MST, a Physiological Caspase Substrate, Highly Sensitizes Apoptosis Both Upstream and Downstream of Caspase Activation*

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The human serine/threonine kinase, mammalian STE20-like kinase (MST), is considerably homologous to the budding yeast kinases, SPS1 and STE20, throughout their kinase domains. The cellular function and physiological activation mechanism of MST is unknown except for the proteolytic cleavage-induced activation in apoptosis. In this study, we show that MST1 and MST2 are direct substrates of caspase-3 both in vivo and in vitro. cDNA cloning of MST homologues in mouse and nematode shows that caspase-cleaved sequences are evolutionarily conserved. Human MST1 has two caspase-cleavable sites, which generate biochemically distinct catalytic fragments. Staurosporine activates MST either caspase-dependently or independently, whereas Fas ligation activates it only caspase-dependently. Immunohistochemical analysis reveals that MST is localized in the cytoplasm. During Fas-mediated apoptosis, cleaved MST translocates into the nucleus before nuclear fragmentation is initiated, suggesting it functions in the nucleus. Transiently expressed MST1 induces striking morphological changes characteristic of apoptosis in both nucleus and cytoplasm, which is independent of caspase activation. Furthermore, when stably expressed in HeLa cells, MST highly sensitizes the cells to death receptor-mediated apoptosis by accelerating caspase-3 activation. These findings suggest that MST1 and MST2 play a role in apoptosis both upstream and downstream of caspase activation.

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The abbreviations used are: MST, mammalian STE20-like kinase; GFP, green fluorescence protein; PCR, polymerase chain reaction; mAb, monoclonal antibody; PAK, p21-activated kinase; TNF, tumor necrosis factor; Z-, benzoyloxycarbonyl; fmk, fluoromethylketone; PBS, phosphate-buffered saline; JNK, c-Jun N-terminal kinase; SAPK, stress-activated protein kinase; PAGE, polyacrylamide gel electrophoresis; aa, amino acids; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside.
study, we investigated the involvement of MST in apoptosis. We clearly show that both MST1 and MST2 are cleaved by caspase in vivo and in vitro. The proteolytic cleavage reveals the activation mechanism of MST and causes its cellular translocation. Most important, we show that MST can caspase-inducently induce morphological changes characteristic of apoptosis and enhances the sensitivity for death receptor-mediated apoptosis in epithelial cells.

EXPERIMENTAL PROCEDURES

Materials—GFPP expression vector pCMX-SAH/Y145F, β-galactosidase expression vector pJH1-LacZ, and Caenorhabditis elegans N2 cDNA library were kindly provided by Dr. K. Umezono (Kyoto University, Japan), Dr. A. Murakami (Kyoto University, Japan), and Dr. A. Sugi-moto (Tokyo University, Japan), respectively. Leptomycin was a generous gift from Dr. M. Yoshida (Tokyo University). Anti-FLAG antibody and cycloheximide were obtained from Sigma; agonistic anti-Fas antibody, CH-11 (0.1 μg/ml) penicillin, and 50 μg/ml streptomycin. CH-11 was produced by recombinant TNF was from Calbiochem.

Expression and Purification of His-tagged Proteins—His-tagged MST was expressed as a kinase-deficient protein because kinase-active MST was poorly expressed in bacteria. Human MST cDNA with a mutation at catalytic amino acid residue (MST1K59R or MST2K56R) was subcloned in frame into His tag expression vector, pQE-30 (Qiagen). For C-terminal His-tagging, MST1D326N was cloned into pET29a (Novagen). To obtain active His-tagged caspase-3, cDNA of human caspase-3 lacking N-terminal 28 amino acid residues was amplified by PCR using 5'-AGGATCTCTCTTGAATATCCCTGGAC-3' and 5'-TGAGCCTTT-GACATGCCCCAAGA-3' as primers (underline indicates BamHI site). PCR product was double-digested with BamHI and PstI and subcloned into pQE30 vector. His-tagged proteins were expressed in BL21(D3)/lysS bacteria and purified using His-Trap affinity column as described previously (28).

Mammalian Expression Constructs—Expression constructs of MSTs were prepared as an N-terminal FLAG tag. We introduced FLAG tag after the initiation codon in pME18S vector (27) and generated a FLAG-tag expression vector, pME18S-FLAG. The cDNA encoding full-length MST1, MST1327–487 (deleting aa 327–487), MST1311–326 (deleting aa 1–326), full-length MST2, MST2323–491 (deleting aa 323–491), and MST21–326 (deleting aa 1–326) was amplified by PCR and inserted in frame into pME18S-FLAG. For GFP fusion, cDNA of MST was subcloned in frame into pCMX-SAH/Y145F. Mutagenesis of the amino acid residue was performed with Quick-Change site-directed mutagenesis kit (Stratagene) and confirmed by DNA sequencing. Human PAK2 cDNA and caspase-8 cDNA were amplified by PCR from an HPB-ALL cDNA library and cloned into pCMX-SAH/Y145F vector.

Preparation of Anti-MST Monoclonal Antibody—For immunization, affinity-purified His-MST1K59R and His-MST2K56R were further loaded onto Mono-Q anion exchange column (Amersham Pharmacia Biotech) and eluted with a linear NaCl gradient of 0.1 to 0.5 M as described (28). Purified fraction, dialyzed against 20 mM Tris (pH 7.5) containing 150 mM NaCl, was used for immunization of BALB/c mice. Establishment and cloning of hybridoma cells were performed as described previously (28). Monoclonal antibodies were purified from hybridoma culture with protein G-affinity column as described (Amersham Pharmacia Biotech) (28).

Cell Culture, Transfection, and Induction of Apoptosis—Human thymoma-derived HPB-ALL, human acute T cell leukemia-derived Jurkat, and murine lymphoma-derived WR19L12a cells were cultured in RPMI 1640 with 10% fetal calf serum, 20 mM Hepes (pH 7.3), 50 μM β-mercaptoethanol, 50 units/ml penicillin, and 50 μg/ml streptomycin. Murine fibroblast NIH 3T3, human adenocarcinoma-derived HeLa, and human embryonic kidney-derived 293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 100 μg/ml kanamycin. Jurkat cells were transfected by electroporation at 300 V with capacitance of 960 microfarads. NIH 3T3 or HeLa cells were seeded on glass coverslides (Nalge Nunc) or 60-mm culture plates 24 h before transfection and then transfected with LipofectAMINE (Life Technologies, Inc.) in accordance with the manufacturer's suggestion. To establish stable HeLa cell lines, pME18S-Neo vector harboring neomycin-resistant gene was co-transfected with expression vectors encoding GFP, GFPP-MST1, or MST1K59R. The transfecants were grown and selected in the presence of 1 mg/ml G418. To induce apoptosis, cells were treated with anti-FLAG antibody, CH-11 (0.1 μg/ml) (27, 30), TNFα (50 ng/ml), or staurosporine (0.5 μM). Where indicated, cells were co-treated with cycloheximide (10 μg/ml).

Preparation of Cell Lysate, Immunoprecipitation, and Immunoblot Analysis—Cells were washed once with PBS and suspended in cold lysis buffer (40 mM Hepes [pH 7.5] with 150 mM NaCl, 1% Triton X-100, 0.5% Nonidet P-40, 150 μM NaCl, 50 mM N-acetyl-L-leucine (in 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, and 0.1 mM vanadate with protease inhibitor mixture (1 μg/ml aprotinin, leupeptin, and pepstatin)). Cell lysates were cleared by centrifugation at 15,000 rpm for 20 min. For immunoprecipitation, cell lysate was incubated with 2 μg of anti-FLAG mAb or anti-MST mAb for 2 h at 4°C and prebound to 1 μg of protein A-agarose (Amersham Pharmacia Biotech). Cell lysate or immunoprecipitates were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membrane (Millipore). The membrane was blocked in TBST buffer (20 mM Tris-HCl (pH 7.5) containing 150 mM NaCl and 0.1% Tween 20) with 5% skim milk at room temperature for 1 h. The membrane was incubated with anti-FLAG mAb or anti-MST mAb for 1 h, washed in TBST, and then incubated for 1 h with horseradish peroxidase-conjugated anti-mouse IgG (Amersham Pharmacia Biotech). After further washing with TBST, peroxidase activity was detected on x-ray films using an enhanced chemiluminescence detection system (DuPont).

In Vitro Cleavage Assay and Cleavage Site Mapping—Purified His-MST1K59R or His-MST2K56R was incubated with the purified recombinant caspase-3 at 30°C for 30 min. The reaction was stopped by adding Laemmli’s sample buffer, resolved on 10% SDS-polyacrylamide gel, and immunoblotted with anti-MST mAb. For purification of C-terminal His-tagged antibody, MST1K59R, or His-MST2K56R was incubated with the purified recombinant caspase-3 at 30°C for 30 min. The reaction was terminated by adding 50 μM Z-VAD-fmk. The sample was mixed with nickel-nitrirotetroic acid agarose (Qiagen) and incubated at 4°C for 2 h. The resin was washed with 10 volumes of lysis buffer containing 5 mM imidazole. Laemmli’s sample buffer was directly added to the resin, and cleaved proteins were separated on 15% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membrane. The membrane was immunoblotted or stained with Coomassie Brilliant Blue. The fragment was cut and analyzed by N-terminal peptide sequencing (27, 28).

Cloning of Murine and C. elegans MST Homologues—MST homologues of mouse and C. elegans were identified by homology search against public data bases and cloned by PCR-based methods as follows. The 5′-end of the identified EST region of murine cDNA cloned with cosmid was sequenced to three regions of human MST1 gene. To obtain a complete cDNA sequence, 5′-rapid amplification of cDNA ends PCR was carried out using a cDNA library of mouse lymphoma WR19L12a cells as template. For cloning of murine MST2 primers, designs based on the reported nucleotide sequence (GenBank™ accession number U28726), 5′-AGGGATCCTCTGGAATATCCCTGGAC-3' and 5′-GACCATGCCCACAGA-3' as primes. The cloned cDNA was compared with a genome data base (ACeDB) and BLAST search against EST or genome data base (ACeDB).

Preparation of N2 cDNA—For immune complex kinase assay, immunoprecipitates were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was incubated with 2 μg of histone H2B in 20 μl of kinase reaction buffer (40 mM Hepes [pH 7.5] with 20 mM MgCl2, 20 mM β-glycerol phosphate, and 0.1 mM vanadate) containing 25 μM ATP and 2.5 μM of γ[32P]ATP for 20 min at 30°C. Reactions were terminated by adding 7 μl of Laemmli’s sample buffer and boiled in boiling water for 5 min. A portion of the sample (15 μl) was analyzed by PhosphorImage analyzer. The cDNAs of murine and C. elegans MST were subcloned pME18S-FLAG and pCMX-SAH/Y145F vector, respectively.

MST Kinase Assay—Endogenous MST or various forms of FLAG-tagged MST were immunoprecipitated with 2 μg of antibody, and the same amounts of immunoprecipitates as analyzed by immunoblot were used for in-gel phosphorylation assay (27) or immune complex kinase assay. For immune complex kinase assay, immunoprecipitates were incubated with 2 μg of histone H2B in 20 μl of kinase reaction buffer (40 mM Hepes [pH 7.5] with 20 mM MgCl2, 20 mM β-glycerol phosphate, and 0.1 mM vanadate) containing 25 μM ATP and 2.5 μM of γ[32P]ATP for 20 min at 30°C. Reactions were terminated by adding 7 μl of Laemmli’s sample buffer and boiled in boiling water for 5 min. A portion of the sample (15 μl) was analyzed by PhosphorImage analyzer. The cDNAs of murine and C. elegans MST were subcloned pME18S-FLAG and pCMX-SAH/Y145F vector, respectively.

β-Galactosidase Staining—NIH 3T3 or HeLa cells were cultured on 6-well plates and transfected with 1 μg of MST kinase expression vector and 0.2 μg of a β-galactosidase expression vector (pLauZ). After 48 h, the cells were washed once with PBS and fixed with 2% formaldehyde and 0.2% glutaraldehyde in PBS for 5 min at 4°C. After being further
FIG. 1. Characterization of anti-MST mAbs. A, FLAG-MST1 or FLAG-MST2 was expressed in HeLa cells on 6-well plates, and apoptosis was induced by 4 h of incubation with CH-11 (0.1 μg/ml) and cycloheximide (10 μg/ml). Cell lysates (20 μg) were resolved on 12% SDS-PAGE gel and immunoblotted with anti-FLAG or anti-MST mAbs as indicated. B, FLAG-MST1 and FLAG-MST2 were immunoprecipitated with anti-FLAG or the indicated anti-MST mAbs. Immunoprecipitates (IP) were resolved on SDS-PAGE gel and immunoblotted with anti-FLAG mAb. C, HPB-ALL cells (2 × 10⁷) were preincubated without (−) or with (+) 25 μM Z-VAD-fmk for 1 h, and apoptosis was induced by incubation with CH-11 (0.1 μg/ml) for the indicated times. Cell lysates (50 μg) were analyzed by immunoblotting with anti-MST mAb, G2B.

FIG. 2. MST1 and MST2 are proteolytically activated by caspase. A–D, HPB-ALL cells (5 × 10⁷) were treated with Z-VAD-fmk and CH-11 as in the legend of Fig. 1. Aliquots of lysate were immunoprecipitated with anti-MST mAb, G2B (A and C), or A8C (B). Immuno blot analysis was performed using G2B (A) or A8C (B). As a control, purified MST from apoptotic HPB-ALL cells (27) was also analyzed and indicated as purified 34-kDa MST (A and B). An aliquot of immunoprecipitate was subjected to in vitro kinase assay using histone H2B as substrate (C). Cell lysates were analyzed for DNA fragmentation as a marker of apoptosis (D). E, bacterially expressed His-MST1 (1 μg) and His-MST2 (1 μg) were incubated with (+) or without (−) recombinant active caspase-3 (50 ng) in the presence (+) or absence (−) of 25 μM DEVD-CHO at 30 °C for 30 min. The samples were resolved on SDS-PAGE gel and analyzed by immunoblotting with G2B.

RESULTS

Proteolytic Cleavage and Activation of Both MST1 and MST2 by Caspase in Fas-mediated Apoptosis—We established several hybridoma cell lines producing anti-MST mAbs, and purified mAbs were characterized by immunoblot analyses for HeLa cells transfected with FLAG-MST1 or FLAG-MST2 (Fig. 1A). Monoclonal antibody G2B equally recognized both MST1 and MST2, whereas A8C selectively recognized only MST1. When apoptosis was induced, G2B and ABC could detect a cleaved 34-kDa fragment of MST. On the contrary, J7B detected the C-terminal 21-kDa fragment of MST2. Other mAbs, DIH, F5C, and H3B, recognized N-terminal 34-kDa fragment of MST1 and MST2.\(^2\)

Immunoprecipitation analysis showed that A8C and DIH selectively immunoprecipitated FLAG-MST1, whereas J7B immunoprecipitated only FLAG-MST2 (Fig. 1B). A8C could not immunoprecipitate N-terminal 34-kDa fragment although A8C recognized it in immunoblot.\(^2\) Both FLAG-MST1 and FLAG-MST2 were efficiently immunoprecipitated by G2B or H3B. With G2B and A8C anti-MST mAbs, we examined the cleavage of endogenous MST1 and MST2 during Fas-mediated apoptosis in HPB-ALL cells, which expressed both MST1 and MST2. G2B recognized a 34-kDa fragment of MST at 2 h after the stimulation of Fas (Fig. 1C). This fragment was also confirmed by immunoprecipitation (Fig. 2A). The cleavage of MST was inhibited when apoptosis was blocked by the pretreatment with Z-VAD-fmk, a broad spectrum caspase inhibitor (Fig. 1C and 2A). The molecular weight of cleaved MST coincided with that of the previously purified protein kinase from apoptotic HPB-ALL lysate, confirming our result that MST is specifically

\(^2\) K.-K. Lee, T. Ohyama, N. Yajima, S. Tsubuki, and S. Yonehara, unpublished observations.
cleaved to a 34-kDa catalytic fragment in apoptosis (Fig. 2A) (27). A8C efficiently precipitated MST1 when apoptosis was not induced or Z-VAD-fmk was pretreated. However, A8C could not immunoprecipitate MST1 when apoptosis was induced (Fig. 2B). These results suggest that MST1 was completely cleaved to a fragment that could not be precipitated by A8C because A8C could immunoprecipitate only uncleaved MST1.2

Next, we examined whether the cleavage of MST induces its activation by immune complex kinase assay using histone H2B as substrate. As depicted in Fig. 2, A–D, the phosphorylation of H2B was substantially increased by Fas ligation with approximately the same kinetics as processing of MST and DNA ladder formation. Preincubation with Z-VAD-fmk effectively prevented MST activation for up to 3 h after Fas ligation. Bacterially expressed His-tagged MST1 and MST2 were completely cleaved to the 34-kDa fragment, and the cleavage was blocked by Z-VAD-fmk (Fig. 2E). These results suggest that both human MST1 and MST2 are physiological substrates of caspase-3 and are proteolytically activated by caspase in Fas-mediated apoptosis.

**Evolutionary Conservation of the Cleavage Site in MST**—To address the functional importance of cleavage sequences in human MST1 and MST2 (hMST1 and hMST2), we cloned murine and *C. elegans* homologues (mMST and cMST, respectively) and compared the polypeptide sequences of hMST1, hMST2, mMST1, mMST2, rat MST2 (rMST2, GenBank™ accession number AJ001529), and cMST (Fig. 3). Murine MST1 and MST2, which show 75% identity to each other, had 97 and 96% homology to human MST1 and MST2, respectively (Fig. 3A). Previously reported cDNA (GenBank™ accession number U28726) that encodes a murine MST2-like polypeptide deleted the C-terminal region, which is caused by single nucleotide deletion. However, we could not obtain MST2 cDNA with nucleotide deletion or alternative splicing from a murine lymphoma WR19L12a cDNA library (28). We obtained only one MST homologue in *C. elegans*, which showed similar homology (50%) to mammalian MST1 and MST2. The kinase domain was most conserved, with 95–100% homology between mammalian homologues. Even the kinase domain of cMST showed 75% homology to mammalian MST1 or MST2. The C-terminal 60 amino acids required for dimerization were also highly conserved in all homologues (23) (Fig. 3B). The putative caspase-cleavable sites (Fig. 3B, indicated by arrow) were con-
served in all MST homologues, and these homologues were actually cleaved when apoptosis was induced. Thus, the functional cleavage sequence for caspase is evolutionarily conserved in MST homologues.

Two Activation Mechanisms of MST1 following Treatment with Staurosporine—Previously, Taylor et al. (16) identified a protein kinase responsive to stress (KRS), which was activated by treatment with staurosporine but not by Fas ligation. Subsequent biochemical purification revealed it as MST (16). However, since our result showed that Fas ligation activated MST (27) (Fig. 2), we compared the MST activation by staurosporine treatment and that by Fas ligation in Jurkat cells that undergo apoptosis following these stimulations. Both staurosporine treatment and Fas ligation effectively induced the cleavage of MST1 to 34 kDa (Fig. 4). Unexpectedly, MST1D326N in which the site of cleavage (Asp326) by caspase-3 is mutated to Asn (27) was also processed to a slower migrating fragment of 40 kDa. In addition, the mobility of the 34-kDa fragment was slightly but significantly different in staurosporine- and Fas-induced apoptosis. The reason for the different mobility might be that staurosporine treatment caused a change of phosphorylation state in MST1, which was not caused by Fas stimulation. We investigated the kinase activity of each MST1 fragment by in-gel phosphorylation assay using histone as substrate. Phosphorylation at 55 kDa corresponding to full-length MST1 was greatly increased at 0.5 h by staurosporine treatment and peaked at 1 h (Fig. 4, lanes 1–4). In contrast, Fas ligation could not induce phosphorylation of full-length MST1 up to 3 h (Fig. 4, lanes 5 and 6). Phosphorylation at 40 kDa was also rapidly induced at 0.5 h by staurosporine, although this fragment was not detected in immunoblot analysis. Fas stimulation did not induce a 40-kDa fragment in immunoblot or kinase assay of 40-kDa in-gel kinase assay. The 34-kDa fragment with histone kinase activity was generated from FLAG-MST1 (Fig. 4, lanes 1–6) but not from FLAG-MST1D326N within 3 h by staurosporine and Fas ligation (Fig. 4, lanes 7–12). These results indicate that staurosporine can activate MST by two mechanisms, caspase-independent early activation and caspase-dependent late activation, whereas Fas ligation activates MST only in a caspase-dependent pathway.

Cleavage of MST1D326N to a 40-kDa fragment indicated that another cleavage site exists in the C-terminal region of Asp326, and this cleavage is also the biochemical event involved in apoptosis. His-tagged MST1K59R,D326N was effectively cleaved to 40 kDa in apoptotic Jurkat cell lysate. To determine the site of this cleavage, we purified a C-terminal 15-kDa fragment derived from MST1K59R,D326N and performed N-terminal peptide sequencing. It was revealed that Asp349 was the cleavage site, and the peptide sequence surrounding Asp349 (TMTD349G) was similar to the identified caspase recognition sequence of MST homologue, particularly that of cMST (Fig. 3B). We mutated both Asp326 and Asp349 to Asn (MST1D326N,D349N) and tested whether MST1D326N,D349N is resistant to proteolytic cleavage during Fas-mediated apoptosis. MST1D326N,D349N was cleaved to neither the 40- nor to the 34-kDa fragment (Fig. 5). MST2D322N was not cleaved to a smaller fragment indicating that MST2 is cleaved only at Asp322 during apoptosis. In contrast, wild type MST and MST1D326N were cleaved to the 34- and 40-kDa fragment, respectively (Fig. 5). Therefore, MST1 is cleaved at two different sites resulting in a 34- or 40-kDa fragment, and the 40-kDa fragment is further cleaved to 34 kDa.

Apoptotic Morphological Changes Induced by MST1—To investigate the possible function of MST in apoptosis, we transfected NIH 3T3 cells with FLAG-tagged MST constructs (Fig. 6A). Expression of the various MST1 constructs was confirmed by immunoblotting with anti-FLAG mAb (Fig. 6B, top). Overexpression did not induce MST1 cleavage. The expression level of MST1Δ327–487, with a C-terminal truncation corresponding to the active MST1 fragment generated by caspase, was very low. In comparison with MST1, MST1Δ327–487 showed enhanced histone phosphorylation activity but little autophosphorylation activity (Fig. 6B, bottom). A similar result was
obtained when FLAG-tagged MST2 was expressed in NIH 3T3 cells. Cells expressing active MST1 showed marked changes resulting in round morphology (Fig. 6C). The morphological change induced by MST1 was indistinguishable from that induced by overexpression of caspase-8, an initiator caspase. However, the round morphology induced by MST1 was not inhibited by the treatment with Z-VAD-fmk, although that by caspase-8 was inhibited by Z-VAD-fmk. We compared the morphological change induced by MST1 with that caused by PAK2 (21). Similar change was observed when PAK2 was transfected into NIH 3T3 cells. Neither kinase-negative PAK2K299R, MST1K59R, nor MST1Δ326–326 deleting N-terminal kinase domain evoked changes in cell morphology indicating that kinase activity of MST1 or PAK2 is required for this morphological change. MST-induced change of morphology was also observed in HeLa cells (Fig. 7). MST-expressing cells were rounded and shrunken with associated destruction of cytoskeletal structure (Fig. 7A). Hyperactive MST1Δ327–487 showed more dramatic change of morphology and resulted in the loss of MST1Δ327–487-expressing cells by detachment from substrate. This result may elucidate why the percentage of the round cells in MST1Δ327–487-transfected cells was reduced in β-galactosidase assay (Fig. 6C). The enhanced morphological change induced by MST1Δ327–487 may be the result from the increased kinase activity because kinase-negative MST1K59RΔ327–487 or MST1Δ327–487 did not induce any morphological changes.

We investigated MST-induced morphological changes in detail. In MST1-expressing HeLa cells at 16 h post-transfection, the cytoplasm was remarkably reduced compared with untransfected cells or transfected cells with MST1K59R (Fig. 7B, top and middle). The morphological change of chromosome was not observed, although the nuclei were slightly reduced and rounded. MST1 and MST1K59R localized in the cytoplasm. At 48 h post-transfection, the cytoplasmic space was completely lost, and cells were eventually detached from the plate (Fig. 7B, bottom). In addition, condensation and fragmentation of chromosome was obvious by Hoechst staining. MST1 appeared to localize in the nucleic area but not overlapped with chromosome. However, these cells were not positive for terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling, and the characteristic DNA ladder in apoptosis was not detected on agarose gel electrophoresis. These observations indicate that chromosome was not cleaved to much smaller fragments generated by caspase-activated nuclease, such as CAD. We conclude that part of apoptosis-related morphological changes are induced by overexpression of MST, which mimics the caspase-dependent activation of MST in apoptosis.

Figure 5. MST is cleaved at Asp326 and Asp349 in apoptosis. HeLa cells on 6-well plate were transfected with FLAG-MST1 or FLAG-MST2 constructs. After 48 h of post-transfection, apoptosis was induced with of CH-11 (0.1 μg/ml) and cycloheximide (10 μg/ml). Immunoblot analysis (20 μg) was performed with anti-FLAG mAb. WT, wild type.

Figure 6. MST kinase activity and its effect on morphological change in NIH 3T3 cells. A, schematic representation of MST1 constructs used in this study. Arrowheads indicate caspase cleavage sites in human MST1. B, FLAG-MST1 constructs were expressed in NIH 3T3 on a 6-well plate and immunoprecipitated with anti-FLAG mAb. Immunoprecipitates were analyzed by immunoblotting with anti-FLAG mAb (top) or in vitro kinase assay (bottom). Kinase assay was performed using histone H2B as substrate at 30 °C for 20 min. The phosphorylated proteins were resolved on 15% SDS-PAGE gel and then analyzed by autoradiography. Arrohead indicates autophosphorylation activity of MST1Δ327–487. C, pJ71-LacZ (0.2 μg) was co-transfected with vector (1 μg) expressing caspase-8, MST1K59R, MST1, MST1Δ327–487, MST1Δ327–487, PAK2K299R, or PAK2. Cells transfected with MST1 were cultured with or without Z-VAD-fmk (100 μM). At 48 h post-transfection, cells were fixed and stained for β-galactosidase expression. At least 100 blue cells/sample were counted, and the number of round blue cells was expressed as a percentage of the total number of blue cells. The means of three independent determinations ± S.D. are shown. WT, wild type.
MST1K59R were exclusively localized in the cytoplasm when expressed as FLAG-tagged or GFP fusion forms (Fig. 7 and 8A). MST1D1–326 deleting N-terminal kinase domain was also localized in the cytoplasm, but MST1D327–487 was distributed in both cytoplasm and nucleus. To test whether the cytoplasmic localization of MST1 is controlled by active nuclear export, HeLa cells expressing MST1 or MST1D1–326 were treated with leptomycin, an inhibitor of CRM1/exportin (31, 32). MST1 or MST1D1–326 rapidly lost cytoplasmic localization (Fig. 8A). These results indicate that cytoplasmic localization of MST1 is controlled by a C-terminal regulatory region through active nuclear export by the CRM1/exportin (33–35).

MST Activation and Involvement in Apoptosis

To test whether the localization of MST is changed in Fas-mediated apoptosis, we established stable HeLa cell lines expressing caspase-cleavable and uncleavable MST1. MST1 was fused at the C-terminal end of GFP for rapid detection of morphological change and translocation. Established cell lines expressing wild type MST did not show significant morphological differences with control cell lines, although transiently expressed MST induced morphological change (Fig. 7). In the stable cell lines, cleavable and uncleavable MST1 showed a similar cytoplasmic localization before induction of apoptosis.2 However, in early apoptotic cells that showed blebbing of membrane but retained their nuclear structure, cleavable MST1 and MST1K59R translocated into the nucleus although the apoptosis was apparently occurring as judged by the apoptotic blebbing of membrane (Fig. 8B, bottom row). In
later apoptotic cells with nuclear fragmentation, even MST1K59R,D1326N,D349N completely lost cytoplasmic localization and was detected in the fragmented nuclei, suggesting the translocation after the breakdown of the nuclear envelope. We conclude that MST1 is cytoplasmic protein and the cleaved MST1 translocates into the nucleus before the nuclear fragmentation is initiated.

**MST Sensitizes Fas- and TNFα-mediated Apoptosis**—To address further the function of MST in apoptosis, we investigated the sensitivity of HeLa cell lines stably expressing MST1 in Fas- and TNFα-mediated apoptosis. The expression of GFP-MST1 and GFP-MST1K59R was confirmed by immunoblotting with G2B or with anti-PARP antibody as a control for loading and transfer (Fig. 9A). All sub-cell lines expressing GFP-MST1 were more sensitive to Fas- or TNFα-mediated apoptosis than parental HeLa cells or GFP-MST1K59R-expressing cells (Fig. 9B). GFP-MST1K59R did not accelerate or inhibit Fas- or TNFα-mediated apoptosis.

Interestingly, GFP-MST1 greatly facilitated the activation of caspase-3-like protease in Fas-mediated apoptosis (Fig. 9C) suggesting that MST1 can accelerate caspase-3 activation. To test this possibility, we investigated the cleavage kinetics of caspase-3 in detail. As shown in Fig. 9D, the p17 subunit of caspase-3 was detected at 1.5–2 h in MST1-expressing cell lines (HL-WT1 and HL-WT2) but p17 was detected after 3 h in control cells. Furthermore, the cleavage kinetics of GFP-MST1 was correlated with that of caspase-3 supporting the accelerated activation of caspase-3, because MST1 was shown to be cleaved by activated caspase-3 (Fig. 2). GFP-MST1 did not accelerate the onset of other apoptosis induced by pharmaceutical drugs such as ceramide, staurosporine, and etoposide. We conclude that stably expressed MST1 sensitizes death receptor-mediated apoptosis through the accelerated onset of caspase-3 activation.

**DISCUSSION**

Cleavage and Activation of MST in Apoptosis—We previously reported that during Fas-mediated apoptosis MST1 and MST2 are proteolytically activated by specific cleavage. However, it remained unclear how MST1 and MST2 are involved in apoptosis. In this study, we clearly show that MST1 and MST2 are physiologically activated by caspase-3-like protease and involved in apoptosis. Caspase cleavage sequences of MST1 (Asp-Glu-Met-Asp326-Ser) and MST2 (Asp-Glu-Leu-Asp322-Ser) are more conserved and optimal than those of other protein kinases known to be activated proteolytically by caspase-3-like proteases including caspase-6 and caspase-7 (27, 36). This probably explains why only MST was purified as a major protein kinase that was activated in Fas-mediated apoptosis (27). Caspase cleavage sequences in MST1 and MST2 are conserved not only in their mammalian but in the nematode homologue, strongly suggesting their biological significance including a role in apoptotic cell death signaling. The cleavage of MST may be a common molecular event in apoptosis since various apoptotic signals induce caspase-dependent MST cleavage, including signals from Fas and TNFα receptor, and chemical apoptotic inducers such as staurosporine, etoposide, and ceramide. Recently bisphosphonates, pharmacological drugs for osteoporosis, and anti-tumor drugs such as cytotoxin A have been reported to induce caspase-dependent cleavage of MST in osteoclast and leukemia cells (37, 38).

The cleavage of MST is closely coupled with its activation. Although MST has basal kinase activity in non-apoptotic cells, caspase-dependent MST cleavage induces significant and strong activation of the catalytic fragment. Furthermore, truncated MST, MST1Δ27–487, has powerful kinase activity supporting the activation by caspase-mediated cleavage. In human MST1, another cleavage site (TMTDGG349) was found in this report, which generates a catalytic fragment of 40 kDa. The 40-kDa fragment is detected early in the in-gel kinase assay,
MST1 is sequentially cleaved at Asp326 and Asp349 in staurosporine-induced apoptosis. However, it is not likely that the cleavage at Asp349 is a prerequisite for MST1 activation because Asp349 is not conserved in murine MST1. In addition, MST1D326N,D349N is processed to the 34-kDa fragment with strong kinase activity. Nevertheless, a specific function of the 40-kDa fragment, particularly in early apoptotic events, cannot be ruled out because it shows distinct catalytic activity from the 34-kDa fragment.

Apoptotic Cleavage of MST Induces Nuclear Translocation—We show that MST is localized exclusively in the cytoplasm. Deletion analysis shows that the C-terminal region of MST is sufficient for cytoplasmic localization that is lost on treatment with leptomycin. Cytoplasmic localization of MST may be regulated by nuclear export signal although the possibility remains that other nuclear export signal-containing proteins or cytoplasmic anchor protein regulates MST localization. MST cleavage by caspase results in the subcellular relocalization of catalytic fragment. In Fas-mediated apoptosis, cleaved MST1 translocates into the nucleus before nuclear fragmentation is initiated. This result suggests additional nuclear functions of cleaved MST1 in apoptosis. Histone H2B is selectively phosphorylated in mammalian apoptotic cells and is associated with chromatin condensation in apoptosis (39). It is of interest to test whether cleaved MST phosphorylates H2B in apoptosis because H2B is an excellent substrate in vitro (Fig. 2 and 6).

Caspase-dependent cleavage might affect other biochemical functions of MST. MST1 and MST2 were reported to form homo- or heterodimers through the C-terminal region (23), and cleavage of MST naturally leads to the dissociation of N-terminal catalytic fragments. Therefore, cleavage of MST during apoptosis results in at least three coupled events, activation, monomerization, and translocation of catalytic fragment into the nucleus. These representative regulatory mechanisms, observed in many signaling molecules, may be involved in the apoptotic function of MST.

MST Is Involved in Apoptotic Events—Overexpression of MST or truncated MST induces morphological changes in cells. These changes are at least partly related to the characteristic morphology of apoptotic cells such as cell rounding, detachment from substratum, and the fragmentation and condensation of nucleus. However, we could not detect other apoptotic characteristics such as externalization of phosphatidylserine, DNA laddering, or formation of apoptotic bodies. These morphological changes induced by MST are similar to those induced by PK2 (21, 22). Overexpression of each kinase-negative, caspase-resistant form of MST1 and MST2 does not inhibit Fas-mediated apoptosis.5 This suggests that other SPS1/STE20 family members are also involved in apoptosis. Recently, STE20-related kinase, SLK, was reported to be cleaved by caspase-3 and to induce cytoskeletal rearrangement (40–42). Caspase-dependent cleavage and effects on apoptotic morphology of MST, PK2, and SLK suggest that SPS1/STE20 family kinases are closely involved in apoptosis, probably targeting similar molecules.

Previously it was suggested that overexpression of MST1 activates caspase through the JNK/SAPK kinase pathway (43). However, others (37) could not detect the SAPK/JNK activation. We detected neither the JNK activation nor the caspase activation by overexpression of MST, and PARP, caspase substrate, was not cleaved in our system.2 In addition, the MST-induced morphological change of the cell was unaffected by preincubation with caspase inhibitor, Z-VAD-fmk (Fig. 6, C and D). Caspase-resistant MST1D326N,D349N still induced similar morphological changes in the presence of caspase inhibitor. Thus, we conclude that morphological changes induced by transient overexpression of MST do not require the activation of caspase, and SAPK/JNK might not be involved in the morphological change.

When stably expressed in the GFP fusion form, MST1 highly sensitizes HeLa cells to Fas- or TNFα receptor-mediated apoptosis. Surprisingly, susceptibility to apoptosis was enhanced by accelerated activation of caspase. This result contradicts the finding of transient expression (Fig. 6) that indicates caspase activation is not required for the MST-induced apoptosis-like morphological changes. One possible explanation is that MST has two distinctive functions, direct induction of morphological changes and sensitization to death receptor-mediated apoptosis. The limited apoptotic change induced by transiently expressed MST may reflect MST function downstream of caspase. This explanation is supported by the fact that MST1 is a substrate of caspase, and truncated MST induces morphological changes that are more dramatic. When stably expressed, MST may accelerate caspase activation through the phosphorylation of pro- or anti-apoptotic regulators or caspases. It cannot be ruled out that MST regulates the expression of apoptotic regulators or caspases. Although it is largely unknown how MST is involved in apoptosis, our result clearly shows that it acts as both an upstream activator and a downstream effector of caspase; MST regulates death receptor-induced caspase activation and amplifies caspase-dependent morphological change.

Our result provides some clue to the regulation and function of MST in non-apoptotic cell signaling. Although no physiological activators are known, the rapid activation of MST by staurosporine indicates that there exists a caspase-independent mechanism of activation, which is regulated by upstream kinase(s) or phosphatase(s). Another critical clue to understanding the function of MST may be obtained from the analysis of subcellular localization since some protein kinases are tightly regulated in intracellular spaces (44–46). We propose the regulation of subcellular localization as a possible mechanism regulating cellular function of MST. The identification of physiological substrate will undoubtedly lead to a better understanding of MST function in apoptosis or other cell signaling.

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