Both Phosphorylation and Caspase-mediated Cleavage Contribute to Regulation of the Ste20-like Protein Kinase Mst1 during CD95/Fas-induced Apoptosis*

Received for publication, December 4, 2000, and in revised form, January 12, 2001 Published, JBC Papers in Press, February 13, 2001

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The serine/threonine kinase Mst1, a mammalian homolog of the budding yeast Ste20 kinase, is cleaved by caspase-mediated proteolysis in response to apoptotic stimuli such as ligation of CD95/Fas or treatment with staurosporine. Furthermore, overexpression of Mst1 induces morphological changes characteristic of apoptosis in human B lymphoma cells. Mst1 may therefore represent an important target for caspasas during cell death which serves to amplify the apoptotic response. Here we report that Mst1 has two caspase cleavage sites, and we present evidence indicating that cleavage may occur in an ordered fashion and be mediated by distinct caspsases. We also show that caspase-mediated cleavage alone is insufficient to activate Mst1, suggesting that full activation of Mst1 during apoptosis requires both phosphorylation and proteolysis. Another role of phosphorylation may be to influence the susceptibility of Mst1 to proteolysis. Autophosphorylation of Mst1 on a serine residue close to one of the caspase sites inhibited caspase-mediated cleavage in vitro. Finally, Mst1 appears to function upstream of the protein kinase MEKK1 in the SAPK pathway. In conclusion, Mst1 activity is regulated by both phosphorylation and proteolysis, suggesting that protein kinase and caspase pathways work in concert to regulate cell death.

Apoptosis, or programmed cell death, is an active process fundamental to the development and homeostasis of multicellular organisms (for review, see Ref. 1). For example, apoptosis of lymphocytes is essential for both eliminating self-reactive cells during development and regulating clonal populations during and after an immune response (for review, see Ref. 2). The decision made by a cell to undergo apoptosis is influenced by many factors. Signals from death receptors such as CD95/Fas and chemical stressors such as staurosporine induce apoptosis, whereas growth factor and cytokine receptors, such as the cytokine interleukin-2 (IL-2)1 promote cell survival (for review, see Refs. 3 and 4).

The critical effectors of the apoptotic response are a family of aspartate-specific cysteine proteases, called caspases (for review, see Ref. 5). Caspases exist as inactive zymogens and are activated in a proteolytic cascade. Recently, it has become clear that caspases target critical components of signaling pathways which normally regulate cell growth and development (for review, see Ref. 6). It is these substrates, and not caspases themselves, that are the true apoptotic effectors. Thus, identifying caspase targets and determining the effect of caspase-mediated cleavage on their activity will be critical in understanding the biochemical mechanisms of apoptosis. Among the caspase substrates that have been identified are several protein kinases, suggesting that phosphorylation/dephosphorylation mechanisms may play an important role in the induction or progression of apoptosis. However, very little is known about protein kinases as potential apoptotic effectors. Even though some protein kinases have been shown to be capable of inducing apoptosis upon overexpression, the precise mechanism by which these kinases induce apoptosis is unclear (for review, see Refs. 7 and 8).

Mst1 (mammalian sterile 20-like kinase 1) is a ubiquitously expressed serine/threonine kinase that belongs to a growing family of protein kinases that are homologous to yeast Ste20 and which regulate diverse cellular processes such as morphogenesis, stress responses, and proliferation (9–11; for review, see Ref. 12). One subfamily of Ste20-like kinases comprises the mammalian p21-activated protein kinases (PAKs), which directly interact with and are regulated by the small GTPases Rac and Cdc42. The other subgroup, which includes Mst1, Mst2, Khs, Gek, Sok1, Nik, Hpk1, and Sps1, lack a domain for interacting with GTPases (for review, see Ref. 13). The carboxyl terminus of Mst1 mediates dimerization and appears to serve an inhibitory function (14). Despite considerable effort on the part of several laboratories, the only stimuli that have been shown to regulate Mst1 are cellular stresses and apoptotic stimuli.

Mst1 is cleaved and activated during apoptosis induced by stimuli that include ligation of CD95/Fas and treatment with staurosporine or genotoxic agents (15–17). We have also shown that Mst1 can induce caspase activity and apoptosis upon overexpression. Mst1 activity, although not required for apoptosis induced by ligation of CD95/Fas, is required for apoptosis in response to certain genotoxic agents (15). Collectively, these

kinase kinase; SAPK, stress-activated protein kinase; MBP, myelin basic protein; HA, hemagglutinin; DT1, dithiothreitol; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; MAPK, mitogen-activated protein kinase.

* This work was supported by National Institutes of Health Grants R01GM58487 and R01AI44250. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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§ The abbreviations used are: IL-2, interleukin-2; Mst1, mammalian sterile 20-like kinase 1; PAK, p21-activated protein kinase; MEKK, mitogen-activated protein kinase/extracellular signal-regulated kinase.
Regulation of Mst1 during CD95/Fas-induced Apoptosis

Reagents and Cells—The anti-Fas monoclonal antibody IPO-4 (22) was kindly provided by Dr. Svetlana Sidorenko. Staurosporine and histone H1 were obtained from Sigma. Myelin basic protein (MBP) was prepared from bovine brain as described previously (23). ZVAD-fmk was purchased from the Kamiya Biomedical Company (Tukwila, WA). Rabbit polyclonal antibodies specific for the amino terminus of Mst1 have been described previously (15). The 9E10 monoclonal antibody specific for the Myc epitope tag was obtained from the American Type Culture Collection (Rockville, MD). The anti-hemagglutinin (HA) monoclonal antibody 12CA5 was obtained from Roche Molecular Biochemicals. The BJAB human B lymphoma line was generously provided by Dr. Virginia Dietz Schenk (Genentech, South San Francisco, CA). The 293T cell line was kindly provided by Dr. Jonathan Cooper (FHCRC, Seattle, WA).

Plasmids—Myc-tagged Mst1 constructs in the pcMV5M expression vector were constructed as described (9, 14, 15). Mst1 D326N, Δ326, Δ349E, Δ326N/Δ349E, S327E, and S327G were constructed by polymerase chain reaction mutagenesis. pSRα456-HA-SAPKα, Ask1, and Tak1 were constructed as described previously (24).

RESULTS

Mst1 Has Two Caspase Sites—We and others have shown that Mst1, which normally migrates at 61 kDa, is cleaved in response to a variety of apoptotic stimuli to generate 36-kDa or 41-kDa cleavage products. The appearance of the 36-kDa Mst1 center panel (Fig. 1, left panels) corresponds to the 41-kDa species. This suggests that Mst contains two caspase cleavage sites that may be catalytically active (Fig. 1, lower panels). These findings suggest that Mst contains two caspase cleavage sites that generate 36-kDa or 41-kDa cleavage products.

To test whether two caspase sites exist in Mst1, we transfected BJAB B cells with either wild-type Mst1 or mutants in which the site responsible for generation of the 36-kDa species had been mutated to render it resistant to caspase-mediated cleavage (Mst1 D326N, Fig. 2A). Fig. 2B shows that transfection of wild-type Mst1, both full-length Mst1 and a band corresponding to the 36-kDa species were observed. The intensity of Western blotting, as described above, or were prepared for immunohistochemical analysis.

Cotransfection and Kinase Assays—293T cells were transfected in 6-well plates with 4 μg of DNA. After incubating appropriate aliquots of LipofectAMINE (Life Technologies, Inc.) for 10 h according to the manufacturer’s instructions, 24 h after transfection, cells were harvested and lysed in buffer (20 mM Tris-Cl, pH 7.5, 10 mM β-glycerophosphate, 5 mM EGTA, 10 mM NaF, 1 mM Na3P04, 150 mM NaCl, 1% Nonidet P-40, 4 mM DTT, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, and 20 mg/ml aprotinin). After being clarified by centrifugation at 15,000 × g for 30 min, the supernatants were incubated with anti-HA or anti-Myc antibody and protein G-Sepharose for 3 h. The beads were washed four times with lysis buffer and once with wash buffer (20 mM Tris, pH 7.5, 2 mM EGTA, 1 mM DTT). Kinase assays were carried out by incubating immunoprecipitates with the appropriate substrate in kinase buffer (20 mM Tris, pH 7.5, 10 mM MgCl2, and 100 μM [γ-32P]ATP, 0.1 μCi) for 10 min at 30 °C. After 20 min, the reactions were stopped by the addition of 4 × Laemmli sample buffer and subjected to SDS-polyacrylamide gel electrophoresis prior to drying and autoradiography.

Data Presentation—All data shown are representative of at least three separate experiments.
the Mst1 D326N mutant was resistant to cleavage at the DEMD326S site, it was still cleaved at a second caspase site. Based on molecular mass we predicted that a likely cleavage site for generation of the 41-kDa band was aspartate 349 within the potential caspase consensus sequence of TMTD349G (Fig. 2A). To test this hypothesis we generated mutants of Mst1 in which this site was mutated alone (Mst1 D349E) or in combination with the first site (Mst1 D326N/D349E). As predicted, the Mst1 D349E mutant behaved like wild-type Mst1, yielding a 36-kDa cleavage product that was stimulated by anti-Fas (Fig. 2B). However, the Mst1 D326N/D349E double mutant was completely resistant to caspase-mediated cleavage in either the absence or presence of anti-Fas. These results confirm that Mst1 contains two caspase cleavage sites at DEMD326S and TMTD349G (Fig. 2A). The fact that Mst1 wild-type, D326N, and D349E are equally sensitive to cleavage argue against a model in which proteolysis at one site is required before cleavage at the other.

Differential Caspase Sensitivity of Mst1 Cleavage Sites—The fact that Mst1 contains two caspase cleavage sites raised the possibility that each site is cleaved by different caspases. To test this possibility we examined the ability of recombinant purified caspase 3, 6, 7, or 9 to cleave in vitro labeled Mst1 with each of the sites. As expected, the Mst1 D349E mutant behaved like wild-type Mst1, yielding a 36-kDa cleavage product that was stimulated by anti-Fas (Fig. 2B). However, the Mst1 D326N/D349E double mutant was completely resistant to caspase-mediated cleavage in either the absence or presence of anti-Fas. These results confirm that Mst1 contains two caspase cleavage sites at DEMD326S and TMTD349G (Fig. 2A). The fact that Mst1 wild-type, D326N, and D349E are equally sensitive to cleavage argues against a model in which proteolysis at one site is required before cleavage at the other.

Role of Phosphorylation in Regulating Mst1 Activity during

FIG. 1. BJAB cells were treated with either 1 μg/ml anti-CD95/Fas (left panels) or 100 nM staurosporine (middle panels). CTLL-2 cells were washed and resuspended in medium without IL-2 (right panels). At the indicated times cell extract was prepared, and Western blotting with Mst1 antiserum (upper panels) or MBP in-gel kinase assays (lower panels) was performed as described under “Experimental Procedures.” The percentage of cells undergoing apoptosis was determined at each time point by annexin V binding assay.

FIG. 2. A, structural organization of Mst1 indicating the positions of the two caspase cleavage sites, the autophosphorylation site, and the various mutants of Mst1 employed in these studies. B, BJAB cells were transiently transfected with 10 μg of Myc-tagged Mst1, Mst1 D326N, Mst1 D349E, or Mst1 D326N/D349E. 12 h after transfection, the cells were either untreated or treated with 1 μg/ml anti-CD95/Fas for a further 10 h. Cell extract was prepared and exogenous Mst1 visualized by Western blotting with anti-Myc. wt., wild-type.

the Mst1 D326N mutant was resistant to cleavage at the DEMD326S site, it was still cleaved at a second caspase site. Based on molecular mass we predicted that a likely cleavage site for generation of the 41-kDa band was aspartate 349 within the potential caspase consensus sequence of TMTD349G (Fig. 2A). To test this hypothesis we generated mutants of Mst1 in which this site was mutated alone (Mst1 D349E) or in combination with the first site (Mst1 D326N/D349E). As predicted, the Mst1 D349E mutant behaved like wild-type Mst1, yielding a 36-kDa cleavage product that was stimulated by anti-Fas (Fig. 2B). However, the Mst1 D326N/D349E double mutant was completely resistant to caspase-mediated cleavage in either the absence or presence of anti-Fas. These results confirm that Mst1 contains two caspase cleavage sites at DEMD326S and TMTD349G (Fig. 2A). The fact that Mst1 wild-type, D326N, and D349E are equally sensitive to cleavage argues against a model in which proteolysis at one site is required before cleavage at the other.

Differential Caspase Sensitivity of Mst1 Cleavage Sites—The fact that Mst1 contains two caspase cleavage sites raised the possibility that each site is cleaved by different caspases. To test this possibility we examined the ability of recombinant purified caspase 3, 6, 7, or 9 to cleave in vitro labeled, [35S]methionine-labeled Mst1 at either site in vitro (Fig. 3). To control for differences in specific activity among the different caspase preparations, we carried out preliminary dose-response experiments to establish conditions under which each caspase had similar activity with respect to wild-type Mst1 (data not shown). All four caspases cleaved wild-type Mst1 and Mst1 D349E to generate a 36-kDa band. However, under these conditions, only caspase 6 and 7 could cleave the Mst1 D326N mutant. Consistent with our expression studies, Mst1 D326N/D349E was completely resistant to cleavage in vitro. These data suggest that caspases differ in their preferences for cleavage at the two sites in Mst1 and support the idea that these sites might be targeted by different caspases in vivo.
was included in the kinase assay buffer. Incubation with prosaurosporine for either 30 min or 8 h. Treatment with anti-Fas in vitro cleavage with caspase 3 further stimulated Mst1 activity by 4-fold compared with untreated cells treated with either anti-Fas or staurosporine for long or short time points (Fig. 4B). In contrast, protein phosphatase 1 had no effect on Mst1 activity, although it did inactivate SAPK in immune complex kinase assays (data not shown). These findings indicate that phosphorylation is absolutely required for Mst1 activity regardless of its state of proteolysis.

To explore further the relationship among cleavage, phosphorylation, and activation of Mst1 during apoptosis, we transiently transfected BJAB cells with various Mst1 mutants and measured their kinase activity in vitro in the presence or absence of anti-Fas. Comparable expression levels of wild-type Mst1, truncated Mst1 D326, Mst1 D326N, Mst1 D349E, and protease-resistant Mst1 D326N/D349E were evident by Western blotting (Fig. 5A, upper panel). Treatment with anti-Fas increased the kinase activity of wild-type Mst1 by about 4-fold (Fig. 5A, lower panel). In contrast, Mst1 D326 exhibited a high basal activity that was not enhanced significantly by Fas ligation. However, the activity of the protease-resistant Mst1 D326N and D349E mutants was stimulated by 3-fold after treatment with anti-Fas. In contrast, the kinase activity of the double protease-resistant form of Mst1 (D326N/D349E) was only weakly activated upon anti-Fas treatment (Fig. 5). These results indicate that cleavage is required for the full activation of Mst1 during apoptosis.

**Autophosphorylation of Mst1**—During these experiments we observed autophosphorylation of Mst1 (Fig. 5B). Interestingly,
Regulation of Mst1 during CD95/Fas-induced Apoptosis

One possible autophosphorylation site is serine 327. Because this residue is adjacent to the DEMD\(^{326}\)S cleavage site, its state of phosphorylation might influence Mst1 cleavage. To test this possibility we constructed mutants of Mst1 in which serine 327 was mutated to either a glycine (Mst1 S327G) or a glutamic acid (Mst1 S327E). Relative to wild-type Mst1, these mutants displayed a reduced ability to autophosphorylate in vitro (Fig. 6B, upper panel). Wild-type Mst1 that had been allowed to autophosphorylate was less sensitive to cleavage in vitro by caspase 3 than Mst1 that was not autophosphorylated (Fig. 6B). In contrast, Mst1 S327G retained its sensitivity to cleavage, whereas Mst1 S327E was constitutively resistant to cleavage. These findings suggest that serine 327 is a major in vitro Mst1 autophosphorylation site and that its state of phosphorylation may influence the sensitivity of Mst1 to cleavage at the DEMD\(^{326}\)S site. Interestingly, the recently identified Ste20 homolog SPAK, which is a close relative of Mst1, contains a putative caspase cleavage site in which the amino acid following the critical aspartate is a glutamic acid (25). Whether SPAK is a less efficient caspase target as a consequence of there being a glutamic acid residue at this position remains to be determined.

**MEKK1 Is a Downstream Target of Mst1**—Another important question concerns the downstream targets of Mst1. Because other mammalian Ste20 homologs have been shown to function as MAPKKKKs in MAPK cascades, we tested the ability of Mst1 to activate components of the SAPK pathway in a transient coexpression system. We have shown previously that coexpression of wild-type Mst1 activated SAPK and the MAPKKK MKK7 (15). To localize more closely the position in the SAPK pathway at which Mst1 functions, we cotransfected Mst1 with SAPK and kinase-dead mutants of several MAPKKKs known to function in this pathway. Interestingly, kinase-dead MEKK1, but not kinase-dead Ask1 or Tak1, blocked the ability of Mst1 to activate SAPK (Fig. 7). These results are consistent with Mst1 functioning as a MAPKKKK, upstream of MEKK1, in the SAPK pathway.

**DISCUSSION**

Previous studies have identified a single caspase cleavage site in Mst1 (DEMD\(^{326}\)S) which yielded a 36-kDa catalytically active fragment (15–17). More recently, several lines of evidence led us to conclude that a second site closer to the carboxyl terminus of Mst1 also exists. For example, withdrawal of IL-2 from CTLL-2 cells, which are dependent on this growth factor for their survival, results in the appearance of a 41-kDa species that preceded that of the 36-kDa cleavage product by about 2–3 h (Fig. 1). A 41-kDa band was also detected at later time points in staurosporine but not anti-Fas-treated BJAB cells. Consistent with a second cleavage site in Mst1 is the observation that a mutant of Mst1 resistant to cleavage at the primary cleavage site (Mst1 D326N) is proteolyzed to generate a band that corresponds to the 41-kDa Mst1 species in response to anti-Fas treatment. Further mutational analyses identified the second cleavage site as being 23 amino acids carboxyl-terminal of the DEMD\(^{326}\)S site at a TMTD\(^{349}\)G sequence (see Fig. 2A). Specifically, a double Mst1 D326N/V349E mutant was found to be completely resistant to cleavage upon expression in and anti-Fas treatment of BJAB cells.

To reconcile these findings with previous results as well as to analyze the caspase specificity of the two sites, we subjected various Mst1 mutants to cleavage by caspase 3, 6, 7, or 9 in vitro. Caspases 3 and 9 efficiently cleaved Mst1 at the DEMD\(^{326}\)S site but were relatively inefficient at cleaving Mst1 at TMTD\(^{349}\)G (Fig. 3). In contrast, caspases 6 and 7 cleaved Mst1 at either site. The TMTD\(^{349}\)G site is closer in sequence to the caspase 6 consensus sequence than the caspase 3 consensus predicted by combinatorial peptide analysis (26). These find-
ings raise the possibility that the two sites in Mst1 may be targeted by different caspases. In addition, the resistance of the TMTD^{449}G site to proteolysis by caspase 3 provides an explanation for our original identification of a single cleavage site.

These results raise the issue of why, if Mst1 contains two caspase sites, is only one cleavage product observed in response to anti-CD95/Fas? There are several possible explanations for this. Because the two cleavage sites may be targeted by different caspases, anti-CD95/Fas may lead to activation of caspases that preferentially generate the 36-kDa species while stimuli such as IL-2 withdrawal may induce a different pattern of caspase specificities. A variation on this hypothesis is that there may be a kinetic difference between cleavage at the two sites such that the 41-kDa species is a transient intermediate. Although the time course of appearance of the 41-kDa band relative to the 36-kDa band in response to withdrawal of IL-2 is consistent with the 41-kDa form being an intermediate cleavage product, we have no definitive evidence that ordered proteolysis occurs at these two sites in response to other stimuli such as anti-CD95/Fas. In addition, the fact that this site is not conserved in Mst2 raises the possibility that Mst1 and Mst2 are targeted differentially during apoptosis.

The carboxyl terminus of Mst1 has been shown to exert a negative regulatory influence upon the kinase domain (14). Although removal of this domain by caspase-mediated cleavage correlates with increased activation of Mst1, we wanted to understand the relative contributions that caspase-mediated proteolysis and phosphorylation/dephosphorylation might have to the activation of Mst1 during apoptosis. This objective was complicated by the fact that no physiological stimulus regulating Mst1 in the absence of caspase cleavage has been identified. However, because Mst1 is activated but not proteolyzed upon short term treatment with staurosporine, we used staurosporine as a pharmacological activator of Mst1. Cleavage with recombinant caspase 3 in vitro enhanced the activity of Mst1 from staurosporine-treated cells but not untreated cells (Fig. 4). These findings suggest that in addition to caspase-mediated cleavage, Mst1 may also require phosphorylation on critical regulatory residues to be fully activated. Consistent with this hypothesis, Mst1 activated by either staurosporine or anti-Fas treatment can be inactivated by treatment with protein phosphatase 2A. Our transfection studies with various Mst1 mutants provide further support for this model. The kinase activities of wild-type Mst1, Mst1 D326N, and Mst1 D349E were all stimulated by anti-Fas. However, the cleavage-resistant mutant (Mst1 D349E) was only slightly stimulated. Thus, both phosphorylation and caspase cleavage contribute to the activation of Mst1 during apoptosis. A similar requirement for both phosphorylation and proteolysis has been shown for γ-PAK/PAK-2 (19).

It is important to note that in addition to contributing to the activation of Mst1, removal of the regulatory domain is likely to influence other biochemical characteristics of the kinase. For example, the carboxyl terminus of Mst1 contains a dimerization domain and a putative nuclear exclusion sequence. Thus, protein-protein interactions, subcellular localization, and substrate specificity of Mst1 are also likely to be altered upon cleavage by caspases. The contribution of these factors to the ability of Mst1 to induce apoptosis is currently being investigated.

Mst1 autophosphorylates in vitro on a site within the regulatory domain, located between the two caspase sites. This region of Mst1 contains several potential phosphorylation sites, including a serine residue (serine 327) within the DEMD^{326}S caspase consensus site. Our analyses with S327E and S327G mutants of Mst1 indicate that serine 327 is a major in vitro autophosphorylation site and that phosphorylation at this residue renders Mst1 resistant to cleavage by caspase 3. A precedent for phosphorylation of a caspase target regulating its cleavage is provided by the inhibitor of nuclear factor-kB (I-κBα). After phosphorylation at sites close to its caspase cleavage site, I-κBα is resistant to caspase cleavage in vitro (27). Although the physiological role of Mst1 phosphorylation at serine 327 is unclear, one possibility is that this might favor generation of 41-kDa Mst1 species rather than 36-kDa form. This may provide an alternative explanation for why the 36 kDa form is observed in response to IL-2 withdrawal but not anti-CD95/Fas treatment. We are currently investigating whether Mst1 is phosphorylated on serine 327 in cells and whether autophosphorylation is mediated in trans within Mst dimers.

Another important question concerns the identity of the downstream targets of Mst1 under apoptotic and/or non-apoptotic circumstances. The ability of Mst1 to activate the SAPK and p38 MAPK pathways upon coexpression suggests that components of these MAPK pathways might be important effectors of Mst1. In this respect, our cotransfection studies indicated that Mst1 may function upstream of MEKK1 to activate SAPK. Interestingly, Cardone et al. (28) showed that caspase cleavage of MEKK1 from non-apoptotic cells did not activate MEKK1 to the same extent as MEKK1 from cells that were undergoing apoptosis. These data are consistent with a requirement for activation of MEKK1 by phosphorylation in addition to caspase-mediated cleavage. Taken together with our previous findings, Mst1 may function as part of an apoptotic Mst1/MEKK1/MAPKK7/SAPK signaling cassette. According to
this model, Mst1 might directly phosphorylate MEKK1 and thereby contribute to its activation by caspase-mediated cleavage. Thus, rather than rendering these protein kinases independent of their normal upstream activators, caspases may exploit the amplification inherent within this protein kinase cascade to transduce their apoptotic signal.

Although Mst1 is capable of inducing caspase activity and apoptosis upon overexpression, its normal cellular role remains unclear. In this respect, a recent study has reported a 33-kDa MBP-in gel kinase activity, referred to as p33 quiescence-activated kinase (p33QIK), which is active during G0/G1 and inactivated as cells enter the cell cycle (29). This kinase is recognized by anti-Mst antibodies and may be either a novel proteolytic product of Mst1/2, an alternative splice product of the Mst1/2 genes, or a separate gene product. The idea that Mst1 might directly phosphorylate MEKK1 and thereby contribute to its activation by caspase-mediated cleavage and phosphorylation, Mst1 would contribute to the induction or progression of apoptosis.

Acknowledgments—We thank Taunya Miller for technical assistance, Jonathan Chernoff for Mst1 reagents and advice, and Mark Mumby for recombinant protein phosphatase 2A.

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J. Biol. Chem. 2001, 276:14909-14915.
doi: 10.1074/jbc.M010905200 originally published online February 13, 2001

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