Caspase cleavage of MST1 promotes nuclear translocation and chromatin condensation

Seiji Ura*, Norihisa Masuyama*, Jonathan D. Graves†, and Yukiko Gotoh*‡§

*Institute of Molecular and Cellular Biosciences, University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan; †Department of Immunology, University of Washington Medical Center, Seattle, WA 98195; and ‡PRESTO Research Project, Japan Science and Technology Corporation, Osaka, 560-0082 Japan

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MST1, mammalian STE20-like kinase 1, is a serine/threonine kinase that is cleaved and activated by caspases during apoptosis. MST1 is capable of inducing apoptotic morphological changes such as chromatin condensation upon overexpression. In this study, we show that MST1 contains two functional nuclear export signals (NESs) in the C-terminal domain, which is released from the N-terminal kinase domain upon caspase-mediated cleavage. Full-length MST1 is excluded from the nucleus and localized to the cytoplasm. However, either truncation of the C-terminal domain, point mutation of the two putative NESs, or treatment with leptomycin B, an inhibitor of the NES receptor, results in nuclear localization of MST1. Staurosporine treatment induces chromatin condensation, MST1 cleavage, and nuclear translocation. Staurosporine-induced chromatin condensation is partially inhibited by expressing a kinase-negative mutant of MST1, suggesting an important role of MST1 in this process. Significantly, MST1 is more efficient at inducing chromatin condensation when it is constitutively localized to the nucleus by mutation of its NESs. Moreover, inhibition of MST1 nuclear translocation by mutation of its cleavage sites reduces its ability to induce chromatin condensation. Taken together, these results suggest that truncation of the C-terminal domain of MST1 by caspases may result in translocation of MST1 into the nucleus, where it promotes chromatin condensation.

Apoptosis, or programmed cell death, is an active process fundamental to development and homeostasis of multicellular organisms. Apoptosis-inducing stimuli such as pro-apoptotic cytokines, UV irradiation, and DNA-damaging drugs induce an apoptotic response characterized by a series of shared morphological changes in the membrane, cytoplasm, and nucleus (1, 2). The activation of a proteolytic cascade, mediated by a family of cysteine proteases called caspases, is critical to the initiation and progression of apoptosis (3–5). Aggregation of the adaptor molecules, FADD and Apaf-1, promotes activation of caspase-8 and caspase-9, respectively. These initiator caspases then activate downstream effector caspases, such as caspase-3 and caspase-6, which are largely responsible for targeting critical substrates (3–5). Although many caspase substrates have been identified, the biological functions of most of these substrates remain largely unknown. Identifying caspase targets and determining the effects of caspase-mediated cleavage on their function will be critical in understanding the mechanisms of apoptosis.

One of the hallmarks of apoptosis is the degradation and compaction of chromatin. The ability of caspase inhibitors to delay or completely abolish chromatin condensation, in response to apoptotic stimuli such as staurosporine or etoposide, indicates that caspases and their downstream targets play an important role in these events (6–8). Among the caspase targets that have been implicated in chromatin condensation are CAD (caspase-activated DNase) (9–11) and acinus (12). However, because the inactivation of these effectors does not completely block chromatin condensation, it appears likely that other caspase targets are also involved (8, 13).

Recently, several protein kinases including PAK2 (14), MEKK1 (15–17), HPK1 (18), ROCK I (19, 20), SLK (21), and mammalian STE20-like kinase 1 (MST1) (22–28) have been identified as caspase substrates, suggesting that phosphorylation processes may play an important role during apoptosis. MST1 is a ubiquitously expressed serine/threonine kinase, structurally related to STE20, a yeast mitogen-activated protein kinase kinase kinase kinase (MAPKKKK) (29, 30). MST1 also appears to function as a MAPKKKK in the c-Jun N-terminal kinase and p38 pathways in mammalian cells (22). Deletion analyses have revealed that the C-terminal domain of MST1 serves an inhibitory function (31). Interestingly, MST1 is activated during apoptosis by a process that involves both phosphorylation and removal of the C-terminal domain by caspases (23). MST1 has been reported to be the most prominently stimulated in-gel kinase activity observed during apoptosis in response to a wide variety of apoptotic stimuli such as staurosporine, engagement of CD95/Fas, etoposide, UV, okadaic acid, serum starvation, anti-tumor drugs, and arsenite (22, 24–28, 30, 32). MST1 is capable of inducing apoptosis upon overexpression (22) and is required for apoptosis induced by certain genotoxic reagents (27, 28). Collectively, these findings suggest that MST1 may be an important caspase effector that contributes to apoptosis.

In this paper, we identify two functional nuclear export signals (NESs) in MST1. Importantly, the caspase-mediated cleavage of MST1 separates the NES-containing C-terminal from the N-terminal catalytic domain, allowing nuclear translocation of the latter and facilitating chromatin condensation. Therefore, we propose that MST1 is a novel mediator of apoptotic signaling from cytoplasm to nucleus and may be one of the inducers of chromatin condensation during apoptosis.

Materials and Methods

Reagents. NES inhibitor leptomycin B (LMB) (33, 34) was a kind gift from Minoru Yoshida (University of Tokyo). Staurosporine was obtained from Sigma. FuGENE6 transfection reagent was purchased from Roche Biochemicals.

Plasmids. Myc-tagged MST1 expression vector (pcMycMST1) was constructed by ligating a *Hin*dIII–*Eco*RI fragment from pJ3 M-MST1 (29) into pcDNA3 (Invitrogen). Kinase-negative mutant of MST1 was made by mutating Lys-59 to Arg. N-terminal domain of MST1 (MST1 1–330, 1–380) was amplified from pcMycMST1 by PCR using T7 primer as a forward primer and 5′-CGAATTCTCACATCGTGCCAGAATCCATTTC-3′ (1–330) or 5′-GCGAATTCTTAAGTTCCTTCCTCTTCCT-

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Abbreviations: NES, nuclear export signal; MST1, mammalian STE20-like kinase 1; ECFP, enhanced cyan fluorescent protein; CLSM, confocal laser scanning microscopy; LMB, leptomycin B; MAPKKKK, mitogen-activated protein kinase kinase kinase kinase.

§To whom reprint requests should be addressed. E-mail: ygotoh@iam.u-tokyo.ac.jp.

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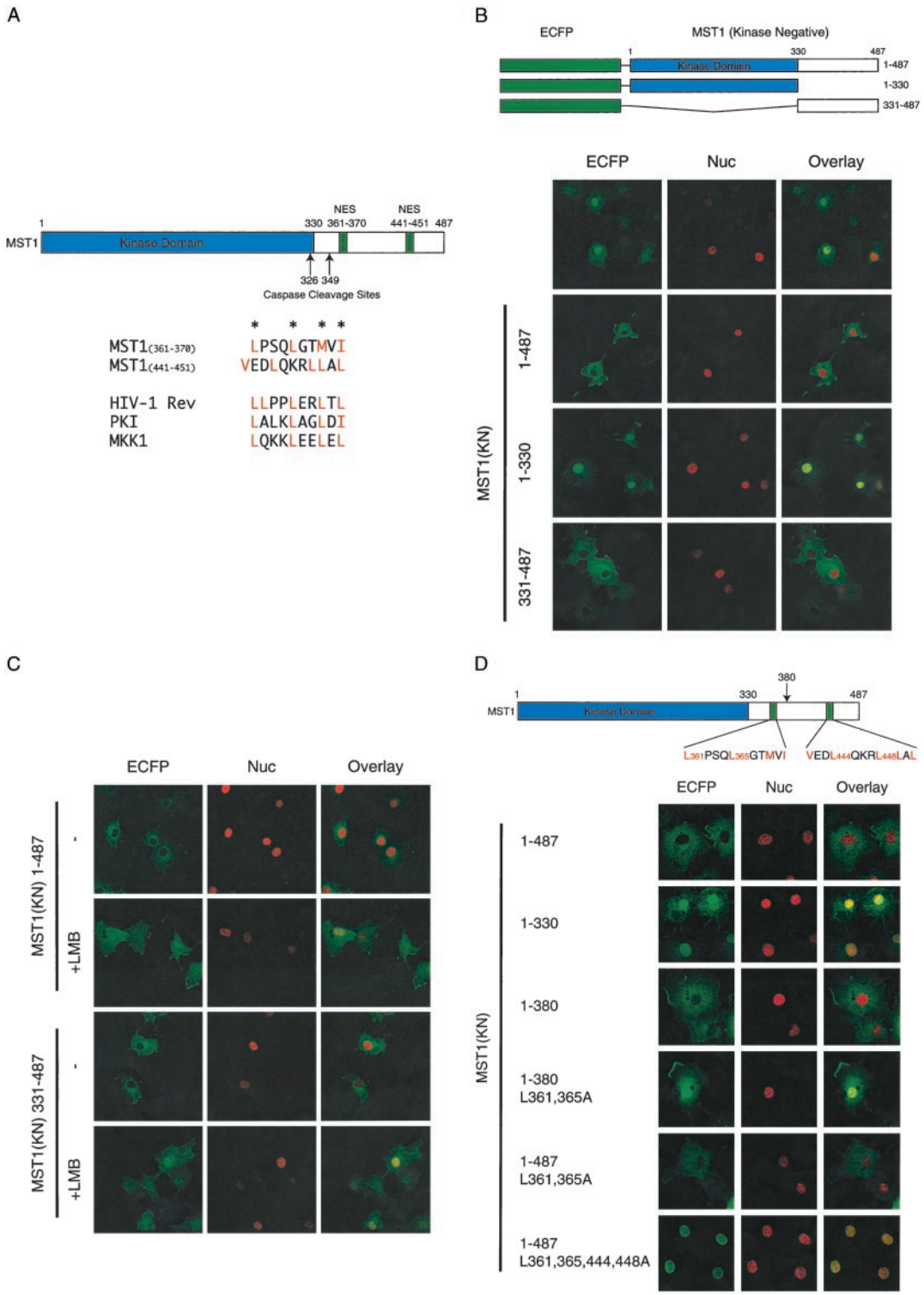

Fig. 1. MST1 has two functional NES sequences in the C-terminal domain. (*A*) The schematic structure of MST1. The location of two putative NES sequences (361–370 and 441–451 aa) are indicated as a green box. The caspase cleavage sites are indicated by arrows. Comparisons of NES sequences in various proteins are shown below. The position of conserved leucines are indicated by *, and corresponding hydrophobic residues are shown in red. (*B*) The structures of ECFP-MST1 fusion constructs are shown (*Upper*). Full-length (1–487), N-terminal domain (1–330), and C-terminal domain (331–487) of kinase-negative MST1 [MST1(KN)] were fused to ECFP as indicated. ECFP alone (−) and ECFP-MST1 fusion constructs (1–487, 1–330, 331–487) were expressed in COS-1 cells and observed by CLSM (*Lower*). MST1 localization was detected by ECFP fluorescence (ECFP), and the nuclei were visualized by histone H2B-DsRed1 fluorescence (Nuc). Overlay images of ECFP and nucleus are also shown. Note that ECFP-MST1 (1–487) and ECFP-MST1 (331–487) were excluded from the nucleus. (*C*) COS-1 cells expressing ECFP fusion constructs of full-length kinase-negative MST1 [MST1(KN) 1–487] or C-terminal domain [MST1(KN) 331–487] were treated with (+LMB) or without (−) LMB (5 nM) for 3 h. Cells were then fixed and observed by CLSM. Note that LMB treatment resulted in nuclear localization of MST1 proteins. (*D*) Full-length (1–487) of kinase-negative MST1 [MST1(KN)], C-terminal deletion constructs (1–330, 1–380), and their mutant constructs, in which indicated leucines (Leu-361 and Leu-365 for the first NES, Leu-444 and Leu-448 for the second NES; positions are shown in the upper panel) were substituted by alanines, expressed in COS-1 cells, and observed by CLSM. Elimination of both NESs resulted in nuclear localization of MST1.

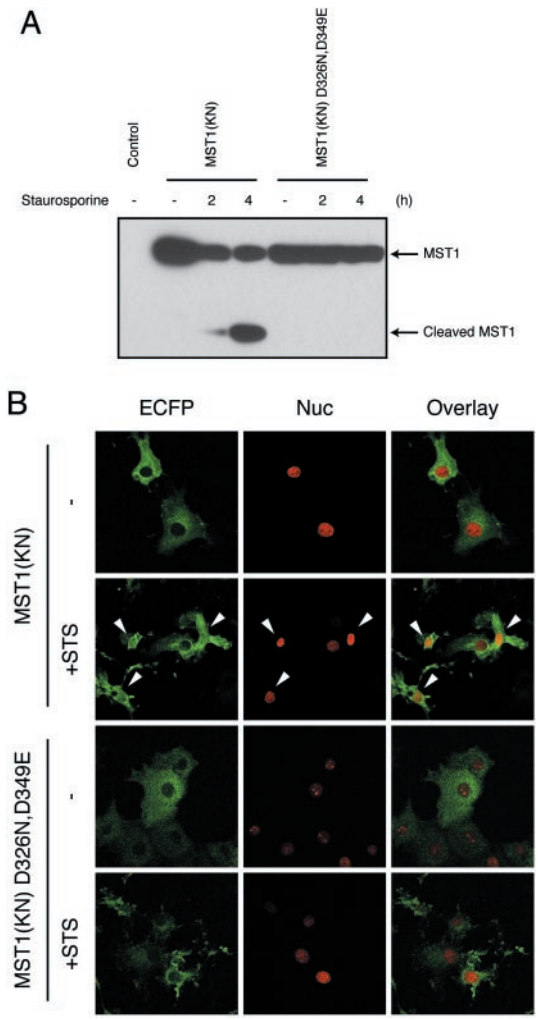

Fig. 2. Staurosporine treatment promotes cleavage and nuclear translocation of MST1. (*A*) Either Myc-tagged kinase-negative MST1 [MST1(KN)] or its cleavage-site mutant [MST1(KN) D326N, D349E] was transfected to COS-1 cells. After 24 h, cells were treated with 0.3 μM staurosporine for indicated hours and subjected to Western blot analysis. Full-length MST1 and a 36-kDa N-terminal fragment (Cleaved MST1) were detected by anti-c-Myc antibody and are indicated by arrows. (*B*) COS-1 cells expressing either ECFP-fused full-length kinase-negative MST1 [MST1(KN)] or its cleavage-site mutant [MST1(KN) D326N, D349E] were treated with (+STS) or without (−) 0.3 μM staurosporine for 3 h. Cells were then fixed and observed by CLSM. MST1 localization was detected by ECFP fluorescence (ECFP), and nuclei were detected by histone H2B-DsRed1 fluorescence (Nuc). Overlay images of ECFP and nucleus are also shown. Cells showing nuclear translocation of MST1 are indicated by arrowheads.

CATCCTC-3′ (1–380) as a reverse primer. The amplified fragment was digested with *Hin*dIII and *Eco*RI and subcloned into pcDNA3. C-terminal domain of MST1 (MST1 331–487) was amplified from pcMycMST1 by PCR using 5′-CGGGATCCGTTCGAGCAGTGGGTGATGAGATGG-3′ as a forward primer and SP6 primer as a reverse primer. The amplified fragment was digested with *Bam*HI and *Eco*RI and subcloned into pcMyc-MST1. A cleavage-site mutant of MST1 was made by mutating Asp-326 and Asp-349 into Asn and Glu, respectively (23). An NES mutant of MST1 was made by mutating leucines critical for NES (Leu-361 and Leu-365 for the first NES, Leu-444 and Leu-448 for the second NES) to alanines. To visualize localization of MST1 in Figs. 1 and 2, each fragment of kinase-negative MST1 was subcloned into *Hin*dIII–*Eco*RI sites of pECFP-C1 (CLONTECH; ECFP, enhanced cyan fluorescent protein) and expressed as an ECFP-fusion protein. pH2B-DsRed1 was constructed by subcloning histone H2B cDNA into pDsRed1-C1 (CLONTECH).

Cell Culture and Transfection. 293T or COS-1 cells were cultured in DMEM (GIBCO/BRL) with 10% FBS in 5% CO_2 humidified atmosphere at 37°C. Cells were plated 18 h before transfection at a density of 1×10^5 cells per 35-mm dish. Culture medium was exchanged to 2 ml of OptiMEM (GIBCO/BRL) just before transfection. Transfection was performed with FuGENE6.

Confocal Laser Scanning Microscopy (CLSM). For CLSM, cells were precultured on coverslips before transfection. Nuclei were visualized by transfecting cells with pH2B-DsRed1, encoding histone H2B-DsRed1 fusion protein. Twenty-four hours after transfection, cells were fixed by 4% formaldehyde. Fluorescence images were recorded by a confocal laser scanning microscope (LSM-510, Zeiss).

Western Blot Analysis. Cell lysates were prepared in a lysis buffer containing 150 mM NaCl, 20 mM Tris·-Cl, (pH 7.5), 10 mM β-glycerophosphate, 5 mM EGTA, 1 mM NaPPi, 5 mM NaF, 0.5% Triton X-100, 1 mM $NaVO_4$, 5 mM DTT, 0.5% (vol/vol) aprotinin, and 1 mM PMSF. Proteins were separated by SDS/PAGE and transferred to nitrocellulose membrane. The membrane was incubated with anti-c-Myc antibody (9E10, Santa Cruz Biotechnology, 1:200) in a blocking solution 5% skim milk in Tris-buffered saline) for 1 h, washed by Tris-buffered saline/Tween 20 (0.05%), incubated with anti-rabbit horseradish peroxidase-conjugated antibody (Amersham Pharmacia, 1:10,000), and washed four times by Tris-buffered saline/Tween 20. The membranes were then subjected to ECL Plus Western blotting detection kit (Amersham Pharmacia).

Chromatin Condensation Analysis. Transfected cells were gently suspended by pipetting in the culture medium and were attached onto poly-D-lysine-coated coverslips by centrifugation. Attached cells were fixed with 4% formaldehyde, and chromatin condensation was observed by CLSM as described above. Typical condensed and noncondensed nuclei are shown in Fig. 3*A*.

Results

MST1 Has Two NESs in Its C Terminus. MST1 contains the sequences LPSQLGTMVI (amino acids 361–370) and VEDLQKRLLAL (amino acids 441–451) that correspond to the NES consensus sequence (Fig. 1*A*) (35, 36). This prompted us to examine whether these putative NESs regulate localization of MST1. To do this, several kinase-negative MST1 mutants were expressed as fusion proteins with ECFP (Fig. 1*B*), and their subcellular localization patterns were examined by CLSM. The full-length MST1 (1–487)-ECFP fusion was excluded from the nucleus and localized exclusively in the cytoplasm (Fig. 1*B*). Likewise, an MST1 C-terminal construct (331–487) fused with ECFP, containing the putative NES sequences, was also exclusively cytoplasmic (Fig. 1*B*). In contrast, the N-terminal half of MST1 (1–330) fused with ECFP, lacking the putative NESs, could be observed in both the cytoplasm and nucleus (Fig. 1*B*). To further confirm that MST1 contains functional NESs, cells expressing ECFP-MST1 (1–487) and ECFP-MST1 (331–487) were treated with LMB, a specific inhibitor of the NES receptor Crm1 (33, 34). LMB treatment resulted in nuclear localization of ECFP-MST1 (1–487) and ECFP-MST1 (331–487) as shown in Fig. 1*C*, whereas it had no effect on the localization of ECFP-MST1 (1–330) or ECFP alone (data not shown). This strongly indicated that nuclear exclusion of MST1 was an NES-dependent event.

To rule out any effect on MST1 localization resulting from the ECFP fusion, we examined the cytoplasmic localization of

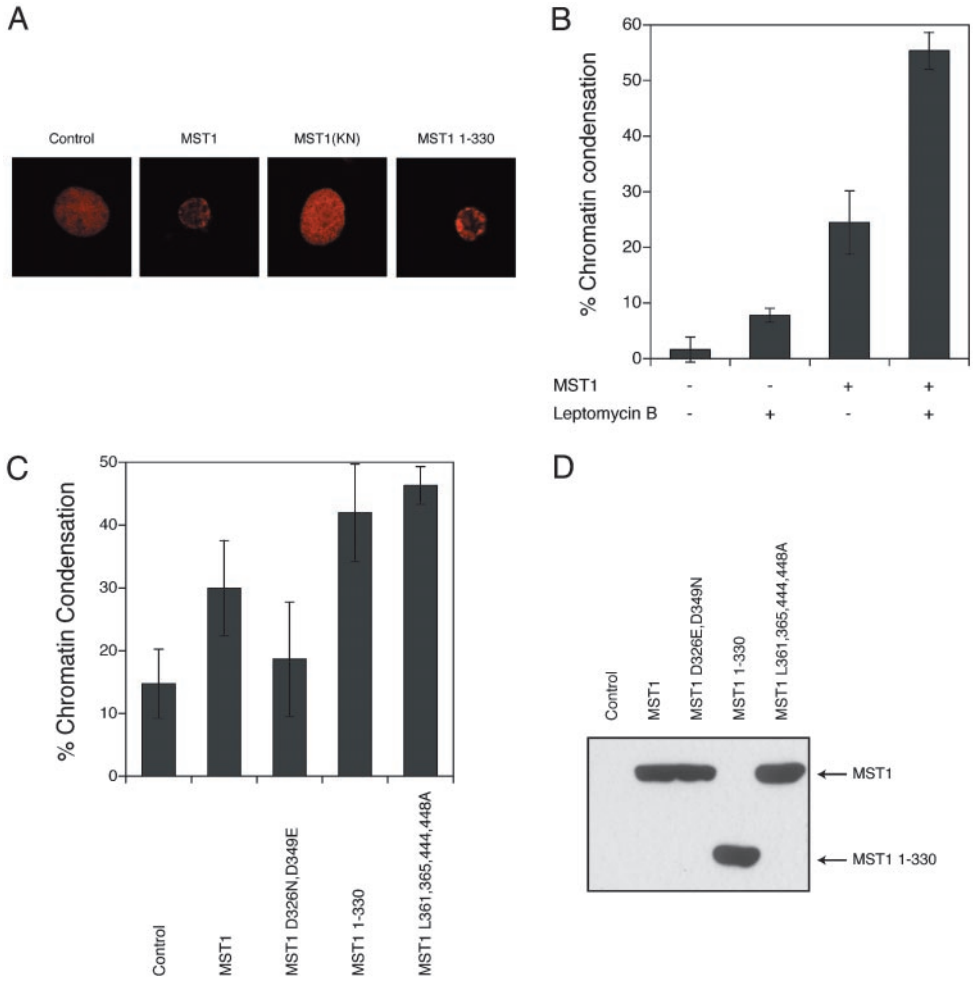

Fig. 3. Nuclear localization of MST1 induces peripheral chromatin condensation. (*A*) Typical nuclear morphologies. Control vector (Control), wild-type MST1 (MST1), kinase-negative MST1 [MST1(KN)], or a cleaved form of MST1 (MST1 1–330) was expressed in 293T cells with pH2B-DsRed1 as a nuclear marker. After 18 h, nuclei were observed by CLSM. Typical noncondensed [Control, MST1(KN)] or condensed (MST1, MST1 1–330) nuclei are shown. (*B*) COS-1 cells were transfected with a control vector or wild-type MST1 in the presence (+LMB) or absence of (−) LMB (5 nM). After 18 h, cells were collected, and the percentage of the cells showing chromatin condensation to the total transfected cells was measured as described in *Materials and Methods*. (*C* and *D*) Control vector, Myc-tagged wild-type MST1, its cleavage-site mutant (D326N, D349E), cleaved form (1–330), or NES mutant (L361,365,444,448A) was expressed in 293T cells. Cells were collected 24 h after transfection. The percentage of the cells showing chromatin condensation is shown (*C*). The expression level of each MST1 mutant was assessed by Western blotting with anti-c-Myc antibody (*D*).

Myc-tagged MST1. Myc-tagged MST1 behaved in a manner identical to ECFP MST1 (1–487) (data not shown). We also attempted to detect the cytoplasmic localization of endogenous MST1 by immunofluorescence cell staining (preliminary experiments, data not shown). However, using the reagents presently available to us, we have not been able to perform immunohistochemical analyses of endogenous MST1.

Because MST1 has two putative NES sequences, we addressed the question of what the relative contributions of these two sequences were to the nuclear exclusion of MST1. When MST1 (1–380), containing only the first putative NES (361–370), was expressed in the cell as an ECFP fusion, it was observed to be localized predominantly in the cytoplasm. In contrast, MST1 1–330, which lacks both putative NESs, was localized both in the cytoplasm and in the nucleus (Fig. 1*D*). To confirm that the first putative NES (361–370) was functional, two critical leucines (Leu-361 and Leu-365) were mutated to alanine (L361,365A) in truncated MST1 (1–380). This mutant (1–380 L361,365A) was observed to be localized in the nucleus as well as in the cytoplasm in a manner similar to MST1 (1–330) (Fig. 1*D*), suggesting that the first putative NES is indeed functional. To address the role of the second NES (441–451), we analyzed the localization of full-length MST1 in which the first NES was mutated (1–487 L361,365A). This mutant, in which the second putative NES remained intact, was still excluded from the nucleus, suggesting that the second NES is also functional. When the critical leucines of both putative NESs were mutated to alanines (1–487; L361,365,444,448A), MST1 was predominantly found localized in the nucleus (Fig. 1*D*). These results clearly demonstrated that MST1 has two functional NESs. Because MST1 accumulates in the nucleus when both NESs are mutated (1–487; L361,365,444,448A) but not when the C terminus is truncated (1–330), it is possible that there is a nuclear localization sequence or a nuclear anchor in the C terminus. Consistent with this hypothesis, MST1 contains a bipartite nuclear localization signal-like motif in the C-terminal domain (469–485; KRQPILDAIEAKKRRQQ) predicted by the PSORTII program (http://psort.ims.u-tokyo.ac.jp/) (37).

Apoptotic Stimuli Induce Nuclear Translocation of MST1. In response to apoptotic stimuli, caspase cleavage of MST1 occurs at Asp-326 and Asp-349, resulting in the separation of its N-terminal kinase

domain from the NES-containing C-terminal domain (22, 23). This fits well with our proposed model that a portion of the liberated MST1 kinase domain may translocate into the nucleus in response to apoptotic stimuli. To test this hypothesis, we treated COS-1 cells that expressed Myc-tagged MST1 with staurosporine. Because wild-type MST1 is capable of inducing significant apoptosis upon overexpression (22), we used kinase-negative MST1. After staurosporine treatment, MST1 was partially cleaved to generate a 36-kDa N-terminal fragment as previously reported, whereas its cleavage-site mutant was not (Fig. 2*A*) (22, 28). Concomitantly, we observed a fraction of MST1 within the nuclei, whereas MST1 was excluded from the nuclei of all untreated cells (Fig. 2*B*). In contrast, a cleavage-site mutant did not show any significant nuclear translocation in response to staurosporine treatment (Fig. 2*B*).

Nuclear Localization of MST1 Enhances Chromatin Condensation. We have previously shown that MST1 is capable of inducing apoptotic nuclear changes such as chromatin condensation (ref. 22; see also Fig. 3*A*). Thus, we addressed the question of whether nuclear translocation contributed to the ability of MST1 to induce these apoptotic events. In initial experiments, we used LMB, an inhibitor of the nuclear export machinery. LMB treatment significantly enhanced the degree of MST1-induced chromatin condensation (Fig. 3*B*). This suggested that nuclear localization of MST1 might augment its ability to promote chromatin condensation. However, it is also possible that other NES-containing proteins contributed to MST1-dependent chromatin condensation. To rule out this possibility, we used MST1 mutants with or without functional NESs. Upon overexpression in COS-1 cells, MST1 (1–330) caused chromatin condensation more efficiently than wild-type MST1. Moreover, MST1 NES mutant (L361,365,444,448A) induced chromatin condensation more efficiently than wild-type MST1 (Fig. 3*C*). In contrast, a cleavage-site mutant (D326N, D349E), which cannot be cleaved by caspases and therefore cannot translocate to the nucleus, was less effective in inducing chromatin condensation (Fig. 3*C*). The expression level of the MST1 mutants was comparable to each other (Fig. 3*D*). Collectively, these results indicate that nuclear translocation of MST1 significantly enhances its ability to induce chromatin condensation.

In addition, we found that a caspase inhibitor did not block the chromatin condensation induced by MST1 (1–330), suggesting the existence of a caspase-independent pathway downstream of MST1 in inducing chromatin condensation (data not shown). However, this does not rule out the possibility that MST1 induces chromatin condensation by activating caspase-dependent pathway(s) after nuclear translocation.

MST1 Is Important for Staurosporine-Induced Chromatin Condensation. Finally, we examined whether MST1 is necessary for inducing chromatin condensation in response to apoptotic stimuli. We chose staurosporine as an apoptotic inducer for this experiment because we observed nuclear translocation of MST1 upon staurosporine treatment, and because staurosporine induces peripheral chromatin condensation which resembles that induced by MST1 expression (8, 13) (Fig. 3*A*). We found that expression of a kinase-negative MST1 effectively suppressed chromatin condensation induced by staurosporine treatment (Fig. 4). For instance, after 6 h of treatment with 0.5 μM staurosporine, 56% of control cells underwent chromatin condensation, whereas only 28% of cells did so when a kinase-negative MST1 was expressed (Fig. 4). Essentially, the same results were obtained in three independent experiments. These results therefore suggest that the activity of endogenous MST1 contributes to chromatin condensation in staurosporine-treated cells.

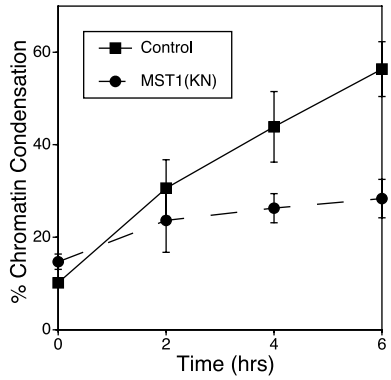

Fig. 4. Kinase-negative MST1 inhibits staurosporine-induced chromatin condensation. 293T cells were transfected with either control vector or kinase-negative MST1, with pH2B-DsRed1 as a nuclear marker. Eighteen hours after transfection, cells were stimulated with or without 0.5 μM staurosporine for indicated hours and then collected. The percentage of the cells showing chromatin condensation to the total transfected cells was measured as described in *Materials and Methods*.

Discussion

In this study, we found that MST1 contains two functional NESs. Full-length MST1 is exclusively localized in the cytoplasm, but this cytoplasmic localization was disrupted by truncation of the NES-containing domain, mutation of NESs, or inhibition of NES-mediated nuclear export by LMB treatment. MST1 is an STE20-like kinase (MAPKKKK) found to contain a functional NES. Importantly, the NESs of MST1 are located in the C terminus, which is separated from the kinase domain upon caspase-mediated cleavage. Thus, caspase cleavage of MST1 serves two purposes: one is activation of MST1 kinase activity and the other is translocation of MST1 into the nucleus (Fig. 5).

There are many ways of nuclear translocation regulated by NES. Some proteins such as Nur77 (38), cyclin B1 (39–41), and NFAT (42) translocate into the nucleus when their NESs are masked upon phosphorylation or dephosphorylation. Other proteins such as NF-κB (43), MAPK (44), and the 14-3-3 binding proteins (45, 46) translocate into the nucleus when they are released from their NES-containing partners (IκB, MEK, and 14-3-3, respectively). The mechanism by which MST1 translocates into the nucleus is a unique one, that is, translocation into the nucleus by being cleaved and separated from its own NES.

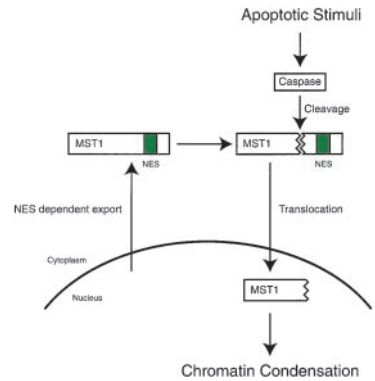

Fig. 5. A model for the role of MST1 translocation in apoptosis. In nonapoptotic cells, MST1 is excluded from the nucleus by its NESs and predominantly distributed in the cytoplasm. In response to apoptotic stimuli, activated caspases cleave MST1 and release its C-terminal regulatory domain containing NESs, which results in nuclear translocation of the N-terminal kinase domain. Nuclear translocation of cleaved MST1 enhances chromatin condensation.

In contrast to nuclear translocation regulated by phosphorylation or subunit binding, the nuclear translocation of MST1 is most likely irreversible as it accompanies cleavage of the polypeptide. It is unclear whether other mechanisms, independent of caspase-mediated cleavage, also exist to regulate the nuclear localization of MST1. It will also be important to characterize the potential nuclear localization signal in MST1 and study the relative contributions of nuclear localization and NES activities to the localization of MST1.

To induce nuclear apoptosis, some apoptotic signal mediators should translocate into the nucleus, as the integrity of nuclear membrane is apparently maintained during the early phase of apoptosis when chromatin condensation begins to take place. In fact, a number of proteins have been suggested to participate in mediating apoptotic signals into the nucleus, such as AIF (apoptosis-inducing factor) (47), caspase-9 (48), and PML (49). Here, we propose that MST1 represents one of these mediators and transmits apoptotic signals from cytoplasm into nucleus, based on several lines of evidence. First, MST1 contains functional NESs, and cleavage of MST1 by caspases releases the MST1 kinase domain from the NESs, resulting in nuclear translocation of the kinase domain. Second, apoptotic stimuli such as staurosporine result in nuclear translocation of MST1. Third, expression of MST1 is sufficient for inducing chromatin condensation. Fourth, expression of a kinase-negative MST1 inhibits staurosporine-induced chromatin condensation. Finally, nuclear localization of MST1 enhances MST1-induced chromatin condensation.

Caspases modulate the functions of their targets by cleavage in various ways. In the case of CAD, caspase-mediated cleavage of its inhibitory partner, ICAD (inhibitor of CAD), activates CAD's DNase activity (9–11, 50). When a caspase (and another unknown protease) cleaves Acinus, it becomes an active short polypeptide capable of condensing nuclei (12). Several kinases including PAK2 (14), MEKK1 (15–17), ROCK I (19, 20), HPK1 (18), SLK (21), and MST1 (22, 24, 25, 31) become active after caspase-mediated cleavage because of separation from their autoinhibitory domains. Caspases also modulate localization of their targets. For example, MEKK1 translocates from detergent-insoluble to detergent-soluble fraction by caspase-mediated cleavage (17). Here, we show that MST1 translocates into the nucleus upon caspase-mediated cleavage. This study presents an example that caspase regulates nuclear translocation of their targets.

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