Caspase-mediated activation and induction of apoptosis by the mammalian Ste20-like kinase Mst1

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Mst1 is a ubiquitously expressed serine–threonine kinase, homologous to the budding yeast Ste20, whose physiological regulation and cellular function are unknown. In this paper we show that Mst1 is specifically cleaved by a caspase 3-like activity during apoptosis induced by either cross-linking CD95/Fas or by staurosporine treatment. CD95/Fas-induced cleavage of Mst1 was blocked by the cysteine protease inhibitor ZVAD-fmk, the more selective caspase inhibitor DEVD-CHO and by the viral serpin CrmA. Caspase-mediated cleavage of Mst1 removes the C-terminal regulatory domain and correlates with an increase in Mst1 activity *in vivo*, consistent with caspase-mediated cleavage activating Mst1. Overexpression of either wild-type Mst1 or a truncated mutant induces morphological changes characteristic of apoptosis. Furthermore, exogenously expressed Mst1 is cleaved, indicating that Mst1 can activate caspases that result in its cleavage. Kinase-dead Mst1 did not induce morphological alterations and was not cleaved upon overexpression, indicating that Mst1 must be catalytically active in order to mediate these effects. Mst1 activates MKK6, p38 MAPK, MKK7 and SAPK in co-transfection assays, suggesting that Mst1 may activate these pathways. Our findings suggest the existence of a positive feedback loop involving Mst1, and possibly the SAPK and p38 MAPK pathways, which serves to amplify the apoptotic response.

Keywords: apoptosis/caspase/protein kinase

Introduction

Apoptosis, or programmed cell death, is an active process that is fundamental to the development and homeostasis of multicellular organisms (reviewed in Jacobson *et al.*, 1997). It is characterized by dramatic cellular alterations,

particularly membrane blebbing, cell shrinkage, chromosome condensation and fragmentation of DNA (reviewed in Dive *et al.*, 1992). Apoptosis can be triggered by a wide variety of cellular stresses, including DNA damage, UV radiation, ionizing radiation, heat shock and oxidative stress, as well as by extracellular stimuli acting through cell-surface receptors (reviewed in Nagata, 1997). Central to the apoptotic execution pathway are a family of cysteine proteases, termed caspases, which are homologous to the *Caenorhabditis elegans* death gene *ced-3* and are expressed as inactive zymogens (Alnemri *et al.*, 1996; reviewed in Henkart, 1996). Ten mammalian caspases have now been identified and classified according to structure and substrate specificity. Overexpression studies have shown caspases to be capable of inducing all the characteristic features of apoptosis. Furthermore caspase inhibitors, such as the cowpox viral serpin CrmA, and cell permeable peptides such as ZVAD-fmk, block cell death induced by a wide variety of apoptotic agents (Ray *et al.*, 1992; Enari *et al.*, 1995, 1996; Los *et al.*, 1995; Tewari and Dixit, 1995).

The mechanism of activation of caspases is probably best understood for the Fas receptor. Fas (CD95/APO-1) is a transmembrane receptor belonging to the tumor necrosis factor (TNF) receptor family (Itoh *et al.*, 1991; Oehm *et al.*, 1992). Cross-linking Fas with Fas-ligand or agonistic antibodies results in rapid apoptosis of many cell types (reviewed in Nagata and Golstein, 1995). Although the role of Fas in other tissues is not clear, Fas-induced apoptosis plays an important role in the development of the lymphoid system and the maturation of the immune response (Brunner *et al.*, 1995; Dhein *et al.*, 1995; Rathmell *et al.*, 1996). The intracellular domain of the Fas receptor contains a death domain, which is required for the induction of programmed cell death (Itoh and Nagata, 1993). The activated Fas receptor recruits other death domain-containing proteins including the adapter protein FADD/MORT-1, which in turn recruits caspase 8 via another dimerization domain termed the death effector domain (Chinnaiyan *et al.*, 1995; Boldin *et al.*, 1996; Muzio *et al.*, 1996). Upon recruitment to activated Fas receptors, caspase 8 undergoes autoproteolytic activation. Active caspase 8 then cleaves and activates downstream caspases including caspases 1, 4 and 5, which in turn activate terminal or effector caspases such as caspases 3, 6 and 7 (Enari *et al.*, 1996; Srinivasula *et al.*, 1996). This cascade of sequential autoproteolytic events apparently transmits and amplifies the apoptotic signal (reviewed in Salvesen and Dixit, 1997).

Identification of caspase substrates is essential in order to understand how these proteases induce the phenotypes associated with apoptosis. Caspase substrates include components of cellular DNA repair mechanisms such as poly ADP-ribose polymerase (PARP) and DNA-dependent protein kinase (DNA-PK) (Lazebnik *et al.*, 1994; Casciola

Rosen *et al.*, 1995; Nicholson *et al.*, 1995); structural proteins such as actin, fodrin, lamin and gelsolin, which are likely to contribute to alterations in nuclear and cellular morphology (Cryns *et al.*, 1996; Orth *et al.*, 1996; Takahashi *et al.*, 1996; Kothakota *et al.*, 1997; Mashima *et al.*, 1997); and I κ B, an endogenous inhibitor of nuclear factor κ B (NF- κ B), suggesting one mechanism by which caspases may influence transcriptional events (Barkett *et al.*, 1997). Furthermore, caspases can cleave components of signal transduction pathways such as D4-GDI, a regulator of Rho family GTPases (Na *et al.*, 1996), the p21 activated kinase-2 (PAK2) (Rudel and Bokoch, 1997), the δ and θ isoforms of protein kinase C (PKC) (Emoto *et al.*, 1995; Datta *et al.*, 1997), PKC-related kinase 2 (PRK2) (Cryns *et al.*, 1997), focal adhesion kinase (FAK) (Crouch *et al.*, 1996) and MEKK1 (Cardone *et al.*, 1997). The fact that caspase cleavage of several of these protein kinases results in stimulation of their kinase activity suggests that, as with other cell fate decisions, protein phosphorylation/dephosphorylation mechanisms may play an important role in the initiation and progression of apoptosis.

Activation of the stress-activated protein kinase (SAPK) and p38 mitogen-activated protein kinase (p38 MAPK) pathways has been observed to correlate with apoptosis induced by a variety of agents, including nerve growth factor withdrawal, B-cell receptor cross-linking and Fas ligation (Xia *et al.*, 1995; Cahill *et al.*, 1996; Graves *et al.*, 1996; Wilson *et al.*, 1996; Juo *et al.*, 1997). Caspase inhibitors can block SAPK and p38 MAPK activation by Fas cross-linking, indicating that these pathways may function downstream of caspase activation (Cahill *et al.*, 1996; Juo *et al.*, 1997). However, overexpression of upstream components of the SAPK and p38 MAPK pathway such as MEKK1, which functions in the SAPK pathway, and MKK6b, an activator of p38 MAPK, can also induce caspase activity and cell death (Cardone *et al.*, 1997; Huang *et al.*, 1997). Although these results suggest that the SAPK and p38 MAPK pathways may function both upstream and downstream of caspases in the apoptotic response, the mechanism by which these pathways might influence caspase activity is unknown. Here we show that the ubiquitously expressed serine-threonine kinase Mst1, a mammalian homolog of the budding yeast Ste20 kinase, is cleaved and activated by caspase-mediated proteolysis in response to apoptotic stimuli. Overexpression of Mst1 induces caspase activity, morphological changes characteristic of apoptosis, and activates the SAPK and p38 MAPK pathways. Thus, Mst1 may function in a positive feedback pathway that amplifies the apoptotic response.

Results

During our analysis of CD95/Fas-induced apoptosis in the human B-lymphoma cell line BJAB, using myelin basic protein (MBP) in-gel kinase assays, we observed the induction of a 36 kDa kinase activity (Figure 1, upper panel). This kinase activity was first stimulated between 1 and 2 h after anti-Fas treatment, increased in a time-dependent fashion and persisted for at least 10 h. The kinetics of induction of this 36 kDa kinase paralleled the onset of apoptosis as determined by annexin V binding. In addition, the appearance of the 36 kDa activity correlated with the disappearance of a 63 kDa MBP in-

Fig. 1. Cleavage of Mst1 during Fas-induced apoptosis. BJAB cells were treated with 1 μ g/ml anti-Fas. Where indicated, cells were pretreated for 3 h with 50 μ M ZVAD-fmk. At the indicated times cell extract was prepared and MBP in-gel kinase assays (upper panel) and Western blotting with anti-Mst1 antiserum (lower panel) performed as described in Materials and methods. The percentage of cells undergoing apoptosis was determined at each timepoint by annexin V binding assay.

gel kinase activity. Based on these observations, we hypothesized that the 36 kDa in-gel kinase activity resulted from cleavage of a 63 kDa kinase by an apoptotic protease. Consistent with this theory, pretreatment of the cells with the cell-permeable cysteine protease inhibitor ZVAD-fmk blocked both the induction of the 36 kDa kinase activity and the decrease in the 63 kDa activity. Staurosporine, a potent inducer of apoptosis in many cells, also stimulated the appearance of a 36 kDa kinase activity in BJAB cells (Figure 2 upper panel). As with the response to anti-Fas, induction of the 36 kDa kinase activity correlated temporally with the onset of apoptosis and could be inhibited by pretreatment with ZVAD-fmk. However, in contrast to the effects of anti-Fas treatment, staurosporine led to an increase in the 63 kDa kinase activity prior to its degradation. This increase in activity was detectable within 1 h of staurosporine treatment and decreased in parallel with the appearance of the 36 kDa kinase activity. In the presence of ZVAD-fmk, persistent activation of the 63 kDa kinase was observed in response to staurosporine treatment.

In order to identify the 63 kDa kinase, we looked for a known kinase that migrated at this molecular mass, was renaturable, stimulated by staurosporine and sensitive to proteolysis yielding a catalytically active fragment of ~36 kDa. One candidate was Mst1, a ubiquitously expressed 63 kDa serine-threonine kinase, which was originally cloned by virtue of its homology to the yeast Ste20 kinase (Creasy and Chernoff, 1995a; Taylor *et al.*, 1996). Extract from BJAB cells was subjected to Western blotting using a polyclonal antibody raised against a peptide from the N-terminus of Mst1. This antibody recognized a single 63 kDa band in extract from unstimu-

Fig. 2. Cleavage of Mst1 during staurosporine-induced apoptosis. BJAB cells were treated with 100 nM staurosporine. Where indicated, cells were pre-treated for 3 h with 50 μ M ZVAD-fmk. At the indicated times cell extract was prepared and MBP in-gel kinase assays (upper panel) and Western blotting with anti-Mst1 antiserum (lower panel) performed as described in Materials and methods. The percentage of cells undergoing apoptosis was determined at each timepoint by annexin V binding assay.

lated BJAB cells (Figure 1, lower panel). Upon anti-Fas treatment, the 63 kDa band diminished in intensity in parallel with the accumulation of a 36 kDa immunoreactive band. These results suggest that Mst1 is rapidly cleaved to generate a 36 kDa fragment upon treatment of BJAB cells with anti-Fas mAb (Figure 1, lower panel). The kinetics of Mst1 proteolysis, as well as the molecular mass of Mst1 and its cleavage product, match those of the 63 kDa and 36 kDa in-gel kinase activities. A similar pattern of Mst1 cleavage was also observed in extract prepared from staurosporine-treated BJAB cells (Figure 2, lower panel). An antibody raised against the C-terminus of Mst1 recognized only the 63 kDa fragment indicating that cleavage of Mst1 results in removal of the C-terminus (data not shown). Pretreatment of cells with ZVAD-fmk, a broad specificity inhibitor of caspases, blocked proteolysis of Mst1 in response to either anti-Fas or staurosporine treatment (Figures 1 and 2, lower panels). The fact that Mst1 exhibited many of the characteristics of the 63 and 36 kDa in-gel kinase activities suggests that they might be identical. Additional studies, have provided confirmation that the 63 and 36 kDa in-gel kinase activities are Mst1 and a 36 kDa proteolytic product. Analysis of anion exchange chromatographic fractions by Western blotting with anti-Mst1 and MBP in-gel kinase assay revealed that immunoreactive Mst1 precisely co-eluted with the 63 and 36 kDa kinase activities (data not shown). Furthermore, the N-terminal Mst1 antibody specifically immunoprecipitates and depletes the 63 and 36 kDa MBP in-gel kinase activities from anti-Fas-treated BJAB extracts (data not shown).

Having established the identity of the MBP in-gel activities as Mst1 and implicated caspases in its proteolysis

during apoptosis, we further investigated the role of caspases in Mst1 cleavage. The cowpox virus serpin CrmA, which binds to and inhibits caspases 1 and 8, has previously been employed to implicate caspases in the cell death program (Ray *et al.*, 1992; Tewari and Dixit, 1995; Tewari *et al.*, 1995; Srinivasula *et al.*, 1996). BJAB cells transfected with either wild-type CrmA, an inactive point mutant termed CrmA p14, or a vector control were treated with anti-Fas. As previously reported, cells expressing CrmA, but not CrmA p14 or vector control, were resistant to anti-Fas-induced apoptosis (Tewari and Dixit, 1995; Figure 3A). Mst1 cleavage induced by Fas cross-linking, as determined by in-gel kinase assay and Western blotting, was also blocked in the CrmA-expressing cells but not those expressing CrmA p14 or vector control (Figure 3). The use of peptide caspase inhibitors that are more selective than ZVAD-fmk has allowed further characterization of the caspases responsible for specific apoptotic events. Such inhibitors include DEVD-CHO, which is relatively selective for caspase 3 and its related subfamily (Nicholson *et al.*, 1995), and YVAD-CHO, which is relatively selective for caspase 1 and its subfamily (Thornberry *et al.*, 1992). Preincubation of BJAB cells with 100 μ M DEVD-CHO almost completely blocked Fas-induced apoptosis and Mst1 cleavage as determined by both in-gel kinase activity and Western blotting (Figure 4). By comparison, YVAD-CHO was an inefficient inhibitor of Mst1 cleavage. Even at a concentration of 100 μ M, YVAD-CHO only partially inhibited Mst1 cleavage induced by anti-Fas (Figure 4). These results suggest that Mst1 is cleaved *in vivo* by a DEVD-sensitive caspase such as caspase 3.

Analysis of the primary structure of Mst1 revealed an amino acid sequence that corresponds to a potential consensus caspase recognition site (DEMD³²⁶S) at the junction of the N-terminal catalytic domain and C-terminal regulatory domain (Figure 5A). To determine whether this site is the main caspase cleavage site in Mst1 we constructed a D326N mutant (DEMN³²⁶S) and used an *in vitro* assay system. Incubation of *in vitro*-translated, ³⁵S-labeled, wild-type Mst1 with either apoptotic extract or caspase 3 resulted in the generation of a 36 kDa fragment that corresponded to the Mst1 fragment observed *in vivo* (Figure 5B left panel). In contrast, the D326N mutant was completely resistant to cleavage *in vitro* by either apoptotic extract or recombinant caspase 3 (Figure 5B right panel). These results establish the caspase cleavage site in Mst1 as being between aspartic acid 326 and serine 327 in the sequence DEMDS. The similarity between the cleavage patterns obtained *in vivo* and *in vitro* with recombinant caspase 3 lends further support to the idea that Mst1 cleavage is mediated by caspase 3 or a member of its related subfamily during apoptosis *in vivo*.

A major question concerns the effect of cleavage at this site on Mst1 catalytic activity. Interestingly, deletion analysis of Mst1 has shown that removal of the regulatory domain results in an activated kinase fragment. Although our in-gel data appear to suggest that the Mst1 cleavage product is more active than full-length Mst1, we wanted to determine more directly whether Mst1 is activated by caspase-mediated cleavage. In order to do this, we immunoprecipitated Mst1 from BJAB cells that were either unstimulated or treated with anti-Fas and performed

Fig. 3. CrmA blocks Mst1 cleavage. BJAB cells expressing either vector control, CrmA p14 or CrmA were treated with 1 μ g/ml anti-Fas for the indicated time. Cell extract was prepared and MBP in-gel kinase assays (upper panel) and Western blotting with anti-Mst1 antiserum (lower panel) performed as described in Materials and methods. The percentage of cells undergoing apoptosis was determined at each timepoint by annexin V binding assay.

Fig. 4. Caspase inhibitors selective for the caspase 3 subfamily block Mst1 cleavage. BJAB cells were treated with the indicated concentration of either DEVD-CHO or YVAD-CHO for 3 h prior to the addition of 1 μ g/ml anti-Fas. Cell extract was prepared and MBP in-gel kinase assays (upper panel) and Western blotting with anti-Mst1 antiserum (lower panel) performed as described in Materials and methods. The percentage of cells undergoing apoptosis was determined at each timepoint by annexin V binding assay.

an immune-complex kinase assay with histone H1 as substrate. An increase in immunoprecipitated Mst1 activity was detected within 2 h of anti-Fas treatment and increased up to at least 8 h after stimulation (Figure 6, lower panel). This increase in Mst1 activity correlated temporally with Mst1 breakdown and induction of apoptosis (Figure 6, upper panel). In addition, preincubation of the cells with ZVAD-fmk blocked Mst1 cleavage and the increase in Mst1 immune-complex kinase activity. These results suggest that caspase-mediated cleavage during apoptosis results in an increase in the catalytic activity of Mst1.

Although the above studies establish that Mst1 is specifically cleaved and activated by caspases during apoptosis, the contribution of Mst1 to the cell death program is unclear. To address this question we transiently transfected BJAB human B lymphoma cells with Myc-tagged wild-type Mst1, Mst1 Δ 330 or kinase dead Mst1 (K59R). Twelve hours post-transfection the cells were fixed for immunostaining and analyzed by fluorescent microscopy. Cells expressing either Myc-tagged wild-type Mst1 or Mst1 Δ 330 displayed a profoundly shrunken morphology and nuclear condensation as determined by Hoechst 33342 (Figure 7A). In contrast, cells expressing Myc-tagged kinase-dead Mst1 were morphologically indistinguishable from cells that did not express Mst1. In repeated experiments, 60–70% of wild-type Mst1 transfectants and 70–80% of Mst1 Δ 330 transfectants exhibited morphological changes characteristic of apoptosis (Figure 7A, right panel). Furthermore, analysis of transfectants at later times following transfection revealed that the proportion of cells positive for either wild-type Mst1 or Mst1 Δ 330 was very significantly reduced relative to those

Fig. 5. Mst1 is cleaved *in vitro* by recombinant caspase 3. **(A)** A diagram showing the functional organization of Mst1. The location of the predicted caspase cleavage site, which resembles the consensus sequence for caspase 3 substrates, is indicated. The amino acid sequence surrounding the cleavage sites of several known caspase 3 substrates is provided for comparison. **(B)** Mst1 was transcribed and translated *in vitro* in the presence of ^{35}S -labeled methionine. Labeled Mst1 was then incubated with either extract from untreated or anti-Fas-treated cells (left panel) or recombinant, purified, caspase 3 (right panel). Where indicated, 1 μM ZVAD-fmk was added simultaneously.

staining for kinase-dead Mst1 (data not shown). Western blotting of extract prepared from cells between 6 and 48 h after transfection revealed that the expression level of both Myc-tagged wild-type Mst1 and the truncated $\Delta 330$ mutant declined rapidly with time and was barely detectable by 48 h after transfection (Figure 7C). In contrast, expression levels of the kinase-dead Mst1 were relatively stable and no evidence of cleavage was observed. Significantly, overexpression of wild-type Mst1 resulted in cleavage of exogenous Mst1 to generate a fragment that corresponded precisely to the 36 kDa fragment observed upon caspase-mediated proteolysis *in vitro* and apoptosis *in vivo*. This observation suggests that overexpression of Mst1 results in activation of caspases and is consistent with the morphological changes observed in such cells being a consequence of apoptosis. These findings also imply that overexpression of either wild-type Mst1 or Mst1 $\Delta 330$ is sufficient to induce caspase activity and apoptosis in BJAB cells and that Mst1 kinase activity is required for this process.

The downstream targets for Mst1 that might function in cell death pathways are unknown. However, since Ste20 functions in yeast MAPK pathways, Mst1 may regulate mammalian MAPK pathways (reviewed in Sells and Chernoff, 1997). In order to test this hypothesis we transiently co-transfected 293T cells with either wild-type Mst1, kinase-dead Mst1, or Mst1 $\Delta 330$ and MAPK, SAPK

or p38 MAPK. 293T cells, which have been widely used for this type of study, have the advantages of being transfectable to high efficiencies and of expressing exogenous proteins at high levels. The MAPK constructs used were epitope-tagged with hemagglutinin to allow immunoprecipitation and measurement of activity by immune-complex kinase assay with the appropriate exogenous substrate. Expression of either wild-type Mst1 or Mst1 $\Delta 330$ in 293T cells induced morphological changes characteristic of apoptosis and similar to those observed upon Mst1 overexpression in BJAB cells (Y.Gotoh and J.Graves, unpublished observations). Co-expression of wild-type Mst1 or Mst1 $\Delta 330$ induced 7- and 5-fold activation of SAPK and p38 MAPK respectively while co-expression of kinase-dead Mst1 was without effect (Figure 8). The level of activation of SAPK and p38 MAPK by Mst1 was comparable to that induced by sorbitol treatment. In contrast, no detectable activation of MAPK in response to co-expression of wild-type or truncated Mst1 was observed. Western blotting with anti-HA confirmed that the expression level of the various MAPKs was not significantly influenced by co-expression of Mst1 constructs. In a continuation of these studies, the relationship between Mst1 and various MAPKs was investigated using the same techniques. Consistent with its effect on SAPK and p38 MAPK, Mst1 activated MKK6 and MKK7 but had little effect on MKK4 and MKK3

Fig. 6. Caspase-mediated cleavage stimulates Mst1 kinase activity. BJAB cells were treated with 1 μ g/ml anti-Fas. Where indicated, cells were pre-treated for 3 h with 50 μ M ZVAD-fmk. At the indicated times cell extract was prepared and anti-Mst1 immune-complex kinase assays performed using histone H1 as substrate as described in Materials and methods (lower panel). Western blotting with anti-Mst1 antiserum was performed in parallel (upper panel). The percentage of cells undergoing apoptosis was determined at each time point by annexin V binding assay.

(Figure 9). As might be predicted, Mst1 had no detectable effect on MKK1 activity. To investigate the role of the p38 MAPK pathway in Mst1-induced apoptotic pathways, the p38 MAPK inhibitor, SB203580, was added at a final concentration of 2 μ M immediately after transfection with wild-type Mst1. In three repeat experiments, SB203580 inhibited Mst1-induced morphological changes in 293T cells, observed 18 h after transfection, by 60–70% relative to untreated control cells. These results suggest that p38 MAPK may constitute one pathway by which Mst1 mediates apoptotic changes.

Discussion

The identification of downstream caspase targets is essential for defining the mechanisms by which these proteases induce the characteristic features of apoptosis. The diversity of these apoptotic changes is reflected in the fact that caspase substrates have been identified which serve both structural and signaling functions within the cell. In this report we establish that the 63 kDa serine–threonine kinase Mst1 is cleaved to generate a 36 kDa active fragment during apoptosis induced by cross-linking CD95/Fas or treatment with staurosporine. This cleavage product is detected within 1 h of anti-Fas treatment, when 80% of the cells are still viable, indicating that Mst1 proteolysis occurs as an early event during the cell death program. Several lines of evidence suggest that Mst1 cleavage is mediated by caspase 3 or a closely related caspase with a similar substrate specificity. First, Mst1 cleavage *in vivo* is sensitive to inhibition by a DEVD-CHO, a peptide that is relatively selective for the caspase 3 subfamily. Secondly, recombinant caspase 3 cleaves Mst1 *in vitro* to

generate a fragment that is identical to that observed upon treatment with extract prepared from anti-Fas-treated cells *in vitro* or upon Fas cross-linking *in vivo*. Finally, mutational analysis indicates that the caspase cleavage site in Mst1 is at a DEMD³²⁶S sequence located between the N-terminal kinase domain and the C-terminal catalytic domain. This sequence is similar to the consensus sequence for caspase 3 (DEVD) and to the sequences that have been identified in putative caspase 3 substrates including PARP, D4-GDI, PKC θ , PRK2 and MEKK1 (Figure 5A). Thus, although we cannot rule out the possibility that Mst1 is also a substrate for related caspases, it appears likely that the proteolysis of Mst1 observed during apoptosis is mediated by caspase 3.

The fact that Mst1 is ubiquitously expressed indicates that its cleavage is likely to be a feature of apoptosis in many types of cells. In this respect we have observed Mst1 cleavage in response to serum withdrawal in NIH 3T3 cells or normal human smooth muscle cells, NGF-withdrawal in PC-12 cells, and BCR ligation in human B lymphoma cells (J.D.Graves, K.E.Draves, J.Chernoff, E.G.Krebs and E.A.Clark, in preparation). Furthermore, Mst1 is cleaved in response to a variety of apoptotic agents including etoposide, UV-irradiation and staurosporine (our unpublished observations). These findings suggest that Mst1 cleavage may be a general feature of the apoptotic response of diverse cells to a wide variety of stimuli. Another question concerns whether Mst2, a 61 kDa isoform of Mst1, is also a caspase substrate. Mst1 and Mst2 are 94% identical within their kinase domains and 74% identical overall (Creasy and Chernoff, 1995b). Mst2 contains a DELD³²³S sequence that closely resembles the DEMD³²⁶S caspase site in Mst1. Based on sequence homology alone, it appears likely that Mst2 is also a target for caspase-mediated cleavage. Our inability to observe a Mst2 breakdown product using MBP in-gel assays may be explained by the fact that, relative to Mst1, Mst2 functions poorly under such circumstances (Taylor *et al.*, 1996). Since our anti-Mst1 antibodies do not cross-react with Mst2, we have no definitive evidence that Mst2 is a caspase substrate.

The location of the caspase cleavage site at the junction of the catalytic and regulatory domains of Mst1 raises the possibility that Mst1 is activated by caspase-mediated cleavage. This hypothesis is supported by data from in-gel and immune-complex kinase assays. Previous structure–function studies of Mst1 have revealed that its C-terminal domain mediates homo- and heterodimerization and inhibits kinase activity. Deletion analysis has revealed that removal of the regulatory domain results in an activated kinase fragment (Creasy *et al.*, 1996). For example, a mutant truncated at amino acid 330 (Δ 330) is ~10-fold more active *in vitro* than the intact kinase. This finding may provide a structural basis for our observation of increased Mst1 activity in apoptotic cells. It is also possible that removal of the regulatory domain influences protein–protein interactions involving Mst1 or its subcellular localization. Although removal of the regulatory domain is likely to prevent Mst1 dimerization, the functional consequences of this are unclear. One possibility, currently under investigation, is that caspase-mediated cleavage may also alter the localization and substrate specificity of Mst1.

Very little is known about the regulation and function

Fig. 7. Overexpression of Mst1 induces caspase activity and morphological changes characteristic of apoptosis. BJAB cells were transiently transfected with either wild-type Mst1, truncated Mst1 Δ 330 or kinase-dead Mst1 K59R. All constructs were Myc epitope-tagged. (A) Twelve h after transfection the cells were fixed and stained with anti-Myc to determine Mst1 expression and Hoechst 33342 to allow analysis of nuclear morphology. Cell staining of a typical field is shown in the left panel and the percentage of cells staining positive for Mst1 that also exhibit a shrunken morphology with nuclear condensation is shown in the right panel. (B) Extract was prepared from transfected cells at the indicated times following transfection and subjected to Western blotting with anti-myc antibody.

Fig. 8. Mst1 activates SAPK and p38 MAPK. 293T cells were transfected with either wild-type Mst1, truncated Mst1 Δ 330 or kinase-dead Mst1 K59R in addition to epitope-tagged MAPK, SAPK or p38 MAPK. Kinase activities were determined by immune-complex kinase assay with the appropriate substrate as described in Materials and methods (upper panel). Western blotting with anti-epitope tag antibodies shows the expression level of exogenous MAPKs (lower panel). The effect of either 10% FCS or 0.5 M sorbitol treatment on MAPK activities is shown for comparison.

of Mst. Mst1 and Mst2 are members of a growing family of enzymes that play regulatory roles in diverse cellular phenomena such as morphogenesis, stress-responses and proliferation (reviewed Sells and Chernoff, 1997). One group of Ste20 homologs are the mammalian p21-activated protein kinases (PAKs) which interact with and are regulated by GTPases. The other branch of this family com-

Fig. 9. Mst1 activates MKK6 and MKK7. 293T cells were transfected with either wild-type Mst1, truncated Mst1 Δ 330 or kinase-dead Mst1 K59R in addition to the indicated epitope-tagged MAPKK constructs. Kinase activities were determined by immune-complex kinase assay with the appropriate substrate as described in Materials and methods (upper panel). Western blotting with anti-epitope tag antibodies shows the expression level of exogenous MAPKs (lower panel). The effect of either 10% FCS or 0.5 M sorbitol treatment on MAPKK activities is shown for comparison.

prises the mammalian Ste20-like (Mst) kinases, which have a long C-terminal extension and which lack a recognizable domain for interaction with GTPases. The Mst subfamily of mammalian Ste20 homologs comprises

at least 10 members including Mst1, Mst2, hematopoietic progenitor kinase (HPK1) (Hu *et al.*, 1996), kinase homologous to Ste20 (KHS) (Tung and Blenis, 1997), germinal center kinase (GCK) (Katz *et al.*, 1994), Ste20/oxidant stress response kinase-1 (SOK-1) (Pombo *et al.*, 1996) and Mst-3 (Schinkmann and Blenis, 1997). Some members of the Mst family have been shown to be responsive to cellular stress. For example, GCK is activated by inflammatory cytokines such as TNF α , and SOK-1 is responsive to oxidative stress (Pombo *et al.*, 1997). Although Mst1 has been reported to be unresponsive to growth factors such as EGF and PDGF, it is weakly responsive to extreme stress such as arsenite and heat shock. Mst1 activity is also stimulated by staurosporine and okadaic acid (Taylor *et al.*, 1996). We show that staurosporine stimulates a biphasic response in which full-length Mst1 is activated prior to its proteolysis at the onset of apoptosis. Furthermore, our results with Fas cross-linking are the first example of the regulation of Mst1 by a physiological stimulus. It is important to emphasize that, in addition to proteolytic activation during apoptosis, Mst1 is likely to be regulated in a caspase-independent manner by stimuli that do not induce apoptosis. The relative contributions of phosphorylation and proteolysis to the regulation of Mst1 activity during apoptosis remain to be determined.

Ectopic expression of either wild-type Mst1 or the truncated Mst1 Δ 330 mutant induce morphological changes characteristic of apoptosis in BJAB cells. Cells overexpressing either Mst1 or Mst1 Δ 330 exhibit nuclear condensation and are profoundly shrunken relative to untransfected cells or cells expressing kinase-dead Mst1. Strong evidence to support the idea that overexpression of Mst1 induces apoptosis may be derived from the fact that wild-type Mst1 is cleaved to generate a fragment that corresponds to the caspase-generated fragment. Thus, in addition to being a target for caspase-mediated cleavage and activation, Mst1 is capable of either directly or indirectly activating the caspase that cleaves it. The fact that no cleavage of kinase-dead Mst1 was observed demonstrates that the kinase activity of Mst1 is required for this effect. Although our results suggest that Mst1 activity may be sufficient to induce apoptosis, they do not address the question of whether Mst1 is necessary for cell death. Preliminary studies, involving the transient expression in BJAB cells, suggest that kinase-dead Mst1 does not block Fas-induced cell death (data not shown). However, we cannot rule out the possibility that kinase-dead Mst1 blocks a subset of Fas-induced apoptotic changes. In this respect, while overexpression of PAK2 is sufficient to induce apoptosis, a dominant-negative mutant blocked only the membrane and morphological changes associated with Fas-induced cell death (Rudel and Bokoch, 1997). It is also possible that the inactive mutant of Mst1 does not effectively compete with endogenous Mst1 and function as a dominant-negative in this system. Since the cellular function of Mst1 has not been clearly defined, careful analysis of potential dominant-negative mutants will be required to determine the precise role of Mst1 amongst the diverse and highly redundant pathways elicited during the apoptotic response.

The identity of the downstream targets of Mst1 are unknown. However, the fact that several other members

of the mammalian family of Ste20 homologs have been shown to activate SAPK and p38 MAPK, suggests that Mst1 may also function in this manner. For example, PAK1 and PAK2 have been implicated in activation of the p38 MAPK and SAPK pathway respectively, while GCK, HPK and KHS can activate the SAPK pathway (Pombo *et al.*, 1995; Hu *et al.*, 1996; Tung and Blenis, 1997). Downstream of these kinases are a diverse group of MAPKKKs, the best characterized of which are MEKK1 and ASK1 (reviewed by Fanger *et al.*, 1997). Although the relationship between the various MKKKs and their respective downstream targets has not been precisely defined, MEKK1 functions in the SAPK pathway while ASK1 can activate both SAPK and p38 MAPK (Minden *et al.*, 1994; Yan *et al.*, 1994; Ichijo *et al.*, 1997). Further downstream, it has been shown that MKK3 and MKK6 activate p38 MAPK (D'Erijard *et al.*, 1995; Han *et al.*, 1996), while SEK1/MKK4 and MKK7 function in the SAPK pathway (Yan *et al.*, 1994; D'Erijard *et al.*, 1995; Lin *et al.*, 1995; Holland *et al.*, 1997). Within these pathways, both PAK2 and MEKK1 have been identified as caspase targets (Cardone *et al.*, 1997; Rudel and Bokoch, 1997). Furthermore, overexpression of either MEKK1, ASK1 or MKK6b is sufficient to induce apoptosis and caspase activity (Cardone *et al.*, 1997; Huang *et al.*, 1997; Ichijo *et al.*, 1997).

Our co-expression data indicate that Mst1 is capable of activating SAPK and p38 MAPK via their respective upstream activators MKK7 and MKK6. Interestingly, MKK7 and MKK6 are also the major MKK activities stimulated during Fas-induced apoptosis (Toyoshima *et al.*, 1997). The fact that little activation of MKK4/SEK1 or MKK3 was observed suggests that, despite being overexpressed, Mst1 demonstrates selectivity for MKK7 and MKK6. These data are consistent with the idea that Mst1 may activate these pathways under physiological circumstances. However, since we do not demonstrate that Mst1 directly phosphorylates and activates a MAPKKK that functions upstream of these kinases, the effect of Mst1 on the SAPK and p38 MAPK pathways may be indirect. One possibility is that activation of the SAPK and p38 MAPK pathways is a consequence rather than a cause of Mst1-induced apoptosis. Although we cannot rule out this possibility, caspase inhibitors such as ZVAD-fmk do not inhibit the ability of Mst1 to activate either SAPK or p38 MAPK (Y.Gotoh, unpublished observations). Alternatively, the ability of Mst1 to activate the SAPK and p38 MAPK pathways may depend on the cell type and/or the level of overexpression. Thus, Mst1 might only activate the SAPK and p38 MAPK pathways under extreme conditions. Fas-induced apoptosis, accompanied by the cleavage and persistent activation of Mst1, may be one such condition. Experiments addressing the question of whether Mst1 might regulate the SAPK and p38 pathways in response to non-apoptotic stimuli await the identification of additional pathways that activate Mst1.

Based on our observations, we propose a model in which Mst1 is activated upon caspase-mediated cleavage and functions as part of a feedback loop that serves to amplify the apoptotic response. Although the mechanism by which Mst1 might influence caspase activity and apoptosis is unclear, the ability of Mst1 to induce SAPK and p38 MAPK activity suggests that one mechanism may

involve the SAPK and p38 MAPK pathways. The ability of SB203580 to inhibit morphological changes induced by Mst1 supports the hypothesis that the p38 MAPK pathway may function downstream during Mst1-induced apoptosis. However, it is important to consider that the effect of Mst1 on the p38 MAPK and/or SAPK pathways may not be direct and that other important targets for Mst1 may remain to be identified. Further studies will be required to determine the precise role of the SAPK and p38 MAPK pathways in Mst1-induced apoptosis. The identification of caspase targets at several distinct levels in the SAPK and p38 MAPK cascades raises important questions about the relationship of caspase cascades to protein kinase cascades. It is intriguing to speculate that caspases might exploit the considerable amplifying potential of the SAPK and p38 MAPK pathways in order to augment their apoptotic signal. A full understanding of the role of Mst1 in apoptosis will require a more detailed definition of its normal physiological regulation and function.

Materials and methods

Reagents and cells

The anti-Fas antibody IPO-4 (Sidorenko *et al.*, 1992) was kindly provided by Dr S.Sidorenko. Staurosporine and histone H1 were obtained from Sigma (St. Louis, MO). Myelin basic protein (MBP) was prepared from bovine brain as previously described (Daum *et al.*, 1997). ZVAD-fmk, DEVD-CHO and YVAD-CHO were purchased from the Kamiya Biomedical Company (Tukwila, WA). Hoechst 33342 was purchased from Molecular Probes (Eugene, OR). The pyridinyl imidazole inhibitor of p38 MAPK, SB203580, was purchased from Calbiochem (San Diego, CA). Rabbit polyclonal antibodies specific for the N-terminus of Mst1 were raised against a peptide corresponding to the first 15 amino acids (ETVQLRNPPRRQLKC). The 9E10 monoclonal antibody specific for the Myc epitope tag was obtained from the American Type Culture Collection (Rockville, MD). The monoclonal anti-hemagglutinin (HA) antibody 12CA5 was obtained from Boehringer Mannheim (Indianapolis, IN). FITC-conjugated anti-mouse secondary antibody was obtained from Biosource International (Camarillo, CA). The BJAB human B lymphoma line and CrmA transfectants were generously provided by Dr V.Dixit (Genentech, South San Francisco, CA) and were maintained as described (Tewari and Dixit, 1995). The 293T cell line was kindly provided by Dr J.Cooper (FHCRC, Seattle, WA).

Plasmids

Myc-tagged Mst1 constructs in the pCMV5M expression vector were constructed as described previously (Creasy and Chernoff, 1995a; Creasy *et al.*, 1996). Mst1 D326N was constructed by PCR mutagenesis. pME18-HA-MKK6 was provided by Drs N.Kuroyanagi and M.Hagiwara. pSR α 456-HA-MKK7 was a kind gift from Drs P.Holland and J.Cooper. pSR α 456-HA-MAPK, p38 MAPK, SAPK α , MAPKK1, SEK1 and MKK3 were constructed as described previously (Ichijo *et al.*, 1997). GST-kinase negative MAPK and His-kinase negative p38 MAPK expression plasmids were prepared as described previously (Ichijo *et al.*, 1997).

MBP in-gel kinase assays

After incubation with the indicated stimuli, $5\text{--}10 \times 10^6$ cells were lysed for 15 min on ice in 1 ml of lysis buffer [20 mM HEPES pH 7.4, 2 mM EGTA, 50 mM β -glycerophosphate, 1% Triton X-100, 10% glycerol, 1 mM dithiothreitol (DTT), 1 mM phenylmethyl sulfonyl fluoride (PMSF), 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, 1 mM Na_2VO_4]. Cell debris was removed by microcentrifugation at 14 000 g for 10 min at 4°C prior to the addition of laemmli sample buffer. Cell extract (10–20 μ g) was used for both Western blotting and MBP in-gel kinase assays. 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 running, the gel was washed twice at room temperature (RT) for 30 min with 100 ml buffer A (50 mM HEPES pH 7.6, 5 mM 2-mercaptoethanol) containing 20% isopropanol. The gel was then washed twice at RT with buffer A

and twice with buffer A containing 6 M urea. Renaturation was achieved by sequentially washing the gel 3 $\times$ for 15 min at 4°C with buffer A containing 3 M urea, 1.5 M urea, 0.75 M urea and buffer A alone. Following an overnight wash in buffer A with 0.05% Tween 20 (v/v) the gel was washed twice and equilibrated at 30°C for 30 min in kinase buffer (20 mM HEPES pH 7.6, 20 mM MgCl_2 , 2 mM DTT) prior to the addition of 20 μ M Mg-ATP and 100 μ Ci 32 P-labeled ATP and incubation for at 30°C for 30 min. The reaction was stopped and unincorporated 32 P-labeled ATP was removed by washing 10 $\times$ for 30 min at RT with 100 ml of 5% TCA (w/v) and 1% NaPPi (w/v). After staining and destaining the gel was dried prior to autoradiography. Quantitation by densitometry was performed using an imaging densitometer (Bio-Rad Laboratories, Hercules, CA).

Apoptosis assays

Apoptotic cells were quantified using annexin V flow cytometry. The annexin V assay exploits the fact that an early event during apoptosis of many cells is a loss of membrane lipid asymmetry resulting in the exposure of phosphatidylserine in the outer leaflet of the plasma membrane. Briefly, cells were incubated with FITC-conjugated annexin V and counterstained with propidium iodide (PI) in order to allow necrotic cells to be excluded (Clontech, Palo Alto, CA). The cells were subsequently analyzed using a Becton Dickinson FACStar Plus flow cytometer (San Jose, CA). Trypan blue dye exclusion was used as a confirmatory cell death assay.

In vitro protease assays

Wild-type Mst1 and Mst1 D326N were translated *in vitro* using a coupled transcription and translation system with T7 polymerase (Promega, Madison, WI). Apoptotic cell extract was prepared as follows. After stimulation, cells were washed once in PBS and resuspended at 2×10^8 /ml in hypotonic lysis buffer (50 mM NaCl, 40 mM β -glycerophosphate, 10 mM HEPES pH 7.0, 5 mM EGTA, 2 mM MgCl_2). The lysate was then subjected to four freeze-thaw cycles prior to centrifugation at 10 000 g for 10 min (100 mM HEPES pH 7.5, 10% sucrose, 0.1% CHAPS, 10 mM DTT, 0.1 mg/ml ovalbumin). Recombinant caspase 3 was expressed and purified as previously described (Han *et al.*, 1997). Cell extract or purified caspase 3 (10 μ g), in 5 μ l of hypotonic lysis buffer, was incubated for 1 h at 37°C with 5 μ l of 35 S-labeled *in vitro*-translated Mst1 or Mst1 D326N. The reaction was stopped by the addition of 4 $\times$ laemmli sample buffer and subjected to SDS-PAGE prior to drying and autoradiography.

Transfections

BJAB cells were transfected by electroporation in a Bio-Rad Gene Pulser II (Bio-Rad Laboratories, Hercules, CA). Briefly cells were washed twice in PBS and resuspended at 2.8×10^7 cells/ml in electroporation buffer (RPMI 1640 with the addition of 1 mM Na pyruvate; 2 mM L-glutamine; 1 $\times$ nonessential amino acids; 15% FCS v/v). pCMV vector (10 μ g) containing the appropriate Myc-tagged Mst1 construct was added to 350 μ l of cells (1×10^7) in a 0.4 cm cuvette and incubated on ice for 10 min. The cells were then resuspended prior to electroporation (230 V, 960 μ F). Following electroporation the cells were incubated on ice for a further 10 min before being resuspended in 20 ml of electroporation buffer and allowed to recover for 3 h at 37°C in a humidified incubator. Following the removal of cell debris by Ficoll density gradient centrifugation, the cells were resuspended at 5×10^5 /ml in RPMI + 10% FCS and returned to the incubator. At the indicated time cells were harvested for the preparation of cell extract for Western blotting, as described above, or were prepared for immunohistochemical analysis.

Immunohistochemical analysis

A quantity of cells (5×10^4) was washed and resuspended in 150 μ l PBS. The cells were then attached to glass slides using a Shandon Cytospin 2 at 1000 r.p.m. for 8 min and air dried overnight at RT. The cells were fixed in acetone at 20°C for 10 min and air dried. Prior to use the cells were rehydrated in PBS at RT for 15 min. The polyclonal anti-myc antibody 9E10 was added at 1 μ g/ml in 100 μ l/slide and incubated at 4°C overnight in a humidified chamber. The slides were washed 5 $\times$ in TBST (150 mM NaCl, 50 mM Tris pH 8.0, 0.1% Tween-20) before incubation at RT for 30 min with FITC-conjugated anti-mouse secondary antibody at a final dilution of 1:1000 in 100 μ l TBST. The slides were then washed twice in ECL wash prior to incubation at 37°C for 30 min with Hoechst 33342 at 10 μ g/ml in 100 μ l of PBS. After washing twice in PBS and twice in double distilled H₂O, the slides were mounted and allowed to dry in the dark before analysis by fluorescent microscopy (Nikon).

Co-transfection and kinase assays

293T cells were transfected in 60 mm dishes with 4 µg of the appropriate plasmids using 24 µl of lipofectamine (Gibco-BRL) for 10 h according to the manufacturer's instructions. Twenty-four hours after transfection, cells were harvested in lysis buffer (20 mM Tris-HCl pH 7.5, 10 mM β-glycerophosphate, 5 mM EGTA, 10 mM NaF, 1 mM NaPPi, 150 mM NaCl, 1% NP40, 4 mM DTT, 1 mM Na vanadate, 1 mM PMSF 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 4× 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-PAGE prior to drying and autoradiography. Morphological analyses of 293T cells were performed 18 h after transfection, using Hoechst 33342, essentially as described above.

Data presentation

All experiments shown are representative of between three and five repeats.

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