Regulation of Mammalian STE20-like Kinase 2 (MST2) by Protein Phosphorylation/Dephosphorylation and Proteolysis*

Received for publication, October 30, 2002, and in revised form, January 9, 2003
Published, JBC Papers in Press, January 27, 2003, DOI 10.1074/jbc.M211085200

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Mammalian STE20-like kinase 2 (MST2), a member of the STE20-like kinase family, has been shown in previous studies to undergo proteolytic activation by caspase-3 during cell apoptosis. A few studies have also implicated protein phosphorylation reactions in MST2 regulation. In this study, we examined the mechanism of MST2 regulation with an emphasis on the relationship between caspase-3 cleavage and protein phosphorylation. Both the full-length MST2 and the caspase-3-truncated form of MST2 overexpressed in 293T cells exist in a phosphorylated state. On the other hand, the endogenous full-length MST2 from rat thymus or from proliferating cells is mainly unphosphorylated whereas the caspase-3-truncated endogenous MST2 from apoptotic cells is highly phosphorylated. Cell transfection studies with mutant MST2 constructs indicate that MST2 depends on the autophosphorylation of a unique threonine residue, Thr180, for kinase activity. The autophosphorylation reaction shows strong dependence on MST2 concentration suggesting that it is an intermolecular reaction. While both the full-length MST2 and the caspase-3-truncated form of MST2 undergo autophosphorylation, the two forms of the phosphorylated MST2 display marked difference in susceptibility to protein phosphatases. The full-length phospho-MST2 is rapidly dephosphorylated by protein phosphatase 1 or protein phosphatase 2A whereas the truncated MST2 is remarkably resistant to the dephosphorylation. Based on the present results, a novel molecular mechanism for MST2 regulation in apoptotic cells is postulated. In normal cells, because of the low concentration and the ready reversal of the autophosphorylation by protein phosphatases, MST2 is present mainly in the unphosphorylated and inactive state. During cell apoptosis, MST2 is cleaved by caspase-3 and undergoes irreversible autophosphorylation, thus resulting in the accumulation of active MST2.

Mammalian STE20-like kinases comprise an extended protein kinase subfamily whose kinase domains show strong homology to that of STE20 kinase of the budding yeast (1, 2). Among 30 members of the protein subfamily known to date, mammalian STE20 kinase 1 (MST1) and 2 (MST2) share the most extensive sequence similarity (3–5). Apoptotic agents including staurosporine, Fas antigen/CD95, okadaic acid, synthetic apoptotic compound MT-21, anti-cancer drug cytostatin A and bisphosphonates, as well as environmental stresses such as heat shock and high concentrations of sodium arsenite have been shown to activate MST1 or MST2 in cultured mammalian cells (6–13). The activation of MST1 or MST2 during apoptosis is generally attributed to the production of an active fragment of the kinase in a caspase-3-catalyzed reaction (7–15). Both MST1 and MST2 undergo phosphorylation in cells but the mechanism of the enzyme regulation by protein phosphorylation is not entirely clear. A number of studies have suggested that protein phosphorylation is accompanied with an activation of the kinase. Using a phosphorylation site-specific antibody, Lee and Yonehara (16) have shown that MST in okadaic acid-treated cells is phosphorylated at a site in the kinase activation loop. The observation suggests that the kinase is activated upon protein phosphorylation. In agreement with such a suggestion, Graves et al. (17) have shown that treatment of the extracts of anti-Fas-treated BJAB cells by protein phosphatase 2A markedly reduced the kinase activity of MST1. On the other hand, evidence suggesting an inhibitory protein phosphorylation mechanism for MST1 has also been reported (3).

The present study examines the regulation of the MST2 kinase activity in detail. Both the native form and the caspase-3-truncated form of MST2 depend on autophosphorylation of a unique threonine residue, Thr180, for kinase activity. The autophosphorylation is an intermolecular reaction, showing strong dependence on the enzyme concentration. Whereas the phosphorylation of the native MST2 can be readily reversed by protein phosphatases, the truncated phospho-MST2 is resistant to protein phosphatases. On the basis of these results, a novel molecular mechanism involving caspase-3 cleavage, MST2 autophosphorylation, and protein phosphatase reaction is postulated to account for the regulation of MST2 activity in normal and apoptotic cells.

EXPERIMENTAL PROCEDURES

Cloning and Site-directed Mutagenesis—Expression constructs of rat MST2 were generated as NH2-terminal Myc-tagged forms using pCMV-Myc vector obtained from Clontech (BD Biosciences). Caspase-3-truncated MST2 (TF-MST2) was constructed by introducing a stop codon after amino acid 322 using PCR amplification. Substitution of Thr180, Thr117, and Thr384 by alanine as well as Lys26 by arginine, was performed with a QuickChange site-directed mutagenesis kit (Stratagene) and confirmed by DNA sequencing.

Cell Culture and Transfection—HeLa and 293T cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 100 unit/ml penicillin-streptomycin (Invitrogen) at 37 °C in a humidified atmosphere with 5% CO2. Transient transfection by DTT, dithiothreitol; JNK, c-Jun NH2-terminal kinase; MAPK, mitogen-activated protein kinase; SAPK, stress-activated protein kinase; MOPS, 4-morpholino propane sulfonic acid.

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‡ The abbreviations used are: MST, mammalian STE20-like kinase; MCT, microcystin-LR; PAK, p21-activated kinase; PKA, protein kinase A; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A; FL-MST2, full-length MST2; TF-MST2, truncated form of MST2; Ab, anti-MMCT, microcystin-LR; PAK, p21-activated kinase; PKA, protein kinase A; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A; FL-MST2, full-length MST2; TF-MST2, truncated form of MST2; Ab, anti-MST2, full-length MST2; TF-MST2, truncated form of MST2; Ab, anti-MST2, full-length MST2; TF-MST2, truncated form of MST2; Ab, anti-MST2, full-length MST2; TF-MST2, truncated form of MST2; Ab, anti-MST2, full-length MST2; TF-MST2, truncated form of MST2; Ab, anti-MST2, full-length MST2; TF-MST2, truncated form of MST2; Ab, anti-MST2, full-length MST2; TF-MST2, truncated form of MST2; Ab, anti-
tion was carried out using LipofectAMINE 2000 reagent (Invitrogen). Cells were incubated for 24 h before harvesting. The cells were washed twice with phosphate-buffered saline and lysed with cold lysis buffer (50 mM Heps, pH 7.2, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 5 mM NaF, 2 mM DTT, 0.1% Nonidet P-40, 0.1 μM microcystin-LR (MCT) (Calbiochem), 20 mM β-glycerophosphate, 1 mM sodium orthovanadate, and the protease inhibitor mixture, CompleteTM (Roche Molecular Biochemicals) for 15 min at 4 °C. Cell lysates were cleared by centrifugation at 14,000 rpm for 10 min. Protein concentration was determined by the Bradford assay (Bio-Rad).

Cross-linking MST2 Antibody to Protein A/G-agarose Beads—2 ml of MST2 antibodies were dialyzed against the cross-linking buffer (0.1 M NaCl, 0.1 mM Na2HPO4, pH 7.2) overnight. Then the antibodies were diluted 1:1 with antibody binding/washing buffer and incubated with protein A/G-agarose beads (50 μl/ml) at room temperature or overnight at 4 °C. After extensive washing with the dephosphorylation buffer (50 mM Tris-HCl, pH 7.4, 10 mM MgCl2, 10 mM NaF, 2 mM DTT, 0.1% Nonidet P-40, 0.1 mM EGTA, 5 mM NaF, 2 mM DTT) the immunoprecipitates were centrifuged at 3000 rpm for 10 min, and the supernatant was further centrifuged at 100,000 × g for 1 h. The 100,000 × g supernatant was diluted with a double volume of buffer B (20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 5 mM NaF) with inhibitor mixture (1× CompleteTM protease inhibitor mixture, 2 mM DTT, and 10% glycerol), and then applied to a 35-ml DEAR-Sepharose Fast Flow column pre-equilibrated with buffer B. The column was washed followed by elution with 200 ml of buffer B with 0−0.5 mM linear NaCl gradient. MST2 was eluted in fractions with 0.075−1 mM NaCl gradient (total 5 fractions, 5 ml/fraction). The elution buffer (50 mM NaCl, pH 8.2) was applied to the beads and mixed with 5 ml of elution buffer (0.1 M ethanolamine, pH 8.2) was applied to the beads and mixed with 5 ml of elution buffer (0.1 M ethanolamine, pH 8.2) was applied to the beads and mixed with 5 ml of elution buffer (0.1 M ethanolamine, pH 8.2) was applied to the beads and mixed with 5 ml of elution buffer (0.1 M ethanolamine, pH 8.2) was applied to the beads and mixed with 5 ml of elution buffer (0.1 M ethanolamine, pH 8.2) was applied to the beads and mixed with 5 ml of elution buffer (0.1 M ethanolamine, pH 8.2) was applied to the beads and mixed with 5 ml of elution buffer (0.1 M ethanolamine, pH 8.2) was applied to the beads and mixed with 5 ml of elution buffer (0.1 M ethanolamine, pH 8.2) was applied to the beads and mixed.
FIG. 1. The full-length and the caspase-3-truncated forms of MST2 overexpressed in cells are phosphorylated and active. A, 293T cells were singly transfected with control vector pCMV-Myc, the full-length MST2 (FL-MST2), or the caspase-3-truncated form of MST2 (TF-MST2) as indicated. Cells were harvested after 24 h and cell lysates were analyzed by immunoblotting with an anti-Myc (A-14) antibody (left panel) or an anti-phosphothreonine antibody (right panel). B, FL-MST2-transfected 293T cells were harvested in lysis buffers with or without the presence of 0.1 mM MST2-transfected 293T cells were harvested in lysis buffers with or without the presence of 0.1 μM MCT. Prolonged incubation was carried out from 30 min up to 9 h and the incubation was stopped at various times indicated with the addition of SDS loading buffer. The phosphorylation level of MST2 was analyzed with anti-phosphothreonine antibody. C, FL-MST2-transfected 293T cells were harvested in lysis buffers with different concentrations of MCT as indicated. The protection effect of different MCT concentrations on MST2 phosphorylation was analyzed by immunoblotting with the anti-phosphothreonine antibody, and the MST2 level in each cell lysate was demonstrated by the anti-Myc (A-14) antibody. D, cell lysates containing equal amounts of FL-MST2 and TF-MST2 were immunoprecipitated by anti-Myc (9E10) antibody and subjected to in vitro kinase assays as described under “Experimental Procedures.”

FIG. 2. Various MST2 mutants show different phosphorylation levels and kinase activities. A, different forms of Myc-tagged MST2, including the wild-type FL-MST2, truncated mutant (TF-MST2), FL-MST2 (T180A) mutant (T180A), truncated MST2 (T180A) mutant (TF-MST2 T180A), FL-MST2 (T117A) mutant (T117A), and kinase dead mutant (K56R), were transfected into 293T cells. The expression levels and the phosphorylation levels of the Myc-tagged MST2 were detected by Western blot using the anti-Myc antibody (left panel) and the anti-phosphothreonine antibody (right panel). B, overexpressed Myc-tagged MST2 mutants were immunoprecipitated by anti-Myc (9E10) antibody, and the immunoprecipitates were subjected to in vitro kinase assays as described under “Experimental Procedures.”

The phosphorylated substrate sites of various protein kinases including PKA, Akt, PKC, MAPK/CDK, and PDK1 were tested for the ability to react with MST2. Among the antibodies tested, the anti-phosho-PKA substrate and anti-phospho-Akt substrate antibodies reacted with MST2 (results not shown). However, co-transfection of a constitutively active Akt with MST2 or treating the MST2-transfected cell culture with dibutyryl-cAMP had no effect on the phosphorylation of MST2 suggesting that neither kinase catalyzes the phosphorylation of MST2 in the cells (data not shown).

Although neither PKA nor Akt was the kinase catalyzing the phosphorylation of Myc-MST2, the recognition of MST2 by antibodies specific for phospho-substrates of PKA and Akt suggested that MST2 was phosphorylated at a site conforming to the phosphorylation motifs of these protein kinases. Three threonine residues, Thr117, Thr180, and Thr384, in MST2 appeared to reside at sites conforming to the consensus phospho-

rylation motifs of PKA or Akt. To investigate whether or not they are the true phosphorylation sites in MST2, we generated mutant constructs of both the full-length and the caspase-3-truncated MST2 with three sites individually mutated: T117A, T180A, and T384A. The mutant constructs were transfected into 293T cells and the cell lysates were analyzed by Western blot using the anti-phosphothreonine antibody. Fig. 2A shows that the phosphorylation was markedly reduced in the full-length MST2 (T180A) mutant and it was completely eliminated in the truncated MST2 (T180A) mutant. In contrast, mutating Thr117 had little or no effect on MST2 phosphorylation. Direct kinase assays using the anti-Myc immunoprecipitates also showed that T180A mutants were inactive whereas T117A mutant had approximately the same kinase activity as the wild type MST2 (Fig. 2B). Similar to the T117A mutant, mutating T384A mutant had little effect on MST2 phosphorylation and kinase activity (results not shown). These results suggest that MST2 depends on the phosphorylation of Thr180 for kinase activity. The observation that the full-length MST2 (T180A) but not the truncated MST2 (T180A) was still phosphorylated, albeit at a much lower level than the wild type MST2, suggests the existence of additional phosphorylation site(s) in the caspase-3-cleaved carboxyl-terminal region of the kinase.

To test the possibility that the phosphorylation of MST2 is an autocatalytic reaction, a kinase dead mutant construct, MST2 (K56R), was transfected into 293T cells and the phosphorylation of the overexpressed protein analyzed by Western blot. As
shown in Fig. 2A, the phosphorylation of the mutant MST2 was much lower than that of the wild type MST2. Significantly, the levels of phosphorylation of MST2 (T180A) and MST2 (K56R) were almost identical. The results suggest that the phosphorylation of Thr180 of MST2 is catalyzed by an autophosphorylation reaction whereas the phosphorylation of the additional site(s) in the carboxyl-terminal region of MST2 was catalyzed by another protein kinase(s).

Characterization of MST2 Autophosphorylation Reaction—

To find out whether MST2 undergoes autophosphorylation and autoactivation, we have undertaken a detailed characterization of the MST2 phosphorylation reaction. Cultured 293T cells with overexpressed FL-MST2 were lysed in the lysis buffer containing no MCT to allow dephosphorylation of the phospho-Myc-MST2 (see Fig. 1B). After immunoprecipitation of MST2 using an anti-Myc antibody, the protein phosphorylation state and the kinase activity of the enzyme were examined. Western blot analysis indicated that MST2 in the immunoprecipitates was almost totally devoid of reactivity toward the anti-phosphothreonine antibody. However, the enzyme exhibited significant kinase activity (Fig. 3A). The observation suggests that the unphosphorylated MST2 may undergo autophosphorylation during the kinase assay.

To determine whether or not MST2 is phosphorylated during the course of the kinase assay, aliquots of the MST2 reaction were withdrawn at intervals and analyzed for both the substrate phosphorylation (i.e., the kinase activity) and MST2 phosphorylation. Fig. 3B shows that MST2 was indeed phosphorylated during the course of the kinase reaction. The increase in MST2 phosphorylation was obvious right from the beginning of the time course and continued throughout the entire course of 20 min. As a control, phospho-MST2 immunoprecipitated from the cell lysates containing MCT was also analyzed and the result indicates that it could be further phosphorylated. The time course of the substrate phosphorylation (Fig. 3A) by dephosphorylated MST2 (in lysates without MCT) displays an upward curvature with a pronounced initial lag suggesting an increase in enzyme activity during the course of the reaction. A slight curvature can also be detected in the time course of the control reaction, but the initial lag is not apparent. These results provide strong support to the suggestion that MST2 depends on phosphorylation for kinase activity.

To further test whether the MST2 autophosphorylation was an intermolecular or intramolecular reaction, the dependence of the protein phosphorylation on MST2 concentration was characterized. Cultured 293T cells containing overexpressed MST2 were lysed in the absence of MCT, the lysates were further incubated at 4°C for 1 h to ensure that MST2 was mostly dephosphorylated. The cell lysates were then diluted with the lysis buffer to different protein concentrations, and the phosphorylation was started at 22°C with the supplement of ATP and Mg²⁺. Three minutes after the start of the phosphorylation reaction, SDS loading buffer was added to stop the reaction. The samples were then analyzed by Western blot using the anti-phosphothreonine antibody. Lysates with a wide range of cellular protein concentrations, from 0.03 to 1 mg/ml, were used in the phosphorylation reactions. A very short reaction time, 3 min, was used in hoping that the phosphorylation could reflect the initial rate of the reaction. As shown in Fig. 4, although equal amounts of cellular proteins were subjected to Western blot analysis, the level of MST2 phosphorylation differed depending on the protein concentration in the phosphorylation reaction. The level of MST2 phosphorylation increased with the protein concentration in the phosphorylation reaction. Densitometric analysis of the immunoblot indicates that the level of MST2 phosphorylation at 0.03 mg/ml was less than 1% that at 1 mg/ml. The observation suggests that autophosphorylation of MST2 is through intermolecular but not intramolecular reaction.

Phosphorylation State of the Endogenous MST2—The observation that autophosphorylation of MST2 shows strong dependence on the enzyme concentration has raised the possibility that the state of phosphorylation of the overexpressed MST2 did not reflect the phosphorylation state of the endogenous MST2. To address such a possibility, we analyzed the phosphorylation status of the endogenous MST2 from rat thymus. Rat thymus contains relatively high amounts of MST2 and was used to find out whether the MST2 autophosphorylation was an intermolecular or intramolecular reaction, the dependence of the kinase activity on MST2 concentration was characterized. Cultured 293T cells containing overexpressed MST2 were lysed in the absence of MCT, the lysates were further incubated at 4°C for 1 h to ensure that MST2 was mostly dephosphorylated. The cell lysates were then diluted with the lysis buffer to different protein concentrations, and the phosphorylation was started at 22°C with the supplement of ATP and Mg²⁺. Three minutes after the start of the phosphorylation reaction, SDS loading buffer was added to stop the reaction. The samples were then analyzed by Western blot using the anti-phosphothreonine antibody. Lysates with a wide range of cellular protein concentrations, from 0.03 to 1 mg/ml, were used in the phosphorylation reactions. A very short reaction time, 3 min, was used in hoping that the phosphorylation could reflect the initial rate of the reaction. As shown in Fig. 4, although equal amounts of cellular proteins were subjected to Western blot analysis, the level of MST2 phosphorylation differed depending on the protein concentration in the phosphorylation reaction. The level of MST2 phosphorylation increased with the protein concentration in the phosphorylation reaction. Densitometric analysis of the immunoblot indicates that the level of MST2 phosphorylation at 0.03 mg/ml was less than 1% that at 1 mg/ml. The observation suggests that autophosphorylation of MST2 is through intermolecular but not intramolecular reaction.
Column chromatography and FPLC Superose 6 gel filtration chromatography as described under "Experimental Procedures." One MST2-containing fraction (fraction 15, about 9 mg of protein) from the DEAE-Sepharose Fast Flow column was concentrated to 200 μl of pooled fractions or 293T cell lysates. The immunoprecipitates and the control samples (Myc-FL-MST2 with or without the presence of MCT, 2 μg of cell lysates) were subjected to Western blot analysis using an anti-MST2 antibody and an anti-phosphothreonine antibody. The observation indicates that anisomycin-treatment induced MST2-phosphorylation in HeLa cells and the floating cells comprise mainly apoptotic cells. To determine the phosphorylation states of MST2 in anisomycin-induced apoptotic HeLa cells, anisomycin-induced apoptotic HeLa cells were immunoprecipitated with an anti-MST2 antibody and the immunoprecipitates were subjected to Western blot analysis using both the anti-MST2 antibody and the anti-phosphothreonine antibody. Western blotting of MST2 showed that a significant amount of MST2 in lysates of anisomycin-treated floating cells was cleaved and displayed as two closely spaced SDS-PAGE bands corresponding to ~34- or 36-kDa protein species. Both cleaved MST2 bands reacted with anti-phosphothreonine antibody, whereas the full-length poly(ADP-ribose) polymerase could be detected in the anisomycin-treated adherent HeLa cells, the majority of the cleaved PAPR was found in the floating cells (Fig. 6A). The observation indicates that MST2 in apoptotic cells appears to exist mainly in a caspase-3-cleaved, phosphorylated and highly active form.

Differential Susceptibility of the Full-length and the Caspase-3-truncated MST2 to Protein Phosphatases—Previous studies showed that MST2 was cleaved by caspase-3 and activated during cell apoptosis. Because both the full-length MST2 and caspase-3-truncated MST2 appear to depend on phosphorylation of MST2 in anisomycin-induced apoptotic HeLa cells. Anisomycin, a protein synthesis inhibitor, strongly activates JNK/SAPK and p38 MAPK and it is also known to induce apoptosis in several mammalian cells (19). HeLa cells were treated with 20 μg/ml anisomycin for 6 h to render a portion of cells detached from the plates. The attached and detached cells, referred to as the adherent and floating cells, respectively, were separately collected and lysed as described in the legend of Fig. 6. Western blotting analysis of the cell lysates showed that a significant portion of poly(ADP-ribose) polymerase, a known substrate of caspase-3, was converted to the caspase-3-cleaved form, an 85-kDa protein species. Although the cleaved poly(ADP-ribose) polymerase could be detected in the anisomycin-treated adherent HeLa cells, the majority of the cleaved PAPR was found in the floating cells (Fig. 6A). The observation indicates that anisomycin treatment could induce the caspase-3-mediated apoptosis in HeLa cells and the floating cells comprise mainly apoptotic cells.

### Table

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<th>Protein conc. (mg/ml)</th>
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**Fig. 4.** MST2 autophosphorylation is concentration dependent. Two forms of FL-MST2 in transfected 293T cell lysates were used in this experiment, including in vivo phosphorylated FL-MST2 that was preserved in lysates with MCT and in vitro dephosphorylated FL-MST2 in lysates without MCT. Two samples were diluted with the lysis buffer (containing MCT) to different concentrations from 0.03 to 1 mg/ml as indicated. Phosphorylation was started with the supplement of ATP and Mg²⁺ at 22°C, the reaction lasted for 3 min and was stopped with the supplement of SDS loading buffer. Equal amounts of the lysates from each reaction were loaded onto SDS-PAGE, and the phosphorylation of MST2 in each concentration was analyzed with an anti-phosphothreonine antibody.
tion of Thr\textsuperscript{180} for kinase activity, we have investigated the possibility that the two forms of MST2 are differentially regulated by phosphorylation/dephosphorylation mechanisms. A caspase-3-truncated MST2 construct was transfected into 293T cells to overexpress the truncated MST2. In an attempt to generate the dephosphorylated TF-MST2, the cells were lysed in buffer without MCT. To our surprise, the TF-MST2, unlike the FL-MST2, was found not to require MCT for the preservation of the protein phosphorylation. The enzyme in lysates with and without MCT displayed essentially identical reactivity toward the anti-phosphothreonine antibody on the Western blot. Fig. 7A shows that the phosphorylation state of the overexpressed TF-MST2 was not significantly changed even after 9 h of incubation in cell lysates. In contrast, greater than 90% loss of FL-MST2 phosphorylation occurred after 30 min of incubation (see Fig. 1B). The observation suggests that the phosphorylation of TF-MST2 is almost irreversible whereas the phosphorylation of FL-MST2 is readily reversed by protein phosphatases.

As described in an early section, overexpressed FL-MST2 in 293T cells was partially phosphorylated because it could be further phosphorylated in the cell lysates with MCT upon ATP/Mg\textsuperscript{2+}/H\textsubscript{11001} supplements (see Fig. 3B). In contrast, overexpressed TF-MST2 in 293T cells appears to be fully phosphorylated. 293T cell lysates with MCT containing overexpressed TF-MST2 was supplemented with ATP/Mg\textsuperscript{2+} and incubated at 30 °C for 30 min. The sample, along with the control sample without the ATP/Mg\textsuperscript{2+} supplements, was analyzed for protein phosphorylation by Western blot. The TF-MST2 that had been subjected to the phosphorylation condition displayed essen-
tially identical reactivity toward the anti-phosphothreonine antibody as the control enzyme (data not shown).

To further test the suggestion that the phospho-TF-MST2 cannot be readily dephosphorylated, both the overexpressed phospho-FL-MST2 and phospho-TF-MST2 were immunoprecipitated using the anti-Myc (9E10) antibody, and then tested for their dephosphorylation by protein phosphatases. Western blot analysis of the immunoprecipitates showed that the full-length MST2 could be readily dephosphorylated by protein phosphatase 1, whereas under the same condition, the truncated MST2 was not dephosphorylated significantly (Fig. 7B). A similar result was obtained if protein phosphatase 2A instead of protein phosphatase 1 was used (data not shown).

To ensure that the differential response of FL-MST2 and TF-MST2 to protein phosphatases is also manifested in the kinase activity, the effect of dephosphorylation on the activities of phospho-FL-MST2 and phospho-TF-MST2 were determined and compared. 293T cells overexpressing FL- or TF-MST2 were lysed in the buffer with or without 0.1 μM MCT. The protein kinases were then immunoprecipitated with an anti-Myc antibody (9E10) and analyzed for kinase activities. The activity of FL-MST2 from lysates with 0.1 μM MCT was about double of lysates without MCT, whereas TF-MST2 samples obtained from different lysis conditions displayed identical kinase activity (Fig. 7C). From these results, we suggest that FL-MST2 and TF-MST2 are differentially regulated by protein phosphorylation/dephosphorylation mechanisms. Both forms of MST2 depend on autophosphorylation for kinase activity but only the FL-MST2 activation can be reversed by protein phosphatases. As a result, TF-MST2 in cells is predominantly in the phosphorylated state, and therefore “constitutively active.”

**DISCUSSION**

In this study, we used deletion and site-directed mutant constructs of MST2, in combination with *in vitro* characterization of the enzyme activation and enzyme phosphorylation/dephosphorylation, to explore the mechanism of MST2 regulation. Our results showed that the phosphorylation of MST2 at a threonine residue, Thr^{180}, in the kinase activation loop was crucial for MST2 kinase activity. Substitution of Thr^{180} by an alanine residue completely abolished the ability of either the full-length or the truncated form of MST2 suggesting that both forms of MST2 depend on Thr^{180} phosphorylation for kinase activity. The suggestion was further substantiated by the observation that dephosphorylation of MST2 caused a significant decrease in kinase activity. A number of studies have suggested that MST2 (or MST1) is activated by a protein phosphorylation mechanism (16, 17). Amino acid sequence at Thr^{180} of MST2, KRXTXXGTP, is conserved in several MST kinase family members including α-PAK, γ-PAK, and MST1, and phosphorylation of this conserved threonine residue in these protein kinases have been shown to result in kinase activation (20, 21). Thus, our result is in agreement with these early findings. On the other hand, Lee and Yonehara (16) suggested in a recent publication that the activation of MST in apoptotic cells does not depend on protein phosphorylation, because both the full-length MST and the caspase-3-truncated form of MST in staurosporine-induced apoptotic cells existed in an unphosphorylated state. However, kinase activity of MST in the staurosporine-treated cells was not determined.

The mechanism of MST2 phosphorylation is complex, and Thr^{180} is likely not the sole phosphorylation site in the enzyme. For example, the MST2 mutant T180A overexpressed in 293T cells still showed immunoreactivity toward anti-phosphothreonine antibody, indicating the existence of the phosphorylation site threonine residue(s) in addition to Thr^{180}. The observation that the truncated MST2 (T180A) mutant was completely devoid of immunoreactivity toward the anti-phosphothreonine antibody indicates that the additional threonine phosphorylation site(s) is localized in the caspase-3-cleaved carboxyl-terminal fragment. The regulatory significance of the additional threonine phosphorylation is not known but it is clearly not essential for the kinase activity. In addition, MST2 may contain phosphoserine residues, which would have eluded detection by the anti-phosphothreonine antibody used in this study. It should also be noted that MST1/2 appears to contain phosphorylation sites that are involved in kinase inhibition; Creasy and Chernoff (3) showed that MST1 from epidermal growth factor-treated COS cells could be activated by protein phosphatase 2A. Graves et al. (17) have identified Ser^{252} as a major phosphorylation site in MST1, the phosphorylation at this site regulates the caspase-3 cleavage of the enzyme. This serine residue is conserved in MST2 as Ser^{253}, its phosphorylation, however, was not investigated in this study.

Several lines of evidence suggest that the phosphorylation of Thr^{180} in MST2 is an autocatalytic reaction. The strongest evidence is that the kinase dead mutant MST2 (K56R), in contrast to wild type MST2 or active MST2 mutants, was poorly phosphorylated when overexpressed in 293T cells. The observation that the dephosphorylated and immunoprecipitated MST2 could be phosphorylated in the presence of ATP/Mg^{2+}, with accompanying kinase activation, further supports such a suggestion. The time course of the MST2-catalyzed reaction showed upward curvature, also characteristic of an autoactivating reaction.

One important feature of the MST2 autophosphorylation reaction is that the reaction showed a strong dependence on the enzyme concentration. The result indicates that the autophosphorylation reaction is an intramolecular reaction. It is well established that both MST1 and MST2 could form homodimers. The protein domain responsible for MST2 dimerization locates in the extreme COOH-terminal 57 amino acids (5). We observed that a dimerization-deficient mutant, MST2 (L448P), had essentially the same level of phosphorylation as wild type MST2 in 293T cells. The result suggests that the enzyme concentration dependence of the autophosphorylation reaction is not related to the dimerization of the enzyme, rather it may be attributed to the interaction between MST2 dimers.

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*Y. Deng, and J. H. Wang, unpublished results.*
During the preparation of the manuscript, a paper (22) on the regulation of MST1 by protein phosphorylation was published. Many of the findings on the regulatory phosphorylation of MST1 are the same as those we have reported in this study. The approaches used in the two studies are significantly different.

The strong protein concentration dependence of the autophosphorylation reaction appears to have important regulatory significance for MST2. In contrast to the transfected MST2, endogenous MST2 in cultured cells or in rat thymus had essentially no reactivity toward the anti-phosphothreonine antibody. This may be attributed to the low cellular MST2 concentration, which can support only very slow autophosphorylation reactions. Thus, one potential mechanism for MST2 activation is to facilitate the autophosphorylation reaction by increasing the enzyme concentration. Lee and Yonehara (16) have observed the shuttling of MST between nuclear and cytoplasmic compartments, and Khokhlatchev et al. (23) have demonstrated the membrane recruitment of MST1 by the Ras effector protein NORE. These results suggest that MST1/2 may be induced to translocate to specific cellular compartments. It is conceivable that compartmentalization of MST1/2 in the cells could markedly increase the local enzyme concentration so as to facilitate the autophosphorylation/autoactivation reaction. In addition to raising local MST2 concentration, the autophosphorylation reaction in the cells may be enhanced by inhibiting protein phosphatases. It has been reported that MST phosphorylation in the activation loop could be induced by introducing the phosphatase inhibitor, okadaic acid, or calyculin A into cultured cells or neutrophils (16, 20). However, physiological stimulus that can activate MST1/2 by inhibiting protein phosphatases has not been reported. In this respect, the observation that the autophosphorylated TF-MST2 is highly resistant to protein phosphatase reactions is significant. We have found, in this study, that the autophosphorylated TF-MST2, in contrast to the autophosphorylated FL-MST2, is highly resistant to dephosphorylation by both protein phosphatase 1 and protein phosphatase 2A. Our result differs from the finding of Graves et al. (17) that MST1 from apoptotic cells could be inactivated by protein phosphatase 2A. The discrepancy may be attributed to a difference in the regulatory properties of MST1 and MST2. The possibility that the discrepancy arises from the differences in experimental conditions and that factors in addition to the molecular properties of TF-MST2 contribute to the remarkable phosphatase resistance cannot be excluded.

It is well documented that MST1 and MST2 are activated and cleaved by caspase-3 during cell apoptosis. Because both the full-length and the caspase-3-truncated forms of MST depend on the phosphorylation of Thr₁⁸⁰ for kinase activity, the question arises as to how the caspase-3 action contributes to the activation of MST2 during apoptosis. Based on results of the present study, we have proposed a molecular model to address this question. As schematically shown in Fig. 8, the model suggests that at the prevailing cytoplasmic MST2 and protein phosphatase concentrations in growing cells, MST2 exists mainly in the unphosphorylated and inactive state. During cell apoptosis, caspase-3 is activated resulting in the conversion of MST2 to the truncated form. Both the full-length and the truncated MST2 can undergo autophosphorylation and autoactivation. The autophosphorylation of the full-length MST2 is readily reversed by protein phosphatases while that of the truncated form is remarkably resistant to protein phosphatases. Thus, through the caspase-3 action, MST2 is converted into a constitutively active kinase. Although, the proposed model of MST2 regulation is far from proven, it is supported by the finding that truncated MST2 in anisomycin-induced apoptotic HeLa cells is present in an active and highly phosphorylated form whereas the full-length MST2 is not phosphorylated and displays very low kinase activity.

Acknowledgment—We are grateful to Dr. Zhenguo Wu for providing anisomycin and preparation of the manuscript.

REFERENCES
