Phosphoinositide 3-Kinase/Akt Inhibits MST1-Mediated Pro-apoptotic Signaling through Phosphorylation of Threonine 120

Zengqiang Yuan1,2, Donghwa Kim3, Shaokun Shu6, Junbing Wu6, Jianping Guo7, Lei Xiao8, Satoshi Kaneko9, Domenico Coppola10, and Jin Q. Cheng10

From the 1National Laboratory of Biomacromolecules, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, China and the 2Department of Molecular Oncology, H. Lee Moffitt Cancer Center and Research Institute, Tampa, Florida 33612

The protein kinase mammalian sterile 20-like kinase 1 (MST1) is a mammalian homologue of the Drosophila hippo and plays a critical role in regulation of programmed cell death. MST1 exerts its pro-apoptotic function by phosphorylating LATS1/2 and Yorkie in the nucleus, leading to its cleavage by PAR-4 protease. MST1 interacts with Akt to regulate cell death via phosphorylation of FOXO3a-Ser207 and the consequent release of BAD and JNK. Further evidence, including the findings that Thr120 of MST1 is phosphorylated by Akt and Thr120 phosphorylation is essential for MST1 activation, suggest that MST1 induces apoptosis by phosphorylation of histone H2B-Ser14 in mammalian cells and Ser10 in yeast cells. These findings indicate that MST1-Thr120 phosphorylation might be a primary autophosphorylation site. The autophosphorylation of Thr120 is essential for MST1 activation (9). This article has been withdrawn by the authors. The same data were used to represent different experimental conditions. Specifically, the HA-Akt1 immunoblot from Fig. 2E was reused as the FLAG-MST1 immunoblot from Fig. 4C. The authors state that they stand by the overall conclusions of the study.
Regulation of MST1 by Akt via Phospho-Thr120

(19, 20), XAIP (21), Par-4 (22), BAX (23, 24), and HtrA2 (25), which leads to direct activation of cell survival pathway. Moreover, Akt has been shown to activate NF-κB pro-survival signaling by phosphorylation of IKKα (26, 27). Although these targets could play important roles in Akt survival signaling, FOXO/DAF-16 is the only major target that has genetically been proven across different species (15).

A previous study showed that epidermal growth factor stimulation caused a transient drop of MST1 kinase activity (28). However, the one or more upstream signaling regulators of MST/Hippo are still largely unknown. In this study, we demonstrate that MST1 is regulated by Akt. Akt phosphorylates MST1 at Thr120 in vitro and in vivo, which leads to inhibition of MST1 cleavage and kinase activity as well as nuclear translocation. We also found that Akt phosphorylation of MST1 reduces caspase 3 activity, JNK activation, and MST1-induced phosphorylated FOXO3-Ser207. Consistent with these results, Akt-null cells or cancer cells with knockdown of Akt show elevated levels of MST1 kinase activity and decreased levels of phospho-MST1-Thr120, when compared with the cells expressing Akt. Further, Akt activation is inversely correlated with autophosphorylation of MST1-Thr183 but paralleled with MST1-Thr120 phosphorylation in cancer cell lines and tumor samples examined. Collectively, our findings suggest that MST1 is a bona fide substrate of Akt and that Akt could play a critical role in regulation of MST/Hippo/MST1/T2-Sav/WW45/Wats/LATS1/2 pathway.

MATERIALS AND METHODS

Reagents and Cell Culture—Dulbecco’s modified Eagle’s medium and fetal bovine serum were purchased from Invitrogen. EDEIA-amide as antigen. Staurosporine, LY294002, and anti-Akt1 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Z-VAD-FMK (caspase inhibitor) was from Calbiochem. COS7, HeLa, and human embryonic kidney (HEK) 293 cells as well as tumor cell lines were cultured at 37 °C and 5% CO2 in Dulbecco’s modified Eagle’s medium and fetal bovine serum were purchased from Invitrogen.

Expression Constructs—MST1 constructs were created by PCR amplification of human fetal Marathon-Ready cDNA library (Clontech). MST1-specific primers were: forward, 5’-CGCAAGCTTATGGAGACGGTACAGCTGAG-3’ and reverse, 5’-CGCTCTAGATGCTCAGAAGTTTTGTTG-3’. The PCR products were cloned into HindIII-BamHI sites of the 3×FLAG-CMV-10 expression vector (Sigma). MST1-T120A, MST1-T120D, wild type-MST1ΔC, MST1-T120ΔC, and MST1-T120ΔC were constructed using mutagenesis kit (Stratagene). Both wild-type MST1 and mutant MST1 were ligated into HindIII-BamHI sites of the 3×FLAG-CMV-10 and GFP-C3 (Clontech) vectors.

Immunoprecipitation and Immunoblotting—Immunoprecipitation and immunoblotting analysis was performed as described previously (27). Briefly, protein lysates were incubated with appropriate antibodies indicated in the figure legends in the presence of 25 μl of protein A-protein G (2:1)-agarose beads for 3 h at 4 °C. After washing three times, the immunoprecipitates were subjected to in vitro kinase assay (see below). Protein expression was determined by probing Western blots of total cell lysate or immunoprecipitates with the appropriate antibodies as noted in the figure legends. Detection of antigen-bound antibody was carried out with the ECL Western blotting Analysis System (Amersham Biosciences).

In Vitro Kinase Assay—Protein kinase assay was performed as previously described (20). Briefly, reactions were carried out in the presence of 10 μCi of [γ-32P]ATP (Amersham Biosciences) and 3 μM cold ATP in 30 μl of buffer containing 20 mM HEPES (pH 7.4), 10 mM MgCl2, 10 mM MnCl2, 1 mM dithiothreitol, and 2 μg of myelin basic protein (MBP) as substrate. After incubation at room temperature for 30 min, the reaction was stopped by adding 8 μl of loading buffer and then separated on a 10% SDS-PAGE gel. Incorporated radioactivity was visualized by PhosphorImager (Amersham Biosciences) and 3 times, and the relative amounts of incorporated radioactivity were quantified and with a phosphorimager.

Cells were seeded in 60-mm plate and transfected with different MST1 constructs. After 36 h of transfection, cells were incubated with a lysis buffer (20 mM Hepes, pH 7.4, 150 mM NaCl, 0.5% Triton X-100, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM sodium vanadate). Cell debris was removed by microcentrifugation for 15 min at 4 °C. 20 μg of cell extract was used for MBP in-gel kinase assay. Cell extract was loaded onto a 10% SDS-PAGE gel that had been polymerized in the presence of 0.2 mg/ml bovine brain MBP. After electrophoresis, SDS-PAGE gel was removed by 3 × 20 min (50 ml) washes with 20% iso-propanol alcohol in 50 mM Tris-HCl, pH 8.0, followed by 3 × 20 min (50 ml) washes in 50 mM Tris-HCl, pH 8.0, 5 mM 2-mercaptoethanol. Proteins were denatured by 2 × 30 min (50 ml) incubations with 6 mM guanidine-HCl, 5 mM 2-mercaptoethanol, 50 mM Tris-HCl, pH 8.0, and subsequently renatured by 5 washes (50 ml of solution containing 5 mM 2-mercaptoethanol, 0.04% Tween 40, and 50 mM Tris-HCl, pH 8.0) overnight. Gels were re-equilibrated by washing 2 × 30 min (50 ml) with a buffer containing 40 mM HEPES, pH 8.0, 10 mM MgCl2, and 2 mM dithiothreitol and then subjected to kinase assay. For the kinase reaction, the gels were incubated for 3 h with 10 ml of 40 mM HEPES, pH 8.0, 10 mM MgCl2, 0.055 mM ATP, 0.1 μM PKA inhibitor, and 25 μCi of [32P]ATP. The reaction was stopped, and excess [32P]ATP was removed by 5 washes in 5% trichloroacetic acid and 1% sodium pyrophosphate over 12 h. The gels were dried and detected by autoradiography.

Cell Death Analysis—TUNEL assay was used to detect apoptosis in situ cell death detection kit (Roche Applied Science). The experiments were performed three times in triplicate. Total cell death was analyzed by trypan blue exclusion assay as previously described (20).
Immunohistochemistry—Immunostaining was performed as previously described (27). Briefly, formalin-fixed, paraffin-embedded sections were subjected to antigen retrieval by boiling in 0.01 M sodium citrate buffer (pH 6.0) in a microwave oven after dewaxing and rehydration. The Vectastain ABC Kit for sheep IgG (Vector Laboratories) was used to immunostain the tissue sections with anti-pAkt-Ser473, -pMST1-Thr 120, and -pMST1-Thr 183 antibodies. Endogenous peroxidase and biotin were blocked, and sections were incubated for 1 h at room temperature with individual antibodies. The remainder of the staining procedure was performed according to the manufacturer’s instructions using diaminobenzidine tetrahydrochloride as the chromogen and hematoxylin for counterstaining.

RESULTS

PI3K/Akt Pathway Inhibits MST1-mediated Apoptosis as Well as MST1 Cleavage and Activation—Previous studies have demonstrated that the MST1 is cleaved and activated upon apoptotic stimuli and that the activation of MST1 is required for DNA damage- and FasL-induced apoptosis (2, 3). However, the regulation of MST1 cleavage and activation still remains elusive. We first examined whether extracellular cell survival signal inhibits DNA damage-induced MST1 cleavage. As shown in Fig. 1A and supplemental Fig. S1, MST1 was cleaved after exposure to STS or CD95/FasL. However, the cleavage was considerably reduced by IGF-1 treatment. Because IGF-1 exerts its cell survival action primarily through the PI3K/Akt pathway (29), we further demonstrated that IGF-1-inhibited MST1 cleavage was abrogated by PI3K inhibitor, LY294002. Consistent with MST1 cleavage status, IGF-1 rescues STS- and CD95/FasL-induced apoptosis, which was inhibited by LY294002 (Fig. 1A). These data suggest that the PI3K/Akt pathway mediates the IGF-1-inhibited MST1 cleavage.

It has been shown that overexpression of MST1 is able to induce its cleavage and cell death (6). To further determine the involvement of PI3K/Akt in regulation of MST1 cleavage and MST1-induced apoptosis, FLAG-MST1 was expressed in COS7 cells, and the p110α, a catalytic subunit of PI3K, or constitutively active Akt significantly reduces MST1 cleavage to the similar extent of Z-VAD treatment, a pan-caspase inhibitor. Further, expression of dominant negative-Akt or treatment with LY294002 or Akt inhibitor API-2 (30) increased the MST1 cleavage and MST1-induced apoptosis (Fig. 1B). Therefore, we conclude that MST1 cleavage is regulated by IGF-1 through a PI3K/Akt-dependent pathway.

The autophosphorylation of MST1 at Thr183 has been shown to be a hallmark of MST1 activation (9). To determine the inhibitory effect of Akt on MST1 activity, we treated Akt1-knockout MEF and Akt1-reconstituted cells with STS and probed with anti-pMST1-Thr183 antibody (Fig. 1C). The results show that autophosphorylation of MST1 is much higher in Akt1+/− cells. To further confirm the inhibition of MST1 by Akt, we knocked down AKT2 in OVCAR8 cells, which overexpress endogenous AKT2 (31). Fig. 1D shows that phospho-MST1-Thr183 was increased when AKT2 was
Regulation of MST1 by Akt via Phospho-Thr\textsuperscript{120}

FIGURE 2. Threonine 120 of MST1 is phosphorylated by Akt \textit{in vitro} and \textit{in vivo}. A, sequence alignment shows a highly conserved Akt phosphorylation consensus site of MST1 among different species. B, Akt phosphorylates MST1 \textit{in vitro}. In vitro Akt kinase assay by incubation of immunoprecipitated HA-Akt and FLAG-MST1 in the presence of \textit{\textsuperscript{32P}}ATP (top). The lower panels are immunoblotting analysis with anti-FLAG or -HA antibody. C, Akt phosphorylates MST1 \textit{in vivo}. Western blot analysis of the MST1 and MST1-T120A immunoprecipitates from COS7 cells co-transfected with indicated plasmids with anti-Akt-phospho-substrate (top), -FLAG (middle), and -HA (bottom) antibodies. D, MST1-Thr\textsuperscript{120} is phosphorylated by Akt \textit{in vivo}. HEK293 cells were transfected with Myr-Akt1 and FLAG-MST1-T120A and immunoprecipitated with anti-FLAG antibody. The immunoprecipitates were immunoblotted with specific anti-phospho-MST1-Thr\textsuperscript{120} antibody (top). Middle and bottom panels show expression of transfected plasmids. E, Akt phosphorylates endogenous MST1-Thr\textsuperscript{120}. Following transfection of HEK293T cells with indicated different Akt1, cells were lysed and immunoblotted with anti-phospho-MST1-Thr\textsuperscript{120} antibody (top). Middle and bottom panels show expression of transfected plasmids. F, phosphorylation of MST1-Thr\textsuperscript{120} is induced by IGF-1. After serum starvation for 12 h, HEK293 cells were stimulated with IGF-1 for 1 h and then immunoblotted with indicated antibodies. G, reconstitution of Akt1 in Akt1-knock-out MEFs increased pMST1-Thr120. Rats were infected with adenovirus expressing Akt1 or control vector and immunoblotted with indicated antibodies. H, interaction of Akt and MST1. HA-Akt1-transfected HEK293 cells were immunoprecipitated with anti-MST1 antibody, and detected with anti-Akt antibody (left panels) and vice versa (right panels). I, N-terminal kinase domain of MST1 binds to Akt1. HEK293 cells were transfected with the indicated plasmids and immunoprecipitated with anti-HA (Akt1). The immunoprecipitates were immunoblotted with anti-GFP (MST1) antibody.

depleted. Together, these results indicate that the PI3K/Akt pathway inhibits the MST1 cleavage and activation as well as MST1-induced cell death.

\textbf{Akt Phosphorylates MST1 at Thr\textsuperscript{120} and Interacts with MST1—}
The majority of Akt substrates contain Akt phosphorylation consensus motif R\textit{\textsuperscript{X}RX\textit{\textsuperscript{X}R\textit{\textsuperscript{X}X}}\textit{(S/T)}} (R = arginine, X = any amino acid, and S/T = serine/threonine). Sequence analysis revealed that threonine 120 (\textit{\textsuperscript{115}R\textit{\textsuperscript{L}R\textit{\textsuperscript{N}K\textit{\textsuperscript{T}120}}}) of human MST1 matches the consensus motif, which is conserved among mouse, rat, Xenopus, Drosophila (Hippo), and yeast (Mess1, Fig. 2A). As constitutively active Akt inhibits, whereas dominant negative-Akt enhances MST1 cleavage, we reasoned that MST1 could be a substrate of Akt. In vitro Akt kinase assay showed that wild-type MST1 but not MST1-T120A mutant, converting Thr120 into alanine, was highly phosphorylated by Akt (Fig. 2B). Further, immunoblotting analysis with anti-Akt substrate antibody revealed that Akt1 phosphorylates wild-type MST1 but not MST1-T120A (Fig. 2C). We next generated an antibody designed to specifically recognize Akt phosphorylation site of MST1-Thr\textsuperscript{120}. Upon immunoblotting of lysates from HEK293T cells transfected with WT-MST1 or MST1-T120A together with Akt1, the pMST1-Thr\textsuperscript{120} antibody recognized the phosphorylated WT-MST1, but not the MST1-T120A (Fig. 2D). Elevated levels of endogenous phospho-MST1-Thr\textsuperscript{120} were also detected in WT-Akt1- and Myr-Akt1- but not kinase-dead Akt1 (KD-Akt1)-transfected HEK293T cells (Fig. 2E). Further, immunoblotting analysis with anti-Akt substrate antibody revealed that Akt1 phosphorylates wild-type MST1 but not MST1-T120A (Fig. 2F). Moreover, re-expression of Akt1 in Akt1-knock-out MEFs increased the MST1-Thr\textsuperscript{120} phosphorylation (Fig. 2G). In addition, we also demonstrated that Akt interacts with the full length and N terminus of MST1 (Fig. 2, H and I). These data indicated that Akt interacts with and phosphorylates MST1 at threonine 120 \textit{in vitro} and \textit{in vivo}.  

\begin{table}[h]
\centering
\begin{tabular}{|c|c|}
\hline
Proteins & Akt phosphorylation sites \\
\hline
Human MST1 & \textit{\textsuperscript{115}RLRNKT\textsuperscript{120}} \\
Mouse MST1 & \textit{\textsuperscript{115}RLRNKT\textsuperscript{120}} \\
Rat MST1 & \textit{\textsuperscript{115}RLRNKT\textsuperscript{120}} \\
Xenopus MST1 & \textit{\textsuperscript{127}RLRKQT\textsuperscript{120}} \\
Drosophila Hippo & \textit{\textsuperscript{127}RLRKQT\textsuperscript{120}} \\
Yeast Mess1 & \textit{\textsuperscript{112}RLRNKT\textsuperscript{117}} \\
\hline
BAD & \textit{\textsuperscript{131}RGRSRS\textsuperscript{136}} \\
GSK3\beta & \textit{\textsuperscript{4}RRPTTS\textsuperscript{9}} \\
\hline
\end{tabular}
\caption{Consensus phosphorylation site of Akt substrates.}
\end{table}
Akt Inhibits MST1 Nuclear Translocation via a Phosphorylation-dependent Mechanism—It has been well documented that cleavage and nuclear translocation are critical steps for MST1-induced programmed cell death (32). Because Akt phosphorylates MST1, we reasoned that Akt phosphorylation of MST1 could regulate its nuclear translocation. To this end, we created GFP-tagged wild-type and mutant forms of MST1, including Akt phosphomimetic (MST1-T120D) and non-phosphorylatable (MST1-T120A) full-length and C-terminal truncated MST1 (Fig. 3A). After transfection of the various forms of GFP-MST1 together with and without constitutively active Akt, subcellular localization of GFP-MST1s was examined under a fluorescence microscope. Constitutively active Akt reduced WT-MST1 nuclear translocation. Phosphomimic MST1-T120D located at the cytoplasm, whereas near 40% of cells expressing MST1-T120A exhibited nuclear localization. Expression of constitutively active Akt had no effect on MST1-T120A (left panels of Fig. 3B). Furthermore, C-terminal truncated MST1-WTΔC and MST1-T120AΔC were highly restricted to the nucleus, whereas MST1-T120DΔC was retained in the cytoplasm. Notably, expression of constitutively active Akt effectively blocked nuclear localization of MST1-WTΔC but not MST1-T120AΔC (Fig. 3C). In addition, STS treatment induced full-length wild-type MST1 and MST1-T120A but not MST1-T120D nuclear translocation (supplemental Fig. S2). Cell fractionation assays also revealed that endogenous nuclear (e.g. cleaved) MST1 induced by STS was largely reduced by expression of constitutively active Akt (Fig. 3D). Thus, we conclude that Akt inhibition of MST1 nuclear translocation is regulated by Akt through phosphorylation of Thr120.

Phosphorylation of Thr120 Is Required for Akt Inhibition of MST1 Cleavage and Kinase Activity—Having demonstrated Akt inhibition of MST1 nuclear translocation through phosphorylation of Thr120, we next examined if Akt inhibition of MST1 cleavage and kinase activity depends on phosphorylation of Thr120. Following transfection with FLAG-tagged wild-type and mutant MST1 together with and without constitutively active Akt, COS7 cells were treated with STS or vehicle. Immunoblotting analysis showed that STS treatment enhanced the cleavage of wild-type MST1 and non-phosphorylatable MST1-T120A, but had no significant effect on the cleavage of phospho-

![Figure 3](http://www.jbc.org/content/285/6/3819/F3)

**Figure 3.** Akt1 phosphorylation of MST1-Thr120 inhibits MST1 nuclear translocation. A, schematic diagram shows GFP-fused different MST1 constructs. Akt retains MST1 in the cytoplasm. COS7 cells were transfected with the indicated plasmids, examined, and quantified under fluorescent microscopy. Constitutively active Akt reduced the nuclear localization of ectopic expression of WT-MST1 (*, p < 0.01) but not non-phosphorylatable MST1-T120A. C, nuclear translocation of MST1ΔC but not MST1-T120AΔC was inhibited by myr-Akt. The experiment was performed as described in panel B. Quantification reveals that myr-Akt1 significantly inhibits MST1ΔC nuclear translocation (*, p < 0.01). D, constitutively active Akt decreased nuclear (e.g. cleaved) MST1 induced by STS. HeLa cells were infected with adenovirus expressing myr-Akt or adenoviral vector. Following 72-h incubation, cells were treated with STS and then fractionated. The nuclear and cytoplasmic MST1 were detected with anti-MST1 antibody and quantified (top). Panels 2 and 3 are the validation of nuclear and cytoplasmic fractionations with indicated antibodies. The bottom panel shows expression of infected myr-Akt.
mimic MST1-T120D. Moreover, ectopic expression of constitutively active Akt reduced STS-stimulated wild-type MST1 but not MST1-T120A cleavage (Fig. 4A). Further, cells expressing MST1-T120D exhibited considerably lower levels of auto-pMST1-Thr183 as compared with those of the cells transfected with MST1-T120A and WT-MST1 (Fig. 4C). In addition, MST1-WTΔC and MST1-T120AΔC had higher levels of kinase activity as compared with MST1-T120DΔC (supplemental Fig. S3). Taking into consideration the findings that knockdown of Akt increases and expression of Akt decreases autophospho-MST1-Thr183 (Fig. 1, C and D), we conclude that Akt phosphorylation of Thr120 represents a key step for the regulation of MST1.

**Akt Inhibits Signaling Downstream of MST1**—We also assessed whether Akt reduces downstream signaling of MST1. Previous studies showed that JNK is a major target of MST1, MST1 activates JNK to induce apoptosis (32). To determine if Akt phosphorylation of MST1 affects JNK activation, COS7 cells were transfected with different forms of MST1. Following 48-h incubation, immunoblotting analysis revealed that JNK activation was induced by expression of wild-type MST1, MST1ΔC, non-phosphorylatable MST1-T120A and MST1ΔC-T120A, but not Akt phosphomimic MST1-T120D and MST1ΔC-T120D (Fig. 5A).

Recently, it has been shown that MST1 promotes cell death through phosphorylation of FOXO3a at residue Ser207 and enhancing its nuclear translocation by disrupting MST1/14-3-3 complex (7). We therefore assessed the phosphorylation of FOXO3a-Ser207 and its capability of binding to 14-3-3 upon the Akt phosphorylation of MST1. As shown in Fig. 5B, ectopic expression of MST1/FOXO3a considerably increased phospho-FOXO3a-Ser207 and reduced FOXO3a/14-3-3 complex when compared with expression of FOXO3a alone (lane 2 versus lane 3). However, constitutively active Akt decreased MST1-induced FOXO3a-Ser207 phosphorylation and, as a result, increased the FOXO3a association with 14-3-3 (lane 4). Further, phosphomimic MST1-T120D significantly reduced its ability to phosphorylate FOXO3a-Ser207, leading to increase of FOXO3a/14-3-3 complex formation.
which resembled the finding of expression of FOXO3a alone (lane 2 versus lane 5). Collectively, these results suggest that MST1 downstream targets are negatively regulated by Akt through phosphorylation of MST1.

In addition, we investigated the effect of Akt phosphorylation of MST1 on the programmed cell death. After transfection and treatment with/without STS, COS7 cells were analyzed by annexin V-fluorescein isothiocyanate/fluorescence-activated cell sorting. Fig. 5C shows that ectopic expression of Akt-phosphomimic MST1-T120D or MST1ΔC-T120D not only failed to induce apoptosis and caspase-3 cleavage but also considerably reduced the programmed cell death and caspase-3 activity induced by STS. In contrast, expression of non-phosphorylatable MST1-T120A or MST1ΔC-T120A induced apoptosis and caspase-3 cleavage but also considerably reduced the programmed cell death and caspase-3 activity induced by STS. Overall survival of the patients with elevated pMST1-Thr120 was significantly lower than those with low pMST1-Thr120/high p-MST1-Thr183 (Fig. 6D). Taken together, we conclude that MST1 is a bona fide substrate of Akt and that pMST1-Thr120 could be a prognostic marker in human cancer.

**DISCUSSION**

Aberrant activation of the PI3K/Akt pathway is one of the most common genetic alterations in human malignancy (33, 34). In this study, we demonstrated that Akt phosphorylates MST1-Thr120 in vitro and in vivo, and inhibits MST1 cleavage, autophosphorylation (e.g. pMST1-Thr183), kinase activity, and nuclear translocation. As a result, MST1 fails to induce apoptosis and activate JNK1 and FOXO3a. Further, the activation of Akt largely correlates with elevated levels of pMST1-Thr120, and an inverse relationship between pMST1-Thr120 and pMST1-Thr183 was detected in human
Ovarian cancer. In addition, elevated levels of pMST1-Thr\textsuperscript{120} are associated with poor prognosis in this malignancy. Our results indicate that Akt is a key upstream regulator of MST1 and that MST1 pro-apoptotic function is regulated by the PI3K/Akt pathway through direct phosphorylation of a highly conserved residue Thr\textsuperscript{120}.

The Hippo pathway was initially identified in the fly to control organ size. Its core components are evolutionally conserved in mammals. Hippo, Sav, Wts, and Mats in the fly are homologous to mammalian MST1/2, WW45, LATS1/2, and Mob1, respectively (35). The pathway impinges on transcriptional co-activator Yorkie in fly and yes-associated protein in mammals to coordinate cell proliferation and apoptosis (13, 14, 36). Previous studies also showed that the Drosophila insulin receptor transduces signals that positively regulate cell and organ growth through its downstream molecule Chico/Dp110/Dakt1 (37, 38). Overexpression of the Dakt1 dramatically increases clonal size in wing imaginal disc through an enlargement of the cells (37). These studies suggest that the cross-talk between Akt and Hippo/MST regulates cell growth and survival. Our study shows that MST1 possesses an Akt phosphorylation site (115RLRNK\textsubscript{Thr\textsuperscript{121}}) within its N-terminal kinase domain that is highly conserved among yeast, Drosophila, Xenopus, mouse, and human. Akt phosphorylates Thr\textsuperscript{120} of MST1 \textit{in vitro} and \textit{in vivo}. Although we did not examine its canonical downstream target LATS1/2, we demonstrated that Akt phosphorylation of MST1-Thr\textsuperscript{120} considerably reduces MST1-induced pFOXO3a-S207 and JNK activation (Fig. 5).

In addition, MST1 has been reported to inhibit Akt activity through direct interaction (39). Thus, this suggests that a feedback regulation loop exists between Akt and MST1, to keep a balance between Akt and MST1 in the cells. Furthermore, a recent study showed that Akt also phosphorylates Thr\textsuperscript{387} at the C-terminal region of MST1 to abrogate MST1 function (40). Thr\textsuperscript{387}, unlike Thr\textsuperscript{120}, is not conserved in Hippo and found only in mammalian cells. Because Akt inhibits the nuclear translocation and the kinase activity of C-terminal-truncated form MST1 (MST1\textsubscript{ΔC}), which lacks Thr\textsuperscript{387}, and because pMST1-Thr\textsuperscript{120} was detected in primary tumor cells expressing hyperactive Akt, we concluded that Akt1 regulates MST1 primarily through phosphorylation of Thr\textsuperscript{120}.

Previous studies have shown that MST1 cleavage is a critical step for the activation of MST1 (6, 41). The two caspase cleavage sites of MST1, Asp\textsuperscript{326} and Asp\textsuperscript{349}, have been reported. The mutation of these cleavage sites attenuates MST1 kinase activity, nuclear translocation, and ability to induce apoptosis (3, 41). Furthermore, it has been reported that MST1 promotes cell death through regulation of multiple targets, including phos-
phorylation of LATS1/2, histone H2B, FOXO3a, as well as induction of JNK and caspase-3 activation (3, 32). Interestingly, caspase-3 also acts as the upstream activator of MST1 through cleavage of the C-terminal regulatory domain primarily at Asp326. As a result, an active catalytic N-terminal region (e.g. MST1/H9004C) is generated and accumulated in the nucleus due to lack of nuclear exporting sequence motifs (Fig. 3A). However, full activation of MST1 requires both caspase-mediated cleavage and autophosphorylation (42). Taken collectively, we proposed the model that the phosphorylation of MST1-Thr120 by Akt inhibits MST1 conformational change and autophosphorylation (e.g. p-Thr183). Therefore, caspase will not be activated by MST1 to feedback cleave MST1 (Fig. 7). With regard to the cleavage form of MST1ΔC, which lacks nuclear export sequence, constitutively activated Akt blocks its nuclear translocation through phosphorylation of Thr120 and inactivation of its kinase activity (supplemental Figs. S2 and S3).

Raf-1 has been shown to bind to and suppress MST2 by preventing MST2 dimerization as well as recruiting a phosphatase in an Raf-1 kinase-independent manner (43). Raf-1 also binds ASK1 (apoptosis signal-regulating kinase-1), another pro-apoptotic kinase, and inhibits its activity independently of its catalytic activity (44). We and others have previously shown that ASK1 activation is inhibited by Akt through direct phosphorylation of ASK1-Ser83 (27). In addition, Thr120 is conserved in MST1 and MST2, and Akt also phosphorylates MST2 (data not shown). Collectively, these studies suggest that Raf-1 functions as a scaffolding protein for MST and ASK1, whereas Akt regulates these proteins through a phosphorylation-dependent mechanism.

Several genetic studies have implicated the involvement of the Hippo/MST pathway in oncogenesis (10, 45). Recent reports have shown down-regulation of MST1 and MST2 in soft tissue sarcoma and colorectal carcinoma (46, 47). However, the total protein expression level of MST1 or MST2 does not correlate with disease progression. Although MST1 kinase activity can be up-regulated by nuclear translocation, the loss of cytoplasmic MST1 is not associated with late stage and shortened survival in colorectal cancer (46). The importance of loss of cytoplasmic MST1 needs to be further examined in a large number of human tumor samples (46). Our study indicates that the correlation between pMST1-Thr120 and pMST1-Thr183 could be a valuable prognostic marker in human ovarian cancer (Fig. 6D). Physiological importance of Akt phosphorylation of MST1-Thr120 needs to be further investigated in a knock-in mouse model.

Acknowledgments—We are grateful for assistance from the Tissue Procurement and DNA Sequence Core Facilities at H. Lee Moffitt Cancer Center. We also thank M. J. Birnbaum for Akt1-KO MEF.

REFERENCES
1. Creasy, C. L., Ambrose, D. M., and Chernoff, J. (1996) J. Biol. Chem. 271, 21049–21053
2. Graves, J. D., Gotoh, Y., Draves, K. E., Ambrose, D., Han, D. K., Wright, M., Chernoff, J., Clark, E. A., and Krebs, E. G. (1998) EMBO J. 17, 2224–2234
3. Lee, K. K., Murakawa, M., Nishida, E., Tsubuki, S., Kawashima, S., Saka- maki, K., and Yonehara, S. (1998) Oncogene 16, 3029–3037
4. Taylor, L. K., Wang, H. C., and Erikson, R. L. (1996) Proc. Natl. Acad. Sci. U.S.A. 93, 10099–10104
Supplemental Figures

Figure S1. CD95/FasL-induced MST1 cleavage and cell death are inhibited by the IGF-I signaling pathway. HeLa cells were treated with or without IGF-1 (50 μM) and/or LY294002 (20 μM) prior to addition of CD95/FasL. Following 3 h incubation, the cells were subjected to immunoblotting analysis with indicated antibodies (top) and annexin V/FACS assay (bottom).

Figure S2. Staurosporine induces WT- MST1 and MST1-T120A but not MST1-T120D nuclear translocation. COS7 cells were transfected with GFP-MST1-WT, -MST1-T120A and -MST1-T120D, and then treated with or without STS (300 μM). Subcellular localization of GFP-MST1s was analyzed and quantified.

Figure S3. Kinase activity of different forms of C-terminal truncated MST1. COS cells were transfected with Flag-WT-MST1ΔC, -MST1-T120AΔC or -MST1-T120DΔC. After 36 h of transfection, cells were lysed and immunoprecipitated with anti-Flag antibody. The immunoprecipitates were subjected to kinase assay using histone H2B as substrate (top). Bottom panel shows expression of transfected plasmids.
Sup. Figure S2
Phosphoinositide 3-Kinase/Akt Inhibits MST1-Mediated Pro-apoptotic Signaling through Phosphorylation of Threonine 120
Zengqiang Yuan, Donghwa Kim, Shaokun Shu, Junbing Wu, Jianping Guo, Lei Xiao, Satoshi Kaneko, Domenico Coppola and Jin Q. Cheng

J. Biol. Chem. 2010, 285:3815-3824.
doi: 10.1074/jbc.M109.059675 originally published online November 24, 2009

Access the most updated version of this article at doi: 10.1074/jbc.M109.059675

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2009/12/15/M109.059675.DC1.html

This article cites 47 references, 22 of which can be accessed free at
http://www.jbc.org/content/285/6/3815.full.html#ref-list-1

WITHDRAWN
September 20, 2016