Phosphorylation and Dimerization Regulate Nucleocyttoplasmic Shuttling of Mammalian STE20-like Kinase (MST)*

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Mammalian STE20-like kinase (MST) is a member of the yeast STE20-related kinase family and proteolytically activated by caspase during apoptosis. However, its other cellular functions are not known, including its activation mechanism, substrate(s), and subcellular localization. In this report, using anti-MST monoclonal antibodies, we clearly show that endogenous MST is localized in cytoplasm in a leptomycin B-dependent manner. Analyses with serial deletions and point mutations show that MST has two functional nuclear export signals and, unexpectedly, another localization motif for nuclear import. When cells are treated with leptomycin, monomeric MST is accumulated more rapidly in the nucleus than dimeric MST, indicating that dimerization contributes to the cytoplasmic retention of MST. Okadaic acid, an inhibitor of phosphatase 2A, induces activation of MST and translocation into the nucleus. Using phosphopeptide-specific antibody, we directly show that okadaic acid induces phosphorylation in the activation loop of MST, and, once phosphorylated, MST is rapidly translocated to the nucleus. However, kinase-deficient MST does not enter the nucleus, indicating that phosphorylation and activation is required for okadaic acid-induced nuclear translocation. In apoptotic cells, the activation of MST does not require phosphorylation in the activation loop and occurs through the release of C-terminal regulatory domain by caspase-dependent cleavage. Kinase-deficient MST functions dominantly-negative and represses okadaic acid-induced morphological change indicating that MST plays a role in okadaic acid-induced cellular shrinkage. Our identification of cytoplasmic and nuclear localization motifs and phosphorylation-dependent translocation of MST suggests that regulation of localization is important to the biological function of MST, including its effects on cellular morphology.

MST1 is a member of a subfamily of kinases that share high similarity in the catalytic domain with STE20. STE20 is a yeast mitogen-activated protein kinase kinase kinase kinase (MAPKKKK) (1–3). However, the cellular function of MST as well as that of most other STE20-like kinases is largely unknown (3, 4). The non-catalytic C-terminal region of MST does not show any significant similarity with that of other family members and may function as a negative regulatory domain, because its removal markedly increases the kinase activity (5, 6). It was reported that MST1 could be activated by stresses such as high temperature heat shock and high concentrations of sodium arsenite (2). In some mammalian cells, MST appears to function as an upstream kinase and be involved in c-Jun N-terminal kinase and p38 pathways (7).

MST1/MST2 is cleaved and activated by various apoptotic stimuli, including death receptor triggering and chemical apoptotic inducers such as staurosporine (STR), etoposide, and ceramide (6, 7). In addition, bisphosphonate, a pharmacological drug for osteoporosis, and cytotoxin A, an anti-tumor drug, have been reported to induce cleavage of MST in osteoclast and leukemia cells (8, 9). MST1/MST2 is a direct substrate of caspase, and truncated MST is greatly activated by the removal of its regulatory region (6, 7, 10). Truncated MST strongly induces cell death, suggesting that some apoptotic events are mediated by cleavage-dependent activation of MST (10). Interestingly, other kinases of the STE20 family such as PAK2, HPK1, and SLK, have been reported as caspase substrates, further implying that some of the family play an important role in apoptosis (11–13).

Small molecules rapidly diffuse through the nuclear pore complex. It is reported that the upper size limit for macromolecules to diffuse through the nuclear pore complex is 50–60 kDa (14, 15). One of the major mechanisms for the cytoplasmic localization of proteins is nuclear export system. Many cytoplasmic proteins involved in cellular signaling are transported actively to the cytoplasm by nuclear export signal (NES) (16–21). In contrast, selective nuclear import of protein is mediated by nuclear localization signal (NLS) and cognate transport factors known as karyopherins or importins (22–25). The classic NLS motif consists of short sequences (5–20 residues) containing several lysine and arginine residues (26, 27).

In this study, we investigated the subcellular localization and analyzed the molecular mechanism that regulates the nucleocyttoplasmic translocation of MST. MST is a cytoplasmic protein that has functional NES and NLS. Using a phospho-specific antibody, we directly show that MST is phosphorylated in an activation loop, and, importantly, the phosphorylated MST is rapidly translocated to the nucleus. We propose a molecular mechanism for the regulation of nucleocyttoplasmic localization and suggest that MST directly transmits signals into the nucleus.

**EXPERIMENTAL PROCEDURES**

Materials—Anti-MST monoclonal antibodies were prepared as described previously (10). Anti-FLAG M2 antibody was obtained from...
Cloning, Vector Construction, and Site-directed Mutagenesis—Expression constructs of MST were prepared as N-terminal FLAG-tagged or GFP-fused forms. We introduced FLAG-tag after the initiation codon in pME18S vector as described previously (10, 28). For the GFP fusion constructs, cDNA of human MST1 or human MST2 was subcloned in-frame into pCMX-SAH/Y145F (10). Substitution of amino acid residues was performed with a QuikChange site-directed mutagenesis kit (Stratagene) and confirmed by DNA sequencing. pEG plasmid, expressing two tandem GFP proteins with a central nuclear export signal (NES) sequence derived from Xenopus MAPKK (16), was generated by subcloning in-frame into pCMX-SAH/Y145F. For the cloning of PAK2, total mRNA from HPB-ALL cells was prepared. Single-stranded cDNAs were synthesized by reverse transcription, using a First Strand cDNA Synthesis kit (Invitrogen), and then used as a template in polymerase chain reactions. Two primers, 5′-ggcctgagctgtgataacggaactg-3′ and 5′-ggcctgagctgataacggaactg-tacctctgactcaac-3′, were used to specifically amplify the coding region of human PAK2. The amplified products were cloned into pME18S-FLAG vector. For kinase-deficient PAK2, Lys399 was changed to Arg by QuikChange site-directed mutagenesis.

Cell Culture and Transfection—HeLa and NIH 3T3 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 100 μg/ml kanamycin. Cells were seeded on glass chamber slides (Nalge Nunc) or plates 24 h before transfection and then transfected with LipofectAMINE (Invitrogen) in accordance with the manufacturer’s recommendation.

Fluorescence Microscopy of Cultured Cells—HeLa and NIH 3T3 cells on chamber slides were washed with PBS and fixed in 3.7% formaldehyde in PBS for 10 min. Fixed cells were permeabilized in PBS containing 0.1% Triton X-100 for 10 min, and blocked in PBS containing 2% bovine serum albumin for 10 min. Cells were treated with anti-MST monoclonal antibody (1 μg/ml), anti-FLAG antibody (1 μg/ml), or anti-phospho antibody for 1 h. Texas Red-conjugated anti-mouse IgG, Alexa Fluor 488-conjugated anti-mouse IgG, and Alexa Fluor 594-conjugated anti-rabbit IgG were used at a dilution of 1:500 for 45 min. Cells were washed with PBS and incubated with Hoechst 33342 (0.2 μg/ml) for 20 min and loaded onto a column of Superdex 200 HR (Amersham Biochemicals). Cell lysates were cleared by centrifugation at 15,000 × g for 20 min. For immunoprecipitation, the lysate was incubated with 2 μg of anti-FLAG or anti-Myc antibody for 2 h and incubated with protein G-Sepharose (Amersham Biosciences, Inc.) for 2 h to overnight. Cell lysates or immunoprecipitates were resolved by SDS-PAGE, transferred to a polyvinyldiene difluoride membrane (Millipore). The membrane was blocked in TBST buffer (20 mM Tris, pH 7.5, 150 mM NaCl, and 0.1% Tween 20) with 5% skim milk at room temperature for 1 h. The membrane was incubated with antibody for 1 h, washed in TBST, and then incubated for 1 h with horseradish peroxidase-conjugated anti-mouse IgG (Amersham Biosciences, Inc.). After further washing with TBST, peroxidase activity was detected on x-ray films using an enhanced chemiluminescence detection system (DuPont).

Size-exclusion Chromatography—Cell lysates were centrifuged at 15,000 × g for 20 min and loaded on a column of Superdex 200 HR 10/30. Chromatography was performed with fast protein liquid chromatography protein purification (Amersham Biosciences, Inc.) at 4 °C. The column was eluted with cold lysis buffer described above. The column was run with a protein standard (Amersham Biosciences, Inc.), including blue dextran, thyroglobulin, ferritin, catalase, bovine serum albumin, and ovalbumin. The eluted fractions were analyzed by Western blotting with anti-FLAG or anti-Myc antibody.

In Vitro Kinase Assay—HeLa cells were transfected with vectors encoding FLAG-MST1 and FLAG-PAK2, and cell lysates were immunoprecipitated with anti-FLAG antibody. Equal amounts of immunoprecipitates as analyzed by immunoblotting were used for immune-complex kinase assay. Immunoprecipitates were incubated with 2 μg of histone H2B in 20 μl of kinase reaction buffer, 40 μM Heps, pH 7.5, 20 mM MgCl2, 20 mM β-glycerol phosphate, and 0.1 mM vanadate containing 25 μM ATP and 2.5 μCi of [γ-32P] ATP. Samples were incubated for 1 h at 30 °C, and their kinase activities were terminated by adding 7 μl of Laemmli sample buffer and boiling for 5 min. A portion of the sample (15 μl) was separated on a 15% SDS-polyacrylamide gel and autoradiographed or analyzed by phosphorimaging.

RESULTS

MST Is Localized to Cytoplasm in a Leptomycin-sensitive Manner—Our previous study revealed that overexpressed MST was localized exclusively in the cytoplasm (10). However, overexpressed proteins are not always localized in the same way as the endogenous proteins. To examine the subcellular localization of endogenous MST, we immunostained NIH 3T3 cells with two antibodies that recognize different epitopes on MST. The cytoplasm was specifically stained by indirect immunostaining with either J7B, which recognized the C-terminal region of MST, or G2B, which recognized the N-terminal catalytic domain of MST (Fig. 1A, top row) (10). A similar pattern of distribution was observed with fluorescein- or Texas Red-conjugated G2B and J7B. To investigate whether the cytoplasmic localization of MST is regulated by a nuclear export pathway, we transfected NIH 3T3 cells with leptomycin (LMB), a specific inhibitor of nuclear export, mediated by leucine-rich NES (29). MST rapidly lost its cytoplasmic localization within 20 min after LMB treatment (Fig. 1A, bottom row). Furthermore, prolonged incubation with LMB for 60 min resulted in the nuclear accumulation of MST, indicating that MST is localized to the cytoplasm in an LMB-dependent manner.

Then, we examined whether MST has a polypeptide region responsible for cytoplasmic localization. Various deletion constructs of MST1 and MST2 were prepared as GFP-fused forms, and their subcellular localization was observed (Fig. 1B). The deletion of 320 N-terminal amino acid residues, including the kinase domain or 60 C-terminal residues contributing to dimer formation did not change the cytoplasmic localization, indicating that the kinase activity and dimerization of MST were not involved in its cytoplasmic localization. Furthermore, further deletion of a central region (323–381) of MST2 resulted in a diffused localization in the cytoplasm and nucleus. When this region was fused to GFP, only a cytoplasmic localization was observed, indicating that a peptide sequence within this region is capable of functioning as a signal for cytoplasmic localization.

Identification of NES and NLS in MST—A closer look at the region corresponding to 323–381 in MST2 revealed that leucine-rich NES-like sequences were present in MST homologues in human, mouse, rat, and even Caenorhabditis elegans, suggesting evolutionary significance (10). This region contained several conserved hydrophobic residues with typical and characteristic spacing, like the well-established NES (Fig. 2, A and B). To test whether the conserved residues are able to function as an NES, we mutated the hydrophobic residues to alanine (361A/365A and 368A/370A), and investigated the subcellular localization. Unexpectedly, FLAG-MST1361A/365A and FLAG-MST1361A/365A were still localized exclusively in the cytoplasm. The cytoplasmic localization did not change even when four residues were replaced with alanine (361A/365A/361A/365A). These results strongly raised the possibility that some other C-terminal region of MST is involved in the localization. Actually, MST1 and MST2 have long clusters of hydrophobic residues in C-terminal proximal regions (430–460) (Fig. 2A). Therefore, we introduced mutations in these hydrophobic residues and investigated the localization.

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As shown in Fig. 2C, when two residues in the C-terminal proximal region were mutated to alanine (439A/441A or 441A/444A), MST1439A/441A and MST1441A/444A still showed a cytoplasmic localization. However, when hydrophobic residues in both regions were mutated (MST1368A/370A/439A/441A and MST1368A/370A/441A/444A), the localization was changed to the nucleus (Fig. 2C). These results indicate that MST has two functional NES sequences (NES1 and NES2, see Fig. 2, A and B), and either NES1 or NES2 is sufficient for the cytoplasmic localization of full-length MST.

The result that MST1 with mutations in both NES sequences (368A/370A/441A/444A, designated MST1-ΔNES) localized only in the nucleus prompted us to investigate whether MST has potential nuclear-localizing activity. This possibility was further supported by the data of Fig. 1A showing that endogenous MST was rapidly accumulated in the nucleus when cells were treated with LMB. We noticed peptide sequences near the C-terminal tail of MST1 and MST2, which is conserved throughout MST homologues and similar to the bipartite NLS consensus sequence (Fig. 2, A and B). To test whether this NLS-like sequence is capable of functioning as a NLS, the C-terminal 25 residues containing this sequence were fused to GFP (GFP-C25), and its subcellular localization was observed. As shown in Fig. 2D, GFP-C25 showed an intense nuclear localization while GFP was distributed throughout the cytoplasm and nucleus. MST1-ΔNES, which is exclusively nuclear (Fig. 2C, M9), was no longer accumulated in the nucleus and showed a diffused localization when lysine residues of the putative NLS were further replaced with asparagine (480N/481N), or when the C-terminal 25 residues were further removed (Fig. 2D). MST1-ΔC25, deleted of the 25 C-terminal residues, did not accumulate in the nucleus when cells were treated with LMB for over 3 h. Thus, the C-terminal NLS-like sequence functions as a NLS and induces the nuclear accumulation of MST.

Dimerization Reinforces the Cytoplasmic Localization of MST—The apparent molecular mass of 60 kDa determined by SDS-PAGE and the value of 55 kDa calculated from the peptide sequence of MST are close to the limit of diffusion through the nuclear pore complex. Because MST was reported to form a dimer (5), we examined the relationship between dimer formation and the kinetics of the nuclear translocation. HeLa cells were co-transfected with wild-type Myc-MST1 and FLAG-MST1444P, mutated at Leu444 to proline, which is not capable of forming a dimer (5). Because the cyclic structure of the proline residue could markedly influence the protein architecture, resulting in a misfolding, we co-transfected wild-type Myc-MST1 and other FLAG-MST1 mutants in which Leu444 was replaced with alanine (444A) or neighboring Leu448 with alanine (448A).

The interaction of Myc-MST1 and FLAG-tagged mutant proteins was analyzed by immunoprecipitation with anti-Myc antibody (Fig. 3A). Wild-type FLAG-MST1 and FLAG-MST1444A were co-immunoprecipitated, whereas FLAG-MST1444P was not detected in the immunoprecipitates. FLAG-MST1444A was co-immunoprecipitated but less efficiently than wild-type FLAG-MST1. We then compared the elution profile of wild-type MST1 and MST1444P by size-exclusion chromatography (Fig. 3B). Wild-type MST1 was eluted at a peak fraction of 250 kDa as calculated from an elution profile of protein standards. However, MST1444P was eluted at the fraction around 130 kDa, being about half the molecular size of wild-type MST1.

Next, we examined the LMB-induced translocation of these mutants in HeLa cells (Fig. 3C). MST1444P lost its cytoplasmic localization within 5 min, and most cells expressing MST1444P showed a nuclear accumulation of MST within 10 min of LMB treatment. In contrast, wild-type MST1 still showed a cytoplasmic localization at 30 min (Fig. 3C) and prolonged incubation induced localization change. The nuclear translocation of MST1444P was much faster than that of wild-type MST1 or endogenous MST1 (Figs. 1A and 3C). MST1444A, which showed strong activity to bind wild-type MST1, was translocated to the nucleus with much slower kinetics than MST1444P and MST1444A, with weak but significant binding activity for wild-type MST1, showed intermediate translocation kinetics between MST1444P and MST1444A. We conclude that monomeric MST is translocated into the nucleus more rapidly upon treatment with LMB, and dimerization facilitates the cytoplasmic retention of MST.

Okaodaic Acid Induces Phosphorylation, Activation, and Nuclear Translocation of MST—A general method to measure the phosphorylation of protein is in vitro kinase assay, but some members of the STE20 kinase family are markedly activated by autophosphorylation in vitro (1, 30, 31). Therefore, the results of this assay greatly depend on the incubation time, significantly masking any difference between control and treated samples. PAK shares a nearly identical activation loop se-
sequence in the kinase domain with MST, and the phosphorylation of the conserved threonine (Thr423 in PAK1, Thr402 in PAK2, and Thr183 in MST1) is required for autoactivation (32–34). In addition, anti-phosphopeptide antibody against the activation loop of PAK was reported to cross-react in vitro with phosphorylated MST (34).

We tested whether a commercially available anti-phosphopeptide antibody to PAK1/PAK2 could cross-react with ac-
tivated MST. We chose as an inducer for the activation of MST, okadaic acid (OA), a specific inhibitor of protein phosphatase 2A, because it activates many protein kinases and because phosphatase 2A is involved in various signaling pathways (35). When HeLa cells were treated with OA, the anti-phosphopeptide antibody specifically recognized a band of around 60 kDa from cell lysates expressing FLAG-MST1 but not from untreated cell lysates (Fig. 4A). Unexpectedly, this band was not detected in cell lysates expressing FLAG-PAK2, whereas both forms of the protein were equally detected with anti-FLAG antibody. When Thr183 in MST1 was mutated to Ala, no band could be detected by OA treatment, indicating that Thr183 was the site of phosphorylation.

The specificity of anti-phosphopeptide antibody to the phosphorylated MST was further examined by immunoprecipitation. Using anti-phosphopeptide antibody, we again observed a band of 60 kDa only from the immunoprecipitate of the OA-treated cell lysate (Fig. 4A, a). When the immunoprecipitate from untreated cells was subjected to in vitro kinase reaction, the autophosphorylation of MST was detected by immunoblotting with anti-phosphopeptide antibody and autoradiography (Fig. 4B, c and d), which could not be detected in immunoprecipitates before the reaction. The induced activation of MST during the kinase reaction was detected as a phosphorylation of the substrate histone H2B. These results indicated that both autophosphorylation and activation of MST were induced during the kinase reaction in vitro in the immunoprecipitates from untreated cells.

In immunoprecipitates from OA-treated cells, FLAG-MST1 showed strong autophosphorylation that was found to have increased about 2-fold by autoradiography. A similar level of phosphorylated MST was observed on immunoblot. The level of phosphorylated H2B was increased 5-fold by OA treatment, indicating that phosphorylation of MST induced its activation in OA-treated cells. However, FLAG-PAK2 showed very little autophosphorylation and the phosphorylation of H2B was not significantly influenced by OA treatment. These results show that the anti-phosphopeptide antibody recognizes the active MST1, which is phosphorylated but not the inactive MST was not.

We tested whether OA could induce the nuclear translocation of the phosphorylated MST, because the inactive MST was

![Fig. 4 OA induces phosphorylation, activation, and nuclear translocation of MST. A, HeLa cells were transfected with vector encoding FLAG-MST1 or FLAG-PAK2. Cells were treated without (−) or with (+) 1 μM OA for 25 min, and cell lysates were analyzed by immunoblotting with anti-phosphopeptide antibody (left) or with anti-FLAG antibody (right). B, FLAG-MST1 and FLAG-PAK2 were immunoprecipitated with anti-FLAG antibody and probed with anti-phosphopeptide antibody (a) or anti-FLAG antibody (b). Immunoprecipitates obtained with anti-FLAG antibody were subjected to immune-complex kinase assay using histone H2B as substrate and autoradiographed (c) or blotted with anti-phosphopeptide antibody (d). C, HeLa cells were transfected with vector encoding kinase-deficient or wild-type MST1 with FLAG tag. Cells were treated with or without 1 μM OA for 25 min. Cells were co-stained with mouse anti-FLAG antibody and rabbit anti-phosphopeptide antibody, followed by Alexa 488 anti-mouse (green) and Alexa 594 anti-rabbit antibody (red). Nuclei appear blue with Hoechst 33342 staining. D, HeLa cells were transfected with vectors encoding wild-type FLAG-MST1, and the time course of the change in localization was observed after 10, 20, and 30 min treatment with OA as in C.]
exclusively localized to the cytoplasmic in OA-untreated cells despite having a functional NLS in the C-terminal tail (Fig. 4C, left panels). In FLAG-MST1-expressing cells, OA treatment induced strong staining with phosphopeptide antibody, but no significant staining was detected in untreated cells (Fig. 4C). Cells expressing kinase-deficient MST1 were not stained with anti-phosphopeptide antibody, irrespective of OA treatment. Importantly, the localization of phosphorylated MST1 changed time-dependently from the cytoplasm to nucleus, whereas that of FLAG-MST1 changed from cytoplasm to a diffused pattern in cytoplasm and nucleus (Fig. 4D). Most of the cells expressing FLAG-MST1 showed a nuclear localization of phosphorylated MST after around 30 min treatment with OA. Further incubation induced cell shrinkage but the nuclear localization continued. In contrast, GFP-NES protein, in which GFP is tandemly fused with an internal NES peptide sequence derived from Xenopus MAPKK, was not translocated into the nucleus on treatment with OA. This result indicated that the OA-induced nuclear translocation of MST was not caused by the inhibition of nuclear export. Furthermore, kinase-negative MST1 was not translocated into the nucleus, indicating that the OA-induced nuclear translocation requires the kinase activity of MST (Fig. 4C). These results clearly show that the OA-induced phosphorylation and activation of MST is needed for its nuclear translocation.

**OA and STR Activate MST by Phosphorylation and Cleavage.**—MST is cleaved by caspase-3, and the cleavage induces a strong activation of the kinase domain of MST in apoptosis (6, 7, 10). We wondered whether the proteolytic activation of MST1 in apoptosis requires the phosphorylation of Thr183 in the activation loop. The activation kinetics of MST was investigated in HeLa cells treated with OA or STR, a well-known chemical inducer for apoptosis (Fig. 5). Phosphorylated MST was detected at 30-min treatment with OA, and the level of phosphorylation did not change significantly after 5-h treatment. When cells were incubated with STR, cleavage of MST was observed at 3 h, which was not detected after OA treatment. However, phosphorylation of Thr183 could be detected in neither full-length nor cleaved catalytic fragments even after 5-h treatment with STR. A similar result was obtained in Fas-mediated apoptosis, indicating that the proteolytic activation of MST in apoptosis does not require phosphorylation in the activation loop.

Kinase-deficient MST Inhibits OA-induced Cellular Morphological Change.—We asked whether activation of MST is required for induction of cellular shrinkage, because overexpression of MST induced cell rounding up and nuclear condensation (10). To test whether kinase-deficient MST is able to function dominantly, HeLa cells stably expressing GFP-MST1 were transfected with increasing amounts of vector encoding kinase-deficient MST and the activation of MST was investigated. As shown in Fig. 6A, the kinase-deficient form efficiently repressed the OA-mediated phosphorylation of MST in a dose-dependent manner. Then, we examined the effect of kinase-deficient MST on the morphological change after 30-min treatment with OA, because the change in both the phosphorylation of MST and cellular morphology occurred simultaneously at around this time (Fig. 6, B and C). Overexpression of kinase-deficient MST markedly decreased OA-induced cell rounding up to less than 25% of kinase-deficient MST1-expressing cells, whereas about 52% of cells not expressing kinase-deficient MST1 showed rounding up. These results strongly suggest that kinase-deficient MST could function dominantly and MST is involved in OA-induced cellular morphological change.

**DISCUSSION**

In this study, we showed that MST is a cytoplasmic protein (Fig. 1). Deletion and mutagenesis experiments revealed that MST has two NESs together with one NLS in its C-terminal regulatory region. It is of note that MST shows an exclusive cytoplasmic localization, although it has a functional NLS (Figs. 1 and 2). This suggests that the nuclear export of MST is more efficient than the nuclear import in unstimulated cells. A similar observation has been reported in a fusion protein carrying the large T NLS of SV40 and the leucine-rich NES of PKI (36), as well as in the transcription factor NF-AT (20). MST may fail to enter the nucleus at a significant rate because of high affinity interaction with a cytosolic anchor protein that does not enter the nucleus. This possibility is supported by the observation that monomeric MST1-144P shows an apparent molecular mass of 130 kDa in size-exclusion chromatography, much larger than the value obtained from SDS-PAGE or calculated from the peptide sequence (Fig. 3). However, MST1-144P enters the nucleus more rapidly than MST1 after treatment with LMB. This observation suggests another mechanism facilitating the cytoplasmic retention of MST; the dimeric form of MST may not be efficiently transported into the nucleus by the nuclear import machinery or passive diffusion because of its large molecular size or its interaction with cytosolic protein.

Using a phospho-specific antibody, we showed that OA induces phosphorylation, activation, and nuclear translocation of MST (Fig. 4). OA activates many protein kinases by inhibiting phosphatase 2A, which is involved in various signaling pathways (35). We speculate that OA induces the activation of MST by directly inhibiting the dephosphorylation of phospho-Thr183 in the activation loop or indirectly activating unknown kinase that phosphorylates Thr183. The phosphorylation and activation of MST are necessary for the OA-induced nuclear translocation of MST. This conclusion is supported by the observation that kinase-deficient MST is neither phosphorylated nor translocated into the nucleus. Our data also show that MST is inactive in untreated cells. This finding is quite surprising, because MST is believed to be constitutively active based on the results of in vitro kinase assays (1, 6, 7, 10).

MAPKAP-2 (37, 38), a kinase that is phosphorylated and activated by p38, has a similar primary structure to MST, an N-terminal catalytic domain and a C-terminal regulatory re-
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**FIG. 6.** Kinase-deficient MST inhibits OA-induced cellular morphological change. A, HeLa cells stably expressing GFP-MST1 were transfected with increasing amounts of vector encoding FLAG-MST1 K59R and then treated with OA for 30 min. The cell lysates were resolved and immunoblotted with anti-MST monoclonal antibody (top) or with anti-phosphopeptide antibody (bottom). B, HeLa cells were transfected with vector encoding FLAG-MST1 K59R. After 16 h, cells were treated with or without 1 μM OA for 30 min, fixed, and stained with anti-FLAG antibody (middle). Representative images of cells are shown. DIC indicates the image of differential interference contrast microscopy (top). Mitotic cells are indicated by the triangle. C, at least 200 cells stained with anti-FLAG antibody were counted, and the number of round cells was expressed as a percentage to the total number of FLAG-MST1 K59R-expressing cells. As a control, the percentage of round cells without anti-FLAG staining to the total number of cells not expressing FLAG-MST1 K59R is shown, because the result was essentially similar to that from mock transfected HeLa cells. The transfection efficiency was 30–50% depending on the experiments. Round cells in the mitotic stage (indicated by open triangle in B), as confirmed by Hoechst staining, were excluded from the counting.

**FIG. 7.** Model for the activation and translocation of MST. In non-apoptotic cells, OA inhibits phosphatase 2A, which otherwise dephosphorylates phospho-Thr183 in the activation loop of MST. Alternatively, OA may induce the activation of unknown kinases that directly phosphorylate Thr183. The phosphorylation of Thr183 induces a conformational change in the catalytic domain and C-terminal regulatory region, thereby switching on the kinase activity and masking the NES or exposing the NLS. Then, the activated MST is translocated to the nucleus, where it undergoes dephosphorylation at phospho-Thr183 by nuclear phosphatase and returns to inactive form. MST with exposed NESs is translocated to the cytoplasm by the nuclear export pathway. In apoptotic cells, the N-terminal catalytic domain is activated by being released from the regulatory domain in a caspase-dependent manner, a process that does not require the phosphorylation of Thr183.

region containing a leucine-rich NES and bipartite NLS (17). In contrast to MST, however, MAPKAP-2 has an NLS that is constitutively active and is localized in the nucleus before its activation. When phosphorylated by p38, the active MAPKAP-2 is exported to the cytosol through the unmasking of NES and acts as a cytosolic tether for p38 (17, 39).

The same mechanism in reverse may be at work in the phosphorylation-dependent translocation of MST (Fig. 7). MST, in an inactive state, is localized to the cytoplasm, because NLS may be masked. When phosphorylated at Thr183 on stimulation by OA, MST may change its conformation to the active form, and the kinase domain may be unmasked from the regulatory domain. Phosphorylation of Thr183 would induce further change in the regulatory region, masking the NESs and exposing the NLS, and phosphorylated MST would be rapidly translocated into the nucleus. In the nucleus, phosphorylated MST1 K59R and then treated with OA for 30 min. The cell lysates were resolved and immunoblotted with anti-MST monoclonal antibody (top) or with anti-phosphopeptide antibody (bottom). B, HeLa cells were transfected with vector encoding FLAG-MST1 K59R. After 16 h, cells were treated with or without 1 μM OA for 30 min, fixed, and stained with anti-FLAG antibody (middle). Representative images of cells are shown. DIC indicates the image of differential interference contrast microscopy (top). Mitotic cells are indicated by the triangle. C, at least 200 cells stained with anti-FLAG antibody were counted, and the number of round cells was expressed as a percentage to the total number of FLAG-MST1 K59R-expressing cells. As a control, the percentage of round cells without anti-FLAG staining to the total number of cells not expressing FLAG-MST1 K59R is shown, because the result was essentially similar to that from mock transfected HeLa cells. The transfection efficiency was 30–50% depending on the experiments. Round cells in the mitotic stage (indicated by open triangle in B), as confirmed by Hoechst staining, were excluded from the counting.
MST may undergo dephosphorylation at phospho-Thr\textsuperscript{183} by nuclear phosphatase. Dephosphorylated MST may then return to the inactive conformation and be translocated to the cytoplasm by the nuclear export pathway.

Cleavage of MST in apoptosis is a good example showing that the localization of MST should be regulated in space and time. Previously, we and others (6, 7, 10) have reported that MST1 and MST2 are substrates of caspase-3 and proteolytically activated by caspase-3 in apoptosis. Once MST is cleaved by caspase-3, the catalytic domain is released from the C-terminal region and hyper-activated (10). We also reported that a truncated but not full-length MST was translocated into the nucleus during Fas-mediated apoptosis (10), implying that the cleaved MST phosphorylates nuclear target proteins that may be required to induce apoptosis. We speculate that the pro-apoptotic function of MST is generated mainly by the caspase-mediated activation of MST.

However, the caspase-mediated activation of MST does not require phosphorylation of Thr\textsuperscript{183} in the activation loop (Fig. 5). This result suggests that MST can be activated in two different ways, phosphorylation-dependently and independently (Fig. 7), depending on the condition of the cells. It should be investigated whether truncated MST is further activated by phosphorylation of Thr\textsuperscript{183}. It is also of interest whether the full-length and truncated MST phosphorylate the same targets or the C-terminal region plays a role in the recognition of substrates.

A pro-apoptotic role for the full-length MST is not excluded, because a stable expression facilitates death receptor-mediated apoptosis upstream of caspase-3 (10). In addition, when overexpressed, MST induces autoactivation and cell death without caspase-dependent cleavage (10). The cell death induced by the overexpression is closely related to the activation of MST, because dead cells show staining with anti-phospho antibody directed against caspase-3, the catalytic domain is released from the C-terminal region and hyper-activated (10). We also reported that a truncated but not full-length MST was translocated into the nucleus via phosphorylation of nuclear proteins. MST can efficiently phosphorylate histones in vitro, but it is remained as a question whether histones are physiological substrates of MST. Rat MST2 was reported to phosphorylate thyroid transcription factor-1 in vitro (42). It is also unknown whether this phosphorylation is a biological event and occurs in the cytoplasm or nucleus.

Our identification of the localization motifs and phosphorylation-dependent nuclear translocation of MST provides critical insight into the cytoplasmic and nuclear function of MST. The identification of cytoplasmic activators and the nuclear substrates remain as further work to be investigated.

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