ATF4.
Deletions were made to further map the mitosin-binding domain of ATF4. We found that deleting residues 296–351 or 185–196 from ATF4 disrupted the binding activity to mitosin \(^{22}\) in yeast two-hybrid assays (Fig. 2A). ATF4 thus bound mitosin through a C-terminal region including the bZip domain responsible for the dimerization and DNA binding \(^{41}\).

We then examined whether mitosin interacted with other members of the ATF family. The homology among the bZip domains of the ATF family members is compared in Fig. 2B. The homologous sequences were roughly equivalent to residues 185–351 of ATF4. We found that deleting residues 296–351 or 185–196 from ATF4 disrupted the binding activity to mitosin \(^{22}\) in yeast two-hybrid assays. The indicated fragments of different ATF proteins were tested for interaction with mitosin \(^{10}\) in the yeast two-hybrid system.

Despite some degradation (Fig. 1C), the mutant ATF4 proteins were still visible (Fig. 3C, lanes 2 and 4). These mutants alone failed to show any binding to the CRE in EMSA (data not shown). When MBP-mitosin \(^{10}\) was added to challenge the ATF4-DNA complex, however, it supershifted the complex in dose-dependent manners (Fig. 3D, lanes 1–4). This result was in agreement with the interaction between ATF4 and this portion of mitosin (Fig. 1) and further indicated that the association with mitosin \(^{10}\) did not alter the DNA binding activity of ATF4. MBP-mitosin \(^{10}\) also supershifted the ATF4-DNA complex (Fig. 3D, lanes 7 and 8), whereas MBP alone had no effect (lane 9), suggesting the existence of an ATF4 binding domain between residues 2484 and 3113 of mitosin. MBP-mitosin \(^{10}\), on the other hand, disrupted the ATF4-DNA complex also in dose-dependent ways (Fig. 3D, lanes 5 and 6), suggesting another ATF4 binding domain between residues 2484 and 2645 of mitosin whose association alone abolished the ATF4-DNA interaction.

**Mitosin** \(^{10/NB}\) **Binds ATF4 through Its Leucine Zipper Motif—**
To corroborate the EMSA results, we further confirmed that...
mitosin<sup>10/10<sub>NB</sub></sup> indeed bound ATF4 in pull-down assays (Fig. 4, lane 1). Like other bZip transcription factors, ATF4 homo- or heterodimerizes through its leucine zipper motif (15, 28, 31, 42). Mitosin<sup>10/10<sub>NB</sub></sup> contained a leucine zipper motif with six consecutive leucine heptad repeats between residues 2557–2592 (Fig. 1A and Fig. 3B). To test whether both proteins interacted by forming leucine zippers, the third and fourth leucine residues (Leu-2571 and Leu-2578) in the leucine motif of mitosin<sup>10/10<sub>NB</sub></sup> were mutated into Ala, and affinity of the mutant (mitosin<sup>10/10<sub>NB</sub></sup>α) to ATF4 was then examined in vitro. Indeed, disruption of the leucine zipper motif in mitosin<sup>10/10<sub>NB</sub></sup> significantly diminished the interaction with ATF4 (Fig. 4, lane 2).

**Mitosin Is a Negative Regulator of ATF4-mediated Transcriptional Activation**

To understand the functional relevance of the interaction between mitosin and ATF4, we examined effects of mitosin overexpression or repression on the transcription activity of ATF4. A plasmid, pUHD-mitosin, was constructed to express FLAG-tagged full-length mitosin. The FLAG-mitosin was indeed full-length according to its comigration with the endogenous one in SDS-PAGE (Fig. 5A). Mitosin<sup>10/10<sub>NB</sub></sup> was cloned to express FLAG-tagged full-length mitosin. ATF4 activation activity of ATF4. A plasmid, pUHD-mitosin, was constructed to express FLAG-tagged full-length mitosin. The FLAG-mitosin was indeed full-length according to its comigration with the endogenous one in SDS-PAGE (Fig. 5A).

Consistently, when pBS/U6/Mi-1 and pBS/U6 were respectively cotransfected into HEK293T cells to assess their effects on the reporter activity. The amount of each plasmid (μg) is also shown. The relative luciferase activity is presented in arbitrary units as mean ± S.D. from two experiments. D, reporter assays for human cyclin A promoter. Results were averaged from two experiments.

**DISCUSSION**

We report that mitosin serves as a negative regulator of the transcription factor ATF4 through direct interaction. First, we cloned ATF4 in yeast two-hybrid screens using mitosin mutant, mitosin<sup>10/10<sub>T</sub></sup>, as bait (Fig. 1). Direct interaction was further confirmed by pull-down assays using bacterially expressed mitosin<sup>10/10<sub>T</sub></sup>, a mutant similar to mitosin<sup>10/10<sub>T</sub></sup> (Fig. 1). On the other hand, ATF4 interacted with mitosin through a C-terminal region between residues 185–351 covering the DNA binding bZip domain in two-hybrid assays (Fig. 2) (28, 31). Its leucine zipper region was required, but not sufficient, for the interaction (Fig. 2). The corresponding regions in other members of the ATF/CREB family, however, failed to interact with mitosin (Fig. 2). Second, mitosin mutants affected the DNA binding activity of ATF4. Both mitosin<sup>10/10<sub>T</sub></sup> and mitosin<sup>10/10<sub>N</sub></sup> were able to supershift the ATF4-DNA complex in EMSA (Fig. 3), while the mutants themselves showed no binding to the DNA probe (data not shown). In contrast, mitosin<sup>10/10<sub>N</sub></sup> disrupted the ATF4-DNA complex, implying that mitosin might regulate ATF4 by regulating its DNA binding activity. Third, mitosin levels significantly affected ATF4-mediated transcription activation. Overexpression of full-length mitosin down-regulated the transactivation activity of ATF4, while mitosin depletion manifested the opposite effect (Fig. 5).

Mitosin appears to contain two distinct ATF4-binding domains between residues 2484–3113. Because mitosin<sup>10/10<sub>N</sub></sup> was...
able to supershift the ATF4-DNA complex in EMSA (Fig. 3), the first domain is located between residues 2645 and 3113, which covered the core region critical for kinetochore targeting (40). Nevertheless, the core region alone was not sufficient for binding ATF4 (Fig. 1). On the other hand, disruption of the ATF4-DNA complex by mitosin10/11/12 suggests existence of another ATF4 binding domain between 2484 and 2645 (Fig. 3), which was further confirmed by pull-down assays (Fig. 4). In contrast to the previous binding domain, this one abolishes ATF4-DNA interaction. Mutagenesis study further suggested that the leucine zipper motif between residues 2557 and 2592 of mitosin is involved in binding to ATF4 (Fig. 4). Persistence of the interaction in high salt conditions (500 mM NaCl) (Fig. 4) further supports hydrophobic interaction between ATF4 and mitosin10/11/12, very likely by forming leucine zippers. Despite this, this second binding domain appears recessive in the presence of the first one because mitosinmt10, which contains both domains, did not abolish the ATF4-DNA complex (Fig. 3). Although we failed to achieve coimmunoprecipitation of ATF4 with full-length mitosin, possibly because of poor solubility of mitosin/CENP-F as a nuclear matrix protein in interphase to sequester ATF4 and possibly other transcription factors (44–47). As a nuclear matrix protein with multiple domains, did not abolish the ATF4-DNA complex (Fig. 3). Alternative, such effect may be due to the specific cell line. Alternatively, such effect may be due to the specific cell line. Possibly, because of poor solubility of mitosin is involved in binding to ATF4 (Fig. 4). Persistence of the interaction in high salt conditions (500 mM NaCl) (Fig. 4) further supports hydrophobic interaction between ATF4 and mitosin10/11/12, very likely by forming leucine zippers. Despite this, this second binding domain appears recessive in the presence of the first one because mitosinmt10, which contains both domains, did not abolish the ATF4-DNA complex (Fig. 3). Although we failed to achieve coimmunoprecipitation of ATF4 with full-length mitosin, possibly because of poor solubility of mitosin/CENP-F as a nuclear matrix protein in interphase to sequester ATF4 and possibly other transcription factors (44–47). As a nuclear matrix protein with multiple domains, did not abolish the ATF4-DNA complex (Fig. 3).