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

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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 using mutant MST2 constructs indicate that MST2 depends on the autophosphorylation of a unique threonine residue, Thr\textsuperscript{180}, 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 differences 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.

**EXPERIMENTAL PROCEDURES**

Cloning and Site-directed Mutagenesis—Expression constructs of rat MST2 were generated as NH\textsubscript{2}-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 Thr\textsuperscript{180}, Thr\textsuperscript{17}, and Thr\textsuperscript{384} by alanine as well as Lys by arginine, was performed with a QuikChange 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% CO\textsubscript{2}. Transient transfection was performed using the calcium phosphate precipitation method to introduce plasmid constructs into HeLa cells.

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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, antibody; DTT, dithiothreitol; JNK, c-Jun NH\textsubscript{2}-terminal kinase; MAPK, mitogen-activated protein kinase; SAPK, stress-activated protein kinase; MOPS, 4-morpholinopropanesulfonic acid.
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 mM microcystin-LR (MCT) (Calbiochem), 20 mM β-mercaptoethanol, 1 mM sodium orthovanadate, and the protease inhibitor mixture, Complete™ (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 (0.15 M NaCl, 0.1 mM Na2HPO4, pH 7.2) overnight. Then the antibodies were diluted 1:1 with antibody binding/washing buffer, 2 ml of blocking buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% Nonidet P-40, 0.1 mM DTT, 1 mM sodium orthovanadate, and 2× Complete™ protease inhibitor mixture). The homogenate was first 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 dialuted 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× Complete™ protease inhibitor mixture, 0.1% 3-mercaptoethanol, 1 mM sodium orthovanadate, 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 0.075–0.1 mM NaCl gradient (total 5 fractions, 5 ml/fraction). The elution buffer (50 ml) was concentrated with Centricon 30 (Amicon) to 0.2 ml and loaded onto the FPLC Superose 6 HR 10/30 gel filtration column, respectively (Amerham Biosciences). The column was pre-equilibrated and run with buffer C (50 mM Tris-HCl, pH 7.5, 1 mM DTT, 1 mM EDTA, 0.01 mM Microcystin-LR, 0.1× Complete™ protease inhibitor mixture). The kinase containing fractions were analyzed by Western blotting using anti-MST2 antibody.

RESULTS

MST2 Overexpressed in 293T Cells Is Phosphorylated and Active—The full-length (FL-MST2) and the caspase-3-truncated form of MST2 (TF-MST2) were subcloned into pCMV-Myc vector and transfected into 293T cells, the cell lysates were analyzed by Western blotting using an anti-Myc antibody, an anti-MST2 antibody, or an anti-phosphothreonine antibody. As shown in Fig. 1A (left panel), the two forms of MST2 were expressed to similar levels in 293T cells. Using an anti-MST2 antibody, we found that the levels of the transfected enzymes were at least 100-fold higher than that of the endogenous MST2 (results not shown). Both the transfected FL-MST2 and TF-MST2 existed in a phosphorylated state as judged by the anti-phosphothreonine blot (Fig. 1A, right panel). The phosphorylation of MST2 underwent rapid dephosphorylation upon cell lysis unless a phosphatase inhibitor, MCT, was added to the lysis buffer, which preserved the phosphorylated MST2 for more than 6 h after cell lysis (Fig. 1B). In a dose-dependent assay, 0.1 mM MCT was found to be effective in keeping MST2 in a phosphorylated form (Fig. 1C). For experiments described in this report, the lysis buffer routinely contained 0.1 mM MCT unless indicated otherwise.

To determine the kinase activity of the full-length and the caspase-3-truncated forms of MST2 overexpressed in the cells, cell lysates containing equal amounts of the two forms of MST2, as judged by Western blotting analysis, were subjected to immunoprecipitation using an anti-Myc antibody. The immunoprecipitates were then assayed for MST2 kinase activity. Fig. 1D shows that both the full-length and the truncated MST2 were active when the kinase dead mutant K65R served as an inactive control. However, the truncated form consistently displayed 2–3-fold higher kinase activity than the full-length MST2.

In addition to MST2, a few other phosphorylated bands were revealed in the lysates of MST2-transfected cells by the anti-phosphothreonine immunoblot (Fig. 1A, right panel). For example, two phosphorylated bands of 46 and 48 kDa were consistently observed in the lysates of cells transfected with either the full-length MST2 or the truncated MST2. Another phosphorylated band migrating at 90 kDa was only observed in lysates of cells transfected with the truncated MST2 but not the full-length MST2. These proteins may represent down-stream targets of MST2.

The Relationship of MST2 Phosphorylation and Kinase Activity, and Determination of the Phosphorylation Site—Although phosphorylation of MST1 and MST2 has been shown previously, the protein kinase responsible for the phosphorylation is not known. In an attempt to determine the kinase catalyzing MST2 phosphorylation, a set of antibodies against...
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-phospho-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 phosphorylation 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-Myc antibody (left panel) and the anti-phosphothreonine antibody (right panel). Fl-MST2-transfected 293T cells were harvested in lysis buffers with or without the presence of 0.1 mM 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 with anti-Myc (9E10) antibody and subjected to in vitro kinase assays as described under “Experimental Procedures.”

**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 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 with 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.”
**Regulation of MST2 Kinase**

**A**

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 lysate buffer to different protein concentrations, and the phosphorylation was started at 22°C with the supplement of ATP and Mg^{2+}. 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 that could be enriched readily on a DEAE ion exchange chromatography column. The endogenous MST2 in rat thymus was purified using DEAE-Sepharose Fast Flow column chromatography column followed by FPLC Superose 6 gel filtration column chromatography. Fractions eluted from the gel filtration column were pooled and subjected to immunoprecipitation using an anti-MST2 antibody, which was cross-linked to protein A/G-agarose beads by disuccinimidyl suberate as described under “Experimental Procedures.” The cross-linked antibodies could not be released from the agarose beads during boiling in the SDS loading buffer, thus the immunoprecipitated MST2 that has a similar mobility as IgGs could be detected clearly by Western blot analysis. As shown in Fig. 5A, the immunoprecipitates of partially purified thymus MST2 fractions displayed intense immunostain by the anti-MST2 antibody, but very faint signals
Regulation of MST2 Kinase

**Lysates without MCT**

| Protein conc. (mg/ml) | 0.03 | 0.075 | 0.15 | 0.3 | 0.5 | 1   | 0.03 | 0.3 | 1   |
|----------------------|------|-------|------|-----|-----|-----|------|-----|-----|

Blot: Anti-P-Thr Ab

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**Lysates with MCT**

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 37 °C, the reaction lasted for 30 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.

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**Experimental Procedures.**

One MST2 containing fraction (fraction 15, about 9 mg of protein) from the DEAE-Sepharose Fast Flow column was concentrated to 200 l of pooled fractions containing fraction (fraction 15, about 9 mg of protein) from the DEAE-Sepharose Fast Flow column was concentrated to 200 l and applied to the FPLC Superose 6 gel filtration column. 400 l of pooled fractions or 293T cell lysates (1 mg of protein) were immunoprecipitated, respectively, overnight by anti-MST2 antibody cross-linked to protein A/G agarose beads. The immunoprecipitates and the control samples (Myc-FL-MST2 with or without the presence of MCT, 2 mg of cell lysates) were applied to a SDS-PAGE gel followed by Western blot analysis using an anti-MST2 antibody and an anti-phosphothreonine antibody.

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**A**

**B**

**Fig. 5.** The endogenous MST2 is unphosphorylated. A, rat thymus MST2 was serially purified using DEAE-Sepharose Fast Flow 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 l and applied to the FPLC Superose 6 gel filtration column. 400 l of pooled fractions or 293T cell lysates (1 mg of protein) were immunoprecipitated, respectively, overnight by anti-MST2 antibody cross-linked to protein A/G agarose beads. The immunoprecipitates and the control samples (Myc-FL-MST2 with or without the presence of MCT, 2 mg of cell lysates) were applied to a SDS-PAGE gel followed by Western blot analysis using an anti-MST2 antibody and an anti-phosphothreonine antibody.

B, 200 l of partially purified thymus MST2 were immunoprecipitated overnight by cross-linked MST2 antibodies. The immunoprecipitates were then subjected to in vitro kinase assays as described under “Experimental Procedures” in the absence of [γ-32P]ATP and the reaction was stopped with the addition of SDS loading buffer. The immunoprecipitates in the beginning (0 min) and end (30 min) of the kinase assay were subjected to Western blot analysis using an anti-MST2 antibody and an anti-phosphothreonine antibody.

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by the anti-phosphothreonine antibody. The lack of staining could not be attributed to low reactivity to the anti-phosphothreonine antibody because the control sample of Myc-MST2 from cell lysates with MCT was intensely stained. Like the recombinant MST2 from the transfected 293T cells, the rat thymus MST2 has kinase activity (data not shown). Analysis of MST2 in the kinase reactions by Western blot showed that MST2 phosphorylation was significantly increased at the end of the reaction (Fig. 5B). Therefore the kinase activity may be attributed to the enzyme autoactivation during the course of the enzyme assay. The observation indicates the endogenous MST2 is capable of autophosphorylation and autoactivation. Similar to thymus MST2, the endogenous MST2 from 293T cells exists mainly in the unphosphorylated state (Fig. 5A).

Because MST1/2 has been documented to undergo activation during cell apoptosis, we examined the phosphorylation status 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 rendered 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 capase-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.

To determine the phosphorylation states of MST2 in anisomycin-induced apoptotic cells, MST2 in the cell lysates were immunoprecipitated using anti-MST2 antibody and the immunoprecipitates were subjected to Western blotting analysis using both the anti-MST2 antibody and the anti-phosphothreonine antibody. Western blotting of MST2 showed that a significant amount of MST2 (Fig. 6). The full-length and the cleaved MST2 species were present predominantly in the adherent and floating cells, respectively (Fig. 6B). Western blotting analysis of the cell lysates showed that a significant amount of protein lysates from the adherent and floating cells was used for Western blotting analysis, the amount of MST2 was significantly lower in the floating cells than in the adherent cells. Unlike MST1, there is only one caspase-3 cleavage site in MST2 (15, 17); the reason for the existence of two SDS-PAGE bands corresponding to ~34- or 36-kDa proteins. Both cleaved MST2 bands reacted with anti-phosphothreonine antibody, whereas the full-length MST2 displayed no such reactivity (Fig. 6B). The full-length and the cleaved MST2 species were present predominately in the adherent and floating cells, respectively (Fig. 6B). The observation, in agreement with early studies (7, 8, 11–13), suggests that MST2 is cleaved by caspase-3 in apoptotic cells. Although the same amount of protein lysates from the adherent and floating cells was used for Western blotting analysis, the amount of MST2 was significantly lower in the floating cells than in the adherent cells. Unlike MST1, there is only one caspase-3 cleavage site in MST2 (15, 17); the reason for the existence of two SDS-PAGE bands of the caspase-3-cleaved MST2 remains unclear. From the relative intensity of the anti-MST2 and anti-phosphothreonine immunostain of the truncated MST2, it may be suggested that the enzyme was highly phosphorylated. Kinase activity determination of the immunoprecipitates indicated that the truncated MST2 was much more active than the full-length MST2 (Fig. 6C). Thus, 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 phosphoryla-
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 essent-
tainly 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 mM 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 mM MCT was about double of lysates without MCT, whereas TF-MST2 samples obtained from the 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 autoactivation 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 activity 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, KRR\(\text{XXX}\)GTP, is conserved in several MST kinase family members including \(\alpha\)-PAK, \(\gamma\)-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\(^{220}\) 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\(^{223}\), 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 autocatalytic 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 intermolecular rather than 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 activity as wild type MST2 in 293T cells.2 The result suggested 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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2 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 for MST2. They include the identification of the activation loop threonine, Thr\(^{185}\), as the essential phosphorylation site and demonstrated that this threonine phosphorylation is via an intermolecular autophosphorylation reaction. Interestingly, 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 phosphatases has not been reported. In this study, we have found, 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 Thr\(^{180}\) 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.

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**REFERENCES**

1. Dan, I., Watanabe, N. M., and Kusumi, A. (2001) *Trends Cell Biol.* 11, 220–230
2. Kyriakis, J. M. (1999) *J. Biol. Chem.* 274, 5259–5262
3. Creasy, C. L., and Chernoff, J. (1995) *J. Biol. Chem.* 270, 21695–21700
4. Creasy, C. L., and Chernoff, J. (1995) *Gene (Amst.*) 167, 303–306
5. Creasy, C. L., Ambrose, D. M., and Chernoff, J. (1996) *J. Biol. Chem.* 271, 21049–21053
6. Taylor, L. K., Wang, H., and Erikson, R. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93, 10099–10104
7. Graves, J. D., Gotoh, Y., Draves, K. E., Ambrose, D., Han, D. K. M., Wright, M., Chernoff, J., Clark, E. A., and Krebs, E. G. (1998) *EMBO J.* 17, 2224–2234
8. Lee, K. K., Murakawa, M., Ishida, E., Tsubuki, S., Kawashima, S., and Yonehara, S. (1998) *Oncogene* 16, 3029–3037
9. Kakeya, H., Onose, R., and Osada, H. (1999) *Ann. N. Y. Acad. Sci.* 866, 273–275
10. Reszka, A. A., Halasy-Nagy, J. M., Masarachia, P. J., and Rodan, G. A. (1999) *J. Biol. Chem.* 274, 34967–34973
11. Watabe, M., Kakeya, H., and Osada, H. (1999) *Oncogene* 18, 5211–5220
12. Kakeya, H., Onose, R., and Osada, H. (1998) *Cancer Res.* 58, 4888–4894
13. Watabe, M., Kakeya, H., Onose, R., and Osada, H. (2000) *J. Biol. Chem.* 275, 8766–8771
14. Ura, S., Masuyama, N., Graves, J. D., and Gotoh, Y. (2001) *Genes Cells* 6, 519–530
15. Lee, K. K., Ohyama, T., Yajima, N., Tsubuki, S., and Yonehara, S. (2001) *J. Biol. Chem.* 276, 19276–19285
16. Lee, K. K., and Yonehara, S. (2002) *J. Biol. Chem.* 277, 12351–12358
17. Graves, J. D., Draves, K. E., Gotoh, Y., Krebs, E. G., and Clark, E. A. (2001) *J. Biol. Chem.* 276, 14909–14915
18. Litwin, C. M. E., Cheng, H., and Wang, J. H. (1991) *J. Biol. Chem.* 266, 2557–2566
19. Hazali, C. A., Le Panse, R., Cano, E., and Mahadevan, L. C. (1998) *Mol. Cell. Biol.* 18, 1844–1854
20. Lian, J. P., Toker, A., and Badwey, J. A. (2001) *J. Immunol.* 166, 6349–6357
21. Pombo, C. M., Bonventre, J. V., Molnar, A., Kyriakis, J., and Force, T. (1996) *EMBO J.* 15, 4537–4546
22. Glantschnig, H., Rodan, G. A., and Reszka, A. A. (2002) *J. Biol. Chem.* 277, 42897–42896
23. Khokhlatchev, A., Rahibadeh, S., Xavier, R., Nedwidok, M., Chen, T., Zhang, X. F., Seed, B., and Avruch, J. (2002) *Curr. Biol.* 12, 253–265

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