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Caspase Activation of Mammalian Sterile 20-like Kinase 3 (Mst3)

NUCLEAR TRANSLOCATION AND INDUCTION OF APOPTOSIS*

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Mammalian Sterile 20-like kinase 3 (Mst3), the physiological functions of which are unknown, is a member of the germinal center kinase-III family. It contains a conserved kinase domain at its NH₂ terminus, whereas there is a regulatory domain at its COOH terminus. In this study we demonstrate that endogenous Mst3 is specifically cleaved when Jurkat cells were treated with anti-Fas antibody or staurosporine and that this cleavage is inhibited by the caspase inhibitor, Ac-DEVD-CHO. Using apoptotic Jurkat cell extracts and recombinant caspases, we mapped the caspase cleavage site, AETD³¹³, which is at the junction of the NH₂-terminal kinase domain and the COOH-terminal regulatory domain. Caspase-mediated cleavage of Mst3 activates its intrinsic kinase activity, suggesting that the COOH-terminal domain of Mst3 negatively regulates the kinase domain. Furthermore, proteolytic removal of the Mst3 COOH-terminal domain by caspases promotes nuclear translocation. Ectopic expression of either wild-type or COOH-terminal truncated Mst3 in cells results in DNA fragmentation and morphological changes characteristic of apoptosis. By contrast, no such changes were exhibited for catalytically inactive Mst3, implicating the involvement of Mst3 kinase activity for mediation of these effects. Collectively, these results support the notion that caspase-mediated proteolytic activation of Mst3 contributes to apoptosis.

Apoptosis (or programmed cell death) is a naturally occurring process that evolved early, and one that has been conserved throughout all the animal kingdoms (1, 2). The dysregulation of apoptotic cell death may be involved in the pathogenesis of a variety of human diseases, including cancers, autoimmune diseases, neurodegenerative disorders, and viral infection (3–6). Growing evidence suggests that although apoptotic stimuli vary from cell to cell, there seems to be a basic

biochemical mechanism underlying this process, which involves the activation of a family of Cys-dependent, Asp-specific proteases known as the caspases (7–9). The activated caspases can cleave downstream substrates, including components of the cellular DNA-repair mechanisms, apoptosis inhibitors, and structural proteins. Furthermore, recent evidence suggests that these caspase substrates are the true apoptotic effectors, not the caspases themselves. Therefore, it is imperative to identify the downstream targets of the caspases, and then to elucidate the effect of this caspase-mediated cleavage to delineate the biochemical mechanisms of apoptosis.

Protein kinases have emerged as direct substrates and effectors of caspases (reviews in Refs. 10 and 11). In a general survey of cleavage products in cells treated with apoptotic agents such as Fas ligand, two anti-apoptotic protein kinases, Raf-1 and AKT, were inactivated as immediate caspase substrates (12). Proteolytic cleavage of ATM generates an inactive kinase, and impairs DNA-repair capability in response to DNA damage (13). Cleavage of focal adhesion kinase by caspases interrupts the assembly of the focal adhesion complex, resulting in cell death (14, 15). In other instances, caspase cleavage engenders the production of a more active kinase by removal of the inhibitory domains. In response to apoptotic stimuli, the cleaved, activated kinases are then serving to propagate the apoptotic signals through phosphorylation of the relevant substrates. Examples include MEKK-1¹ (16), hPAK2/hPAK65 (17, 18), protein kinase C isoforms δ (19–21) and θ (22), PKN (23), Etk (24), Mst1 (25–28), Mst2 (26, 27), ROCK1 (29, 30), SLK (31), and HPK1 (32). Several of the aforementioned kinases, including Mst1, Mst2, SLK, and HPK1, belong to the Sterile 20 family of serine/threonine kinases. Together, these observations imply that protein phosphorylation and/or dephosphorylation may play an essential role in apoptotic signal transduction.

Sterile 20 was originally discovered as a component of the pheromone-response pathway in budding yeast (Ref. 33; reviewed in Ref. 34). The mammalian Sterile 20-related kinases represent a rapidly growing kinase family, with 28 members identified in humans at the time of writing. Some of these

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¹ The abbreviations used are: MEKK, mitogen-activated protein kinase kinase; MEK/ERK, mitogen-activated protein kinase/extracellular signal-regulated kinase; GCK, germinal center kinase; GFP, green fluorescent protein; HA, hemagglutinin; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonic acid; PIPES, 1,4-piperazinediethanesulfonic acid; NLS, nuclear localization signal; Mst, mammalian Sterile 20-like kinase; HEK, human embryonic kidney.

kinases activate mitogen-activated protein kinase cascades, and serve as cellular sensors that respond to numerous stimuli (reviewed in Refs. 34 and 35). Furthermore, they are traditionally divided into the p21-activated kinase and germinal center kinase (GCK) families, which are distinguished by the relative location of their kinase domains. The p21-activated kinase domains were located at their COOH termini, whereas the GCK homologs are at their NH₂ termini. Four Mst family kinases are already known and can be divided into two subgroups, GCK-II (Mst1 and Mst2) and GCK-III (Mst3 and Mst4), based on their sequence homology within and outside their kinase domains, and additional information obtained from the *Drosophila* and/or *Caenorhabditis elegans* orthologs (34).

While sharing considerable homology in the kinase domain, the Mst kinases have different biological activities. For instance, Fas-mediated cleavage of Mst1/Mst2 is required for full activation of these kinases during apoptosis (26, 27). Overexpression of either full-length wild-type Mst1 or its truncated form in BJAB cells induces morphological changes characteristic of apoptosis (25), suggesting that Mst1 is involved in this process. On the other hand, it has been demonstrated that Mst4, a newly identified Sterile 20-related kinase that shares 88% sequence similarity to Mst3 and SOK1 (36), mediates the growth and transformation of the tumor cell lines, HeLa and Phoenix, via the MEK/ERK pathway (37); whereas Mst1-mediated apoptosis occurs via the c-Jun NH₂-terminal kinase pathway (38). These findings indicate that the Mst family members may recognize distinct downstream targets, and hence, act through distinct signaling pathways. Analysis of the data raises interesting questions with respect to identification of the structural features that make Mst4 act differently to Mst1, and whether Mst3 might have a role in apoptosis (like Mst1), or cell transformation (like Mst4).

In this study, we report that similar to Mst1 and Mst2 but not Mst4, Mst3 can be cleaved by caspases during apoptosis. Mst4 is resistant to cleavage, which may be the reason for cell transformation. The cleavage of Mst3 generates two fragments, 35 and 15 kDa, respectively. Unlike the conserved caspase-cleavage sites in Mst1 and Mst2, the caspase-cleavage site in Mst3 is mapped to AETD^{313G}. Finally, ectopic expression of the truncated Mst3 results in kinase activation, nuclear translocation, and the induction of apoptosis. Taken together, these observations implicate the involvement of Mst3 in the process of apoptosis.

EXPERIMENTAL PROCEDURES

Materials—All restriction enzymes were purchased from New England BioLabs. Fetal bovine serum, Dulbecco's modified Eagle's medium, penicillin, streptomycin, and LipofectAMINETM were purchased from Invitrogen. The $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was also from New England BioLabs. The Ac-DEVD-CHO was purchased from Calbiochem. Monoclonal antibody against GFP (CLONTECH) was used to detect the presence of the enhanced green fluorescent protein (EGFP)-tagged proteins. The bacterially expressed carboxyl terminus of Mst3 (amino acids 310–432) was prepared from pET21-Mst3. Recombinant Mst3 protein was purified, and rabbit antiserum against the Mst3 carboxyl terminus was generated.

Cell Lines and Cell Culture—All cell lines were purchased from ATCC and maintained in a humidified incubator at 37 °C in the presence of 5% CO₂. HeLa, COS-1, and 293T cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin. Human embryonic kidney 293 (HEK293) cells were maintained in Dulbecco's modified Eagle's medium containing 10% horse serum, 100 units/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin. Transfection of cells was performed with LipofectAMINETM according to the manufacturer's instructions.

Construction of Plasmids and Site-directed Mutagenesis—The cDNAs, encoding full-length (amino acids 1–431) and truncated (amino acids 1–313) forms of Mst3, were amplified by reverse transcriptase-PCR using HeLa cell messenger RNA as the template. These constructs

are referred to as Mst3^{WT} and Mst3^{WTΔ314}, respectively. The Mst3 cDNA fragments were inserted in-frame into the HA-tagged (at the COOH terminus) expression vector, pCDNA 3.0 (Invitrogen), and the EGFP-tagged expression vector, pEGFP-C2 (CLONTECH). The catalytically inactive mutants, Mst3^{KR} and Mst3^{KRA314} (with Lys⁵³ to Arg⁵³ mutation on the full-length and truncated Mst3), as well as caspase-cleavage site mutants (Mst3^{D301N}, Mst3^{D302N}, Mst3^{D307N}, Mst3^{D309N}, Mst3^{D313N}, Mst3^{D321N}, Mst3^{D324N}, and Mst3^{D333N}) of Mst3 were generated by site-directed mutagenesis using Mst3^{WT} and Mst3^{WTΔ314} as the templates (QuikChangeTM site-directed mutagenesis kit, Stratagene).

Preparation of Cell-free Apoptotic Extracts—To induce apoptosis, Jurkat cells were treated with 250 ng/ml anti-Fas monoclonal antibody (clone CH11, Upstate Biotechnology Inc.) (39) or 1 μM staurosporine (Calbiochem) (40) at 37 °C for 5 h. To prepare the cell-free extracts, cells were lysed in lysis buffer (20 mM PIPES, pH 7.2, 100 mM NaCl, 1 mM EDTA, 0.1% CHAPS, 10% sucrose, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 1 mM Na₃VO₄, and 10 $\mu\text{g}/\text{ml}$ each of leupeptin, aprotinin, chymostatin, and pepstatin) (24). After incubation at 4 °C for 30 min, cellular debris was removed by centrifugation.

In Vitro Cleavage with Apoptotic Cell Extracts or Recombinant Caspases—The TNT quick coupled transcription/translation system (Promega) was used to synthesize [³⁵S]methionine-labeled proteins. The ³⁵S-labeled Mst3 or Mst4 proteins were incubated with various apoptotic extracts (150- μg extracts per reaction) or recombinant caspases (BD PharMingen) at 37 °C for 1 h. For caspase-inhibitor treatment, apoptotic extracts were preincubated with Ac-DEVD-CHO (Calbiochem) at 37 °C for 15 min before ³⁵S-labeled Mst3 proteins were added.

Immunoprecipitation, Kinase Reaction, and Western Blot Analysis—COS-1 cells were transiently transfected with plasmids encoding various HA-tagged Mst3 proteins. Forty-eight hours after transfection, cells were lysed with modified RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 6 mM deoxycholate, 1% Igepal CA-630 (Sigma), 1 mM Na₃VO₄, 2 mM EGTA, 0.5% aprotinin, and 1 mM phenylmethylsulfonyl fluoride). Equal amounts of total lysates were immunoprecipitated with anti-HA tag monoclonal antibody (3F10; Roche Molecular Biochemicals) and Protein A-agarose beads (Upstate Biotechnology Inc.) following standard procedures. Immune complexes were resuspended in kinase reaction buffer (20 mM PIPES, pH 7.4, 2 mM MnCl₂, 10 mM MgCl₂, 20 μM ATP, 10 μCi of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, and 20 μg of histone H1) at 30 °C for 10 min. The kinase reaction mixtures or cell lysates were resolved using 12% SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membranes and detected with autoradiography or anti-HA antibody. The complexed IgGs were detected by incubation with secondary antibodies conjugated to horseradish peroxidase, and developed using the ECL system (Amersham Biosciences).

Fluorescence Microscopy—The expression of EGFP-Mst3 in living cells was monitored using an inverted fluorescence microscope (Leica). The HEK293, 293T, and HeLa cells were cultured on chamber slides (LAB-TEK, Nalgel Nunc) to about 70% confluency and transfected with pEGFP-Mst3 or its mutants. Twelve hours after transfection, the chamber slides were fixed with 5% paraformaldehyde containing 2% sucrose in phosphate-buffered saline at 4 °C for 20 min, followed by staining with 200 ng/ml 4',6'-diamidino-2-phenylindole at 4 °C for 30 min. The fluorescence was analyzed using 4',6'-diamidino-2-phenylindole (excitation/emission for 4',6'-diamidino-2-phenylindole, 372/456 nm) and fluorescein isothiocyanate fluorescence filter cubes (488/510 nm). Photomages were acquired using a Kodak digital camera, and then processed using Free PLUS software (Media Cybernetics, Silver Spring, MD).

Apoptosis Assay—The β -galactosidase co-transfection assay for determination of cell death was performed as described earlier (41, 42). Briefly, HEK293 cells in 35-mm culture dishes (about 70% confluency) were transiently transfected with a pCMV/LacZ plasmid (0.5 μg) together with 1 μg of pEGFP-Mst3, pEGFP-Mst3^{WTΔ314}, pEGFP-Mst3^{KRA314}, or pEGFP-C2 (vector) constructs. Thirty-six hours after transfection, cell lysates were prepared by incubating with 200 μl of lysis buffer at room temperature for 15 min, as has been described. To detect the β -galactosidase activity, 50 μl of cell lysates were mixed with 200 μl of PM2 buffer (0.1 M Na₃PO₄, 1 mM MgSO₄, and 0.2 mM MnSO₄) and 200 μl of *o*-nitrophenyl- β -D-galactopyranoside (4 mg/ml) for 3 h, or until a yellow color developed, at 37 °C. The reaction was stopped by adding 0.5 ml of 1 M Tris base (pH 7.0) and the β -galactosidase activity was determined by reading the optical density at 420 nm.

DNA Fragmentation Analysis—DNA fragmentation analysis of the HEK293 cells was performed as described previously (43). Total DNA of the HEK293 (3 \times 10⁶) cells, with or without transfection of the various pEGFP-Mst3 plasmids, was isolated using a Puregene DNA Isolation

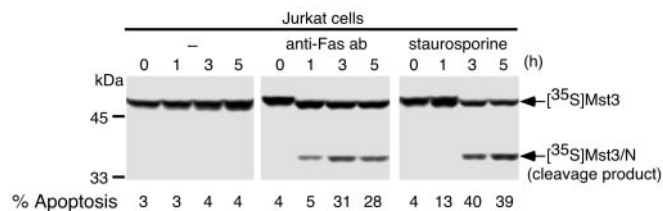


FIG. 1. Mst3 degradation in apoptotic extracts. To induce apoptosis, Jurkat T cells were either left untreated, or were treated with 250 ng/ml anti-Fas monoclonal antibody (CH11) or 1 μ M staurosporine at various time points. The apoptosis percentage for these samples is shown at the bottom of the figure. After treatment, the apoptotic extracts were incubated with [³⁵S]methionine-labeled Mst3, which was obtained by coupled *in vitro* transcription/translation. The cleavage of [³⁵S]methionine-labeled Mst3 was carried out for 1 h at 37 °C. Cleavage products were analyzed using SDS-PAGE and visualized by autoradiography. Arrows indicate full-length Mst3 (50 kDa) and one of the cleaved products, Mst3/N (35-kDa band), respectively. N, NH₂ terminus.

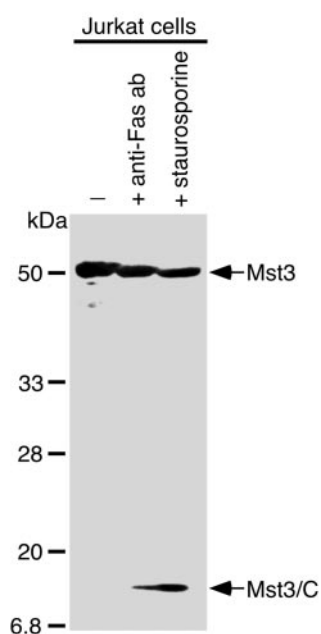


FIG. 2. Proteolytic cleavage of endogenous Mst3 during apoptosis in Jurkat cells. To induce apoptosis, Jurkat cells were either left untreated, or treated with 1 μ M staurosporine or 250 ng/ml anti-Fas monoclonal antibody (CH11) for 5 h. The cell lysates were then separated using SDS-PAGE, transferred to polyvinylidene difluoride membrane, and probed with an antibody generated against the COOH terminus of Mst3. Arrows indicate endogenous full-length Mst3 (50 kDa) and one of the cleavage products, Mst3/C (15 kDa band), respectively. C, COOH terminus.

Kit (Gentra Systems), as described in the manufacturer's instructions. The DNA pellet was redissolved in 50 μ l of DNA-hydration solution. Equal amounts of each isolated DNA sample were separated on a 2% agarose gel and visualized under UV light.

RESULTS

Mst3 Is Proteolytically Cleaved *In Vitro* and *In Vivo* during Apoptosis—To examine whether Mst3 might serve as a caspase substrate during apoptosis, cell-free apoptotic extracts were first prepared from Jurkat T cells as a source of caspases. It has been demonstrated that Jurkat T cells are hypersensitive to a wide variety of apoptotic inducers, such as anti-Fas antibody (11, 39) and staurosporine (40). Treatment of Jurkat T cells with either anti-Fas antibody (CH11) or staurosporine for 5 h resulted in ~30–40% cell death (Fig. 1), with a concurrent elevation of the activities of caspase 3, 6, 8, and 9 (3–5-fold; data not shown). The *in vitro* translated [³⁵S]methionine-labeled Mst3 was proteolytically degraded in both apoptotic ex-

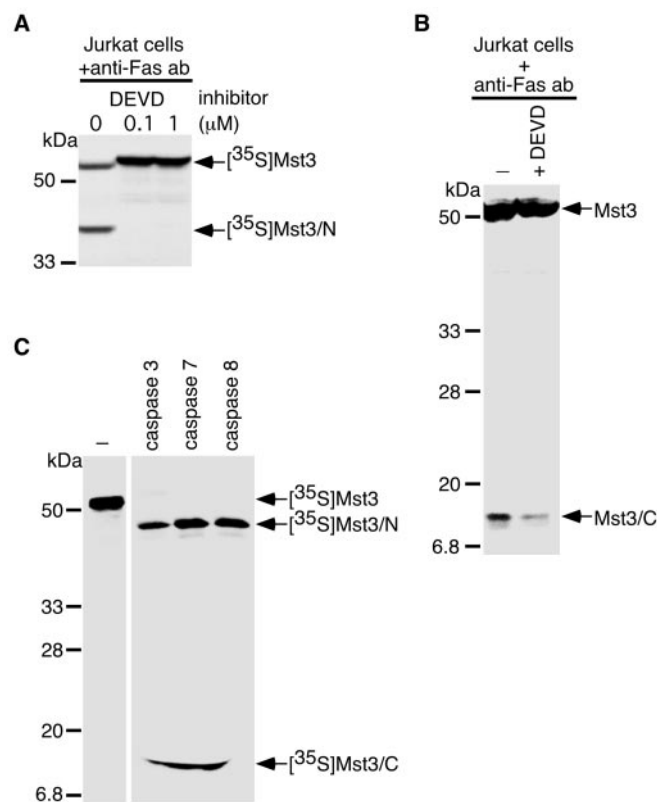


FIG. 3. Sensitivity of Mst3 to caspase cleavage. A, anti-Fas antibody-treated Jurkat cell extracts (250 ng/ml for 5 h) were preincubated for 15 min at 37 °C with or without 0.1 or 1 μ M of the caspase tetrapeptide inhibitor, Ac-DEVD-CHO. Cell extracts were then incubated with [³⁵S]methionine-labeled Mst3 for 1 h at 37 °C. Cleavage products were separated using SDS-PAGE and visualized by autoradiography. Arrows denote full-length Mst3 and a cleavage fragment, Mst3/N. B, Jurkat cells were treated with 250 ng/ml anti-Fas monoclonal antibody for 5 h in the absence or presence of 1 μ M of the caspase inhibitor, Ac-DEVD-CHO. Cell lysates were then separated using SDS-PAGE, transferred to polyvinylidene difluoride membrane, and probed with an antibody against the COOH terminus of Mst3. Arrows indicate the endogenous full-length Mst3 and one of the cleavage products, Mst3/C. C, [³⁵S]methionine-labeled Mst3 was incubated with 1 ng of recombinant caspase 3, 7, or 8 for 30 min at 37 °C. Cleavage products were separated using SDS-PAGE and visualized by autoradiography. Caspase treatments generated two cleavage products (Mst3/N and Mst3/C) as indicated by arrows. N, NH₂ terminus; C, COOH terminus.

tracts, but remained intact when untreated extracts were used (Fig. 1). In addition, Mst3 cleavage followed the same time course as that for the activation of the caspases (data not shown), suggesting that caspase activity in these apoptotic extracts was likely responsible for the Mst3 cleavage.

To test whether the cleavage of endogenous Mst3 also occurs in apoptotic cells, Jurkat T cells were treated with either anti-Fas antibody or staurosporine to induce apoptosis. Cell lysates were prepared, and immunoblot analysis was performed using an antibody against the Mst3 COOH terminus. Compared with the untreated Jurkat cell lysates, the data revealed a decrease in the amount of full-length Mst3 and the appearance of Mst3/C in the anti-Fas antibody and staurosporine-treated cells (Fig. 2). We were unable to detect the Mst3 NH₂ terminus cleavage product because of the poor specificity of antibody against the Mst3 NH₂ terminus. Similar cleavage patterns were also observed when HeLa cells were treated with staurosporine or tumor necrosis factor- α plus cycloheximide (data not shown).

Mst3 Is a Substrate for Caspases—Several lines of evidence suggest that caspases are capable of Mst3 cleavage. First, the cleavage of [³⁵S]methionine-labeled Mst3 in anti-Fas antibody (Fig. 3A) or staurosporine-induced apoptotic extracts (data not

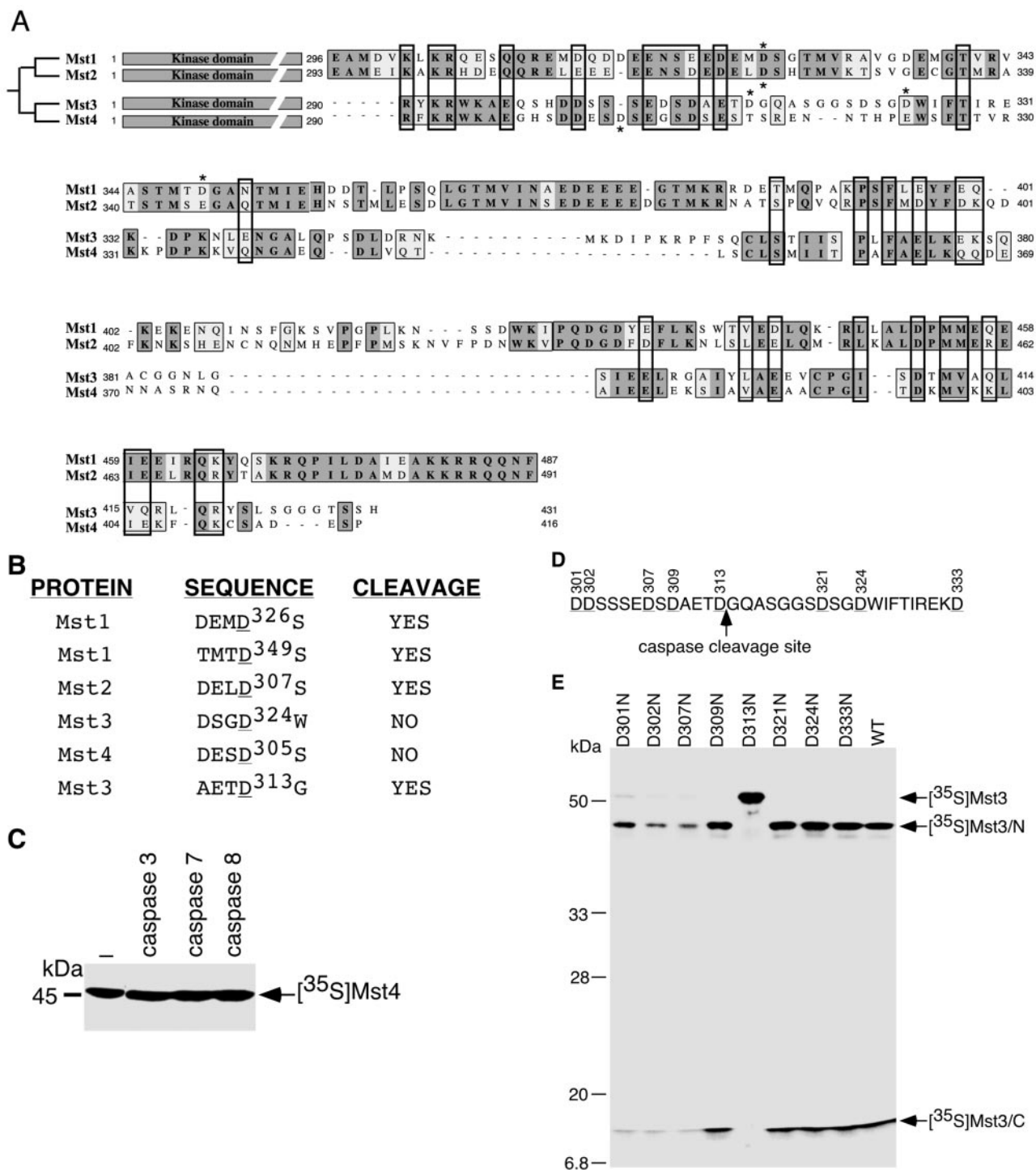


FIG. 4. Identification of the Mst3 caspase cleavage site. *A*, sequence alignment of the regulatory domains of Mst1, Mst2, Mst3, and Mst4. *B*, a list of the amino acid sequences surrounding the experimentally proven caspase-cleavage sites of Mst1 and Mst2 and the predicted caspase-cleavage sites of Mst3 and Mst4, which resemble the consensus sequence for the caspase 3 cleavage motif (DXXD), is shown. The Mst3 caspase cleavage site identified in this study is provided for comparison. The caspase cleavage sites are also shown in *A* by stars. *C*, [³⁵S]methionine-labeled Mst4 was incubated with 20 ng of recombinant caspase 3, 7, or 8 for 30 min at 37 °C. Cleavage products were resolved on SDS-PAGE and visualized by autoradiography. No cleavage product was visible, as indicated by the similar intensity of [³⁵S]methionine-labeled full-length Mst4. *D*, Mst3 amino acid residues 310–333 are shown. Single mutations of Asp (D) to Asn (N) at amino acids 301, 302, 307, 309, 313, 321, 324, and 333 were introduced into Mst3 as indicated by the *underlines*. The predicted caspase 3 recognition motif (DXXD³²⁴) is indicated in *bold*. The caspase-cleavage site (Asp³¹³) identified in this study is indicated by an *arrow*. *E*, wild-type (WT) or mutant forms of [³⁵S]methionine-labeled Mst3 were incubated with 1 ng of recombinant caspase 3 for 30 min at 37 °C. Degradation products at 35 and 15 kDa were observed in all cases except in the D313N mutant.

shown) was inhibited by Ac-DEVD-CHO, an inhibitor for caspase 3-like caspases. Second, proteolytic cleavage of the endogenous Mst3 was also partially blocked in the presence of Ac-DEVD-CHO when Jurkat T cells were treated with anti-Fas

antibody (Fig. 3B) or staurosporine (not shown). Finally, [³⁵S]methionine-labeled Mst3 was cleaved by purified recombinant caspases 3, 7, and 8 (Fig. 3C), generating two fragments (Mst3/N and Mst3/C) that mirror those observed for the apo-

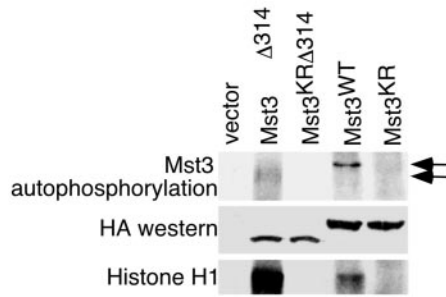


FIG. 5. Activation of Mst3 kinase activity via caspase-mediated cleavage. COS-1 cells were transiently transfected with either empty vector or various HA-tagged Mst3 constructs. These HA-tagged Mst3 proteins were immunoprecipitated from cell lysates using anti-HA antibody. Immunoprecipitated Mst3 was incubated with myosin basic protein in the presence of [γ - 32 P]ATP. Mst3 autophosphorylation (*top*) and Histone H1 phosphorylation (*bottom*) were analyzed using SDS-PAGE and autoradiography. To ensure that all of the immunoprecipitated samples were at approximately equivalent levels, the relative amounts of immunoprecipitated HA-tagged Mst3 used in the kinase reactions were monitored by immunoblotting with anti-HA antibody (*middle*).

ptotic extracts. These results suggest that proteolytic cleavage of Mst3 is a general feature of apoptotic cells.

It has been shown that the phosphorylation of presenilin-2 (45) and Mst1 (46) regulates their cleavage by caspases. To verify if the kinase activity of Mst3 or its autophosphorylation were required for caspase-mediated cleavage, the invariant lysine (Lys⁵³) in the ATP-binding pocket of Mst3 was mutated to arginine. The resultant mutant, Mst3^{KR}, has neither kinase activity nor autophosphorylation ability, as determined by an *in vitro* kinase activity assay (will be discussed later). This mutant was transcribed/translated *in vitro* and treated with either apoptotic extracts or recombinant caspases. The kinetics of Mst3^{KR} degradation were identical to that of the Mst3^{WT} (data not shown), indicating that the kinase activity and/or autophosphorylation of Mst3 were not required for caspase-mediated cleavage.

Mapping the Caspase-cleavage Site in Mst3—Previous studies have demonstrated that caspase 3 cleaves Mst1 (25, 27, 28, 44) and Mst2 (26, 27) at the consensus caspase-cleavage sites, DEMD³²⁶ and DELD³⁰⁷, respectively (Fig. 4, A and B). We have noticed that Mst3 also contains a potential caspase 3-recognition motif, DSGD³²⁴/W, in a similar region (Fig. 4, A and B; the slash indicates the potential cleavage site in the amino acid sequence), suggesting that Mst3 may serve as a substrate for caspase 3 (or a caspase 3-like activity) during apoptosis. All the DXXD motifs of the Mst family members are located within the regulatory domain (Fig. 4A). Based on the size of the Mst3-cleavage products, it seems probable that D³²⁴/W is the candidate cleavage site (Fig. 4, A and B). The Asp³²⁴ site was therefore mutated to asparagine, and its susceptibility to cleavage by recombinant caspases was tested. Both the [³⁵S]methionine-labeled Mst3^{WT} and Mst3^{D324N} mutants were cleaved by recombinant caspases 3, 7, and 8, indicating that D³²⁴/W was not an acceptor site for these caspases (Fig. 4E). Furthermore, [³⁵S]methionine-labeled Mst4 was not cleaved by any of the recombinant caspases, including caspase 3, 7, and 8 (Fig. 4C). These results suggest that the DXXD motif was not always the preferred cleavage site for caspases (especially the caspase 3) on their substrates, and that the cleavage properties of Mst3 and Mst4 (the GCK-III subfamily) are intrinsically different from that of Mst1 and Mst2 (the GCK-II subfamily). It was noted that there are several aspartic acids, such as Asp³⁰¹, Asp³⁰², Asp³⁰⁷, Asp³⁰⁹, Asp³¹³, Asp³²¹, and Asp³³³ (Fig. 4D), in the vicinity of DSGD³²⁴. To identify the cleavage site for Mst3, these aspartic acids were individually mutated to asparagines,

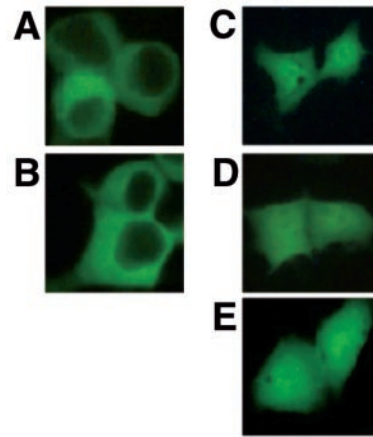


FIG. 6. Relocation of truncated Mst3 to the nucleus. HEK293 cells were transiently transfected with pEGFP-Mst3^{WT} (A), pEGFP-Mst3^{KR} (B), pEGFP-Mst3^{WTΔ314} (C), pEGFP-Mst3^{KRΔ314} (D), and pEGFP vector (E). Twelve hours after transfection, cells were fixed and the location of the proteins was identified under a fluorescence microscope (Leica).

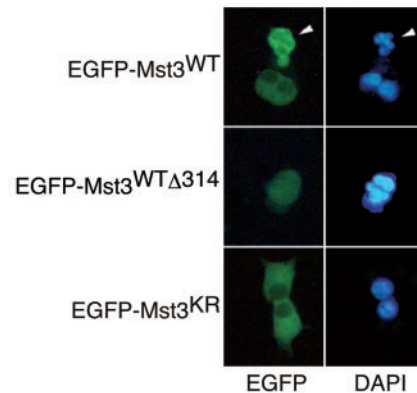
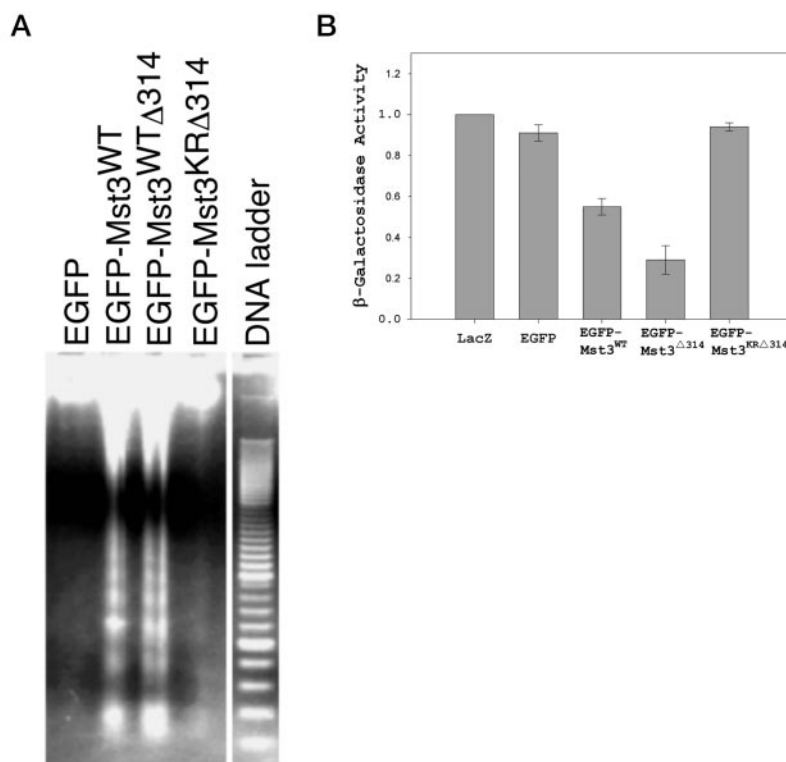


FIG. 7. Overexpression of EGFP-Mst3^{WT} and EGFP-Mst3^{WTΔ314} induces nuclear condensation of cells. HEK293 cells were transiently transfected with pEGFP-Mst3^{WT} (*top*), pEGFP-Mst3^{WTΔ314} (*middle*), or pEGFP-Mst3^{KR} (*bottom*). Eighteen hours after transfection, cells were fixed and stained with 4',6'-diamidino-2-phenylindole (DAPI). Fluorescent images were observed under a fluorescence microscope (Leica). The *arrowhead* in the *top* panel indicates the apoptotic cells.

and their potential for caspase cleavage was tested by recombinant caspases 3, 7, and 8. Of all the mutants investigated, the Asp³¹³ mutation blocked cleavage by recombinant caspases 3 (Fig. 4E), 7 (data not shown), and 8 (data not shown). Similar to Mst3^{WT}, the rest of the mutants were cleaved by these recombinant caspases (Fig. 4E and data not shown). Similar experiments were carried out using cell-free apoptotic extracts and confirmed our findings (data not shown). Therefore, it is demonstrated that the caspase-cleavage site of Mst3 is at AETD³¹³/G. Interestingly, there is no homologous site in Mst4, which may explain why Mst4 is not cleaved by caspases (Fig. 4C).

Activation of Mst3 after Caspase-mediated Proteolysis—Caspase-mediated cleavage of Mst3 generates two fragments (Fig. 3C). To test whether Mst3 caspase cleavage changes its kinase activity, COS-1 cells were transiently transfected with HA-tagged Mst3 constructs encoding Mst3^{WTΔ314}, Mst3^{KRΔ314}, Mst3^{WT}, or Mst3^{KR}. Each sample was immunoprecipitated with anti-HA antibody and the immunocomplexes were subjected to a kinase-activity assay using histone H1 as the substrate. The kinase activity of Mst3^{WTΔ314} was about 10-fold

FIG. 8. Induction of apoptosis by ectopic expression of EGFP-Mst3. A, DNA fragmentation was induced in HEK293 cells by EGFP-Mst3^{WT} and EGFP-Mst3^{WTΔ314}. The HEK293 cells were transfected with pEGFP vector (lane 1), pEGFP-Mst3^{WT} (lane 2), pEGFP-Mst3^{WTΔ314} (lane 3), or pEGFP-Mst3^{KRΔ314} (lane 4). Lane 5 represents a 100-bp DNA ladder marker. B, exogenous β -galactosidase activity assay. Various plasmids were co-transfected with pCMV-LacZ into HEK293 cells as described under "Experimental Procedures." Thirty-six hours after transfection, cells were harvested and assayed for β -galactosidase activity. Results are presented as mean \pm S.E. from three independent experiments.



higher than that of Mst3^{WT} and no detectable kinase activity was observed for the catalytically inactive mutants of Mst3 (Fig. 5). The most plausible explanation for these results is that caspase cleavage removes an inhibitory COOH-terminal regulatory domain from Mst3, thereby generating an active kinase. This is consistent with earlier data showing that deletion of the regulatory domain leads to a constitutively active Mst1 (25, 47).

Truncated Mst3 Translocates from the Cytosol to the Nucleus—To investigate the cellular localization of full-length and proteolytically cleaved Mst3 in cells, a series of constructs was generated encoding Mst3 fused to the EGFP, which is commonly used as a marker to monitor gene expression, vesicular trafficking, and protein localization *in vivo*. Four EGFP-Mst3 constructs, including EGFP-Mst3^{WT}, EGFP-Mst3^{KR}, EGFP-Mst3^{WTΔ314}, and EGFP-Mst3^{KRΔ314}, were transiently transfected into the human embryonic kidney 293 (HEK293) or HeLa cells. Fig. 6, A and B, reveal that both EGFP-Mst3^{WT} and EGFP-Mst3^{KR} predominated in the cytoplasm, which is consistent with the finding of Schinkmann and Blenis (48). Interestingly, the truncated forms of Mst3, EGFP-Mst3^{WTΔ314} and EGFP-Mst3^{KRΔ314}, were translocated to the nucleus (Fig. 6, C and D), suggesting that proteolytic cleavage results in the nuclear translocation of Mst3.

Eighteen hours following transfection of the various Mst3 constructs, a profoundly shrunken morphology was noted for cells expressing either EGFP-Mst3^{WT} or EGFP-Mst3^{WTΔ314}, with nuclear condensation visualized by green fluorescence and 4',6'-diamidino-2-phenylindole staining (*top* and *middle panels*, Fig. 7). By contrast, no morphological abnormalities were observed for cells expressing EGFP-Mst3^{KR} (*bottom panel*, Fig. 7). Together, these results suggest that, as well as for kinase activity, the Mst3 translocation may be required for induction of the morphological changes characteristic of apoptosis.

Overexpression of Mst3 Results in Characteristics of Apoptosis—To further examine the role of Mst3 in apoptosis, DNA fragmentation, one of the hallmarks of cell death, was ana-

lyzed in cells expressing Mst3. Overexpression of EGFP-Mst3^{WT} and EGFP-Mst3^{WTΔ314}, but not of EGFP and EGFP-Mst3^{KRΔ314}, resulted in DNA fragmentation in the HEK293 cells (Fig. 8A). Additionally, because the intracellular content of β -galactosidase reflects the intactness of the cells, the extent of cell death was determined by measuring the activity of ectopically expressed β -galactosidase inside the tested cells (41, 42). HEK293 cells were co-transfected with a pCMV/LacZ reporter plasmid and the various EGFP-Mst3 constructs. Cells transfected with pCMV/LacZ reporter alone were used as the control, with the β -galactosidase activity from these cells defined as 1. Compared with the control, cells expressing EGFP-Mst3^{WT} and EGFP-Mst3^{WTΔ314} exhibited significantly lower β -galactosidase activities (~55 and 28% of control, respectively; Fig. 8B). By contrast, similar β -galactosidase activity was detected for cells expressing EGFP or EGFP-Mst3^{KRΔ314} when compared with the control (Fig. 8B). These results suggest that Mst3 kinase activity is required for the induction of apoptosis. It is not clear, however, whether the relatively stronger apoptotic tendency of cells in the presence of EGFP-Mst3^{WTΔ314} is because of its nuclear localization, elevated kinase activity, or a combination of both of these factors. Altogether, these findings suggest that the truncated form of Mst3 is proapoptotic.

DISCUSSION

Four Mst family kinases have been reported so far (sequence alignments of the regulatory domain of these members are shown in Fig. 4A). The physiological functions and the downstream substrates of these four Mst family kinases are largely unknown. Although Mst1 and Mst3 are generally thought as the ubiquitously expressed protein kinases, they exhibit distinct cofactor requirements and biological properties (25, 48). Previous studies have shown that both Mst1 and Mst2 are the substrates for the caspases during apoptosis. In this study, we provide evidence that Mst3 is cleaved by caspases during apoptosis induced by either Fas ligation or staurosporine treat-

ment. Furthermore, Mst3 is activated by caspase cleavage, implicating proteolysis as the Mst3-activation mechanism. This is the first report of Mst3 regulation by a physiological stimulus. Interestingly, Mst4 is resistant to caspase cleavage under the conditions tested, suggesting the selective involvement of Mst family members in apoptosis, despite the overall sequence similarity. The molecular mechanism and the structural-functional relationship underlying this process, however, remain to be delineated.

Central to the apoptosis-execution pathway is the activation of caspases. The identification of caspase substrates and elucidation of the biochemical functions of these substrates provide the means to further understanding of the often complex and diverse apoptotic pathways. Both Mst3 and Mst1 (46) are direct substrates for several caspases, suggesting that the functional redundancy of the caspases, one or more of which may be involved in proteolysis of the same targets (49). Mutational analysis unambiguously identifies the cleavage site for Mst3 as AETD³¹³/G. This sequence does not match the caspase 3-cleavage sequence, DXXD, but bears some similarity to the caspase 8 consensus recognition motif, IETD/X (50). This observation is also consistent with our finding that recombinant caspase 8 can efficiently degrade Mst3 (Fig. 3C). Interestingly, of the Mst kinases, the AETD sequence is present only in Mst3, despite the overall structural homology of these family kinases (Fig. 4A). Conversely, there are caspase 3 consensus cleavage motifs (DXXD) present in all Mst family kinases. Under the conditions used in this study, however, Mst3 and Mst4 do not undergo proteolytic cleavage at this consensus motif, suggesting that distinct caspases may exhibit specificity among the same Mst family members in cells.

It has been reported that the COOH-terminal domains of Mst1 (47) and Mst4 (34) are needed for dimerization and regulation of the activity of their NH₂-terminal kinase domains. The removal of the COOH-terminal domain through caspase cleavage results in kinase activation of Mst1, Mst2, and Mst3. Collectively, these findings suggest that the COOH-terminal domain is an inhibitory domain. The finding that overexpression of either full-length or truncated mutants of Mst1/Mst3 induces the characteristics of apoptosis further supports the notion of a role for these kinases in the mediation of apoptotic events. Through the activation of caspases, which cleave these kinases, further activation may result. This may form a feedback loop that serves to amplify the apoptotic response.

The active transport of proteins between the nucleus and cytoplasm is an important process in eukaryotic cells. The majority of proteins required for these nuclear functions are transported by importins through the recognition of a nuclear localization signal (NLS) domain (51). Truncated forms of Mst3 are localized mainly in the nucleus, suggesting that Mst3 translocation may alter substrate specificity, which may in turn be important for apoptosis. Similarly, recent findings suggest that caspase-mediated cleavage of Mst1 promotes translocation into the nucleus (52, 53). These findings raise the possibility that Mst3 may contain a putative NLS within the NH₂-terminal cleaved fragment (amino acids 1–313), and that this NLS may be shielded by its COOH-terminal regulatory domain. Alternatively, the existence of a nuclear export sequence in the COOH-terminal regulatory domain of Mst3 can also be postulated. Caspase-mediated cleavage of Mst3 may expose the putative NLS and/or remove the putative nuclear export sequence, resulting in promotion of cytosol to nucleus translocation for the truncated form of Mst3. The notion of the presence of the nuclear export sequence in the COOH-terminal domain of Mst family members has been supported by the recent study of Mst1 where the existence of two nuclear export

sequences has been determined (52). On the other hand, a short basic residue-rich region, ²⁷⁸KKTSYLTELIDRYKRWK²⁹⁴, resembling the bipartite-type NLS sequence of human ProT-β (KKAEDDEDVDTKKQKTD) (54) and nucleoplasmin (KKPAATKKGQAKKKK) (55) has also been found in Mst3. Deletion and point mutation analysis of the putative NLS on Mst3 suggest that it is necessary for the translocation of the truncated Mst3.²

In summary, we have identified that Mst3 is a caspase substrate. Proteolytic cleavage of Mst3 results in kinase activation, nuclear translocation, and the induction of apoptosis. The exact biological response and signaling pathways remain largely unidentified, however. To delineate the role of the cleaved Mst3 in apoptosis, therefore, it is imperative to identify its downstream targets in both the cytosol and nucleus.

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