Mapping of MST1 Kinase Sites of Phosphorylation

ACTIVATION AND AUTOPHOSPHORYLATION*

Helmut Glantschnig, Gideon A. Rodan, and Alfred A. Reszka‡

From the Department of Bone Biology and Osteoporosis, Merck Research Laboratories, West Point, Pennsylvania 19486

MST1 is a member of the Sterile-20 family of cytoskeletal, stress, and apoptotic kinases. MST1 is activated by phosphorylation at previously unidentified sites. This study examines the role of phosphorylation at several sites and effects on kinase activation. We define Thr<sup>183</sup> in subdomain VIII as a primary site of phosphorylation. Thr<sup>187</sup> is also critical for kinase activity. Phosphorylation of MST1 in subdomain VIII was catalyzed by active MST1 via intermolecular autophosphorylation, enhanced by homodimerization. Active MST1 (wild-type or T183E), but not inactive Thr<sup>183</sup>/Thr<sup>187</sup> mutants, was also highly autophosphorylated at the newly identified Thr<sup>177</sup> and Thr<sup>387</sup> residues. Cells expressing active MST1 were mostly detached, whereas with inactive MST1, adhesion was normal. Active MKK4, JNK, caspase-3, and caspase-9 were detected in the detached cells. These cells also contained all autophosphorylated and essentially all caspase-cleaved MST1. Similar phenotypes were elicited by a caspase-insensitive D326N mutant, suggesting that kinase activity, but not cleavage of MST1, is required. Interestingly, an S327E mutant mimicking Ser<sup>327</sup> autophosphorylation was also caspase-insensitive, but only when expressed in caspase-3-deficient cells. Together, these data suggest a model whereby MST1 activation is induced by existing, active MST kinase, which phosphorylates Thr<sup>183</sup> and possibly Thr<sup>187</sup>. Dimerization promotes greater phosphorylation. This leads to induction of the JNK signaling pathway, caspase activation, and apoptosis. Further activation of MST1 by caspase cleavage is best promoted by caspase-3, although this appears to be unnecessary for signaling and morphological responses.

In mammalian cells, Sterile-20 (Ste20)-related kinases participate in the regulation of the cytoskeleton that controls cell morphology and motility, and in the regulation of apoptosis (1). These kinases share a conserved catalytic (kinase) domain at the amino terminus and a C-terminal regulatory region of great structural diversity, which interacts with signaling molecules that regulate the cytoskeleton. Currently, four closely related MST kinases have been described (5–9). Most Ste20 group kinases activate mitogen-activated protein kinase (MAPK)<sup>1</sup>

* 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.

‡ To whom correspondence should be addressed: Dept. of Bone Biology and Osteoporosis, Merck Research Laboratories, WP26A-1000, Summitney Pike, West Point, PA 19486. Tel.: 215-652-1410; Fax: 215-652-4328; E-mail: Alfred_Reszka@merck.com.

1 The abbreviations used are: MAPK, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; MEKK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase

cascades in the signaling pathways between the cellular membrane and the nuclear compartment (2). In yeast, the mating pheromone receptor Ste20p phosphorylates and activates a MAPK kinase, Ste11p, raising the possibility that mammalian homologs of Ste20p (e.g. MST1 kinase) also function as MAPK kinase kinase kinases (3, 4). MST1 was shown to act upstream of MAPK kinases that regulate p38 and JNK activities, probably acting via the MAPK kinase kinase MEKK1 (10).

There is substantial evidence that MST1 promotes apoptosis, although its role in this process may vary in different cell types. Overexpression of MST1 can induce apoptosis and nuclear condensation in BJAB, 293T and COS-1 cells (4, 14, 15), whereas MST1 promotes nuclear condensation without apparent chromosomal cleavage in HeLa cells (13).

A consistent feature of MST1 in all tested cell types is its proteolytic cleavage by caspase, in response to apoptotic stimuli, to a 34–36-kDa product (hereafter referred to as 36-kDa MST1). Cleavage increases MST1 kinase activity severalfold (4, 16–19) and influences its subcellular localization (13, 15). Recently, a second caspase cleavage site was identified in the human MST1 sequence that is absent in mouse MST1 and in MST2 from several species (10). Mutation of this site has no impact on accumulation of the 36-kDa species.

Expression of a kinase-dead MST1 mutant (K59R) can partially or fully suppress apoptosis or chromatin condensation in HL-60 and 293T cells treated with chemical apoptotic stimuli (15, 18). This protective effect is associated with suppression of both JNK activity and DNA fragmentation. However, the K59R mutant fails to suppress apoptosis in BJAB and HeLa cells treated with Fas ligand or TNF-α (4, 16). The basis for the cell type- and stimulus-specific differences in MST function has not yet been elucidated.

Interestingly, like PAK proteins (11, 12), MST1 (full-length or cleaved) can have a profound effect on cell shape (cell rounding and detachment) independent of caspase activation and prior to nuclear condensation (13). This action and the high basal activity of MST1 kinase suggest a possible function unrelated to apoptosis.

Stress-inducing agents such as staurosporine and sodium arsenite (9) can increase MST1 kinase activity; however, no physiological activator of MST1 has been identified, and little is known about the endogenous activation of MST1. Recently, it was proposed that in addition to caspase cleavage, MST1 phosphorylation at yet unidentified sites contributes to kinase activation (10), as has been observed for PAK2 (20, 21). Creasy kinase; TNF, tumor necrosis factor; PAK, p21-activated kinase; MKK, mitogen-activated protein kinase kinase; WT, wild-type; β-HBS, β-glycerophosphate/HEPES-buffered solution; SEK, SAPK/Erk kinase; SAFK, stress-activated protein kinase; PARP, poly(ADP-ribose) polymerase; DEVase, combined caspase-3, -6, and -7 activities; ERK, extracellular signal-regulated kinase; CHX, cycloheximide.
and Chernoff (5) showed that dephosphorylation of MST1 with protein phosphatase-2A 

in vitro results in 3-4-fold stimulation of activity, suggesting that phosphorylation sites are involved in suppression of activity. The physiological occurrence of this type of phosphoregulation of MST1 has not been reported.

To address some of the questions related to MST regulation, we performed a structure-function analysis of MST1 phosphorylation sites. We identified novel phosphorylation sites and, by mutational analysis, demonstrated a role for intermolecular autophosphorylation of the activation loop of MST1 in subdomain VIII. We identified Thr\textsuperscript{183} and Thr\textsuperscript{187} as essential for kinase activity and for effects on farther downstream targets such as MKK4 and JNK. Phosphorylation at Thr\textsuperscript{177} altered the electrophoretic mobility of MST1, suggesting an influence on the conformation of the activation loop. We also found that MST1 phosphorylation status had no effect on MST1 cleavage by caspase-3 during apoptosis, but might influence susceptibility to cleavage by other caspases.

**MATERIALS AND METHODS**

**Cell Culture**—Mouse fibroblast NIH-3T3 cells and African green monkey kidney cells were obtained from American Type Culture Collection (Manassas, VA). Cells were cultured at 37 °C in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 1 mM l-glutamine, 100 units/ml penicillin G, and 100 μg/ml streptomycin in a humidified atmosphere with 5% CO\textsubscript{2}. MCF7 cells (MCFT/pBI pool) and caspase-3-expressing WT3C/MCF7 cells were kindly provided by Sophie Roy (Mercer Frosst, Kirkland, Canada) and cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin G, 100 μg/ml streptomycin, 100 μg/ml Geneticin, and 150 μg/ml hygromycin B (Invitrogen). Apoptosis of MCF7 cells was induced by treatment with TNF (35 ng/ml) and cycloheximide (50 μM) for 6–7 h.

**MST1 Cloning and Site-directed Mutagenesis**—Mouse osteolast mRNA was reverse-transcribed with PowerScript reverse transcriptase (Clontech, Palo Alto, CA), and full-length MST1 was amplified with F1 primers, Turbo DNA polymerase (Stratagene, La Jolla, CA) and mouse MST1-PCR2 primers (5’-CCGCGGATCCGGAGGACCGTGCAGCTGAGGAACCCACCA 3’-CATGAGGATCCCGGATCCGGAGGCATGGAGACCGTAGCCATGGAGACCGTGCAGCTGAGGAACCCACCA) and Primer 3-primers containing an Sfi I site (5’-CTTATGGCCATGGAGACCGTGCAGCTGAGGAACCCACCA and Primer 3’-GGGAGGATCCGGAGGACCGTGCAGCTGAGGAACCCACCA) and Primer 3’-GGGAGGATCCGGAGGACCGTGCAGCTGAGGAACCCACCA) and Primer 3’-GGGAGGATCCGGAGGACCGTGCAGCTGAGGAACCCACCA. Kinase assays were performed in-gel using the In-gel Kinase Assay—Kinase assays were performed in-gel using the method described previously (17) with one modification: the kinase buffer contained 20 mM MgCl\textsubscript{2} in place of 5 mM MnCl\textsubscript{2}. Lanes were loaded with equivalent amounts of protein lysate (5–10 μl/gene) and were separated by SDS-10% polyacrylamide gels. Proteins were isolated 2 h after transfections, and the expression levels of diverse Myc-MST1 protein variants were assessed by Western blotting with anti-Myc antibody. Myc-tagged MST1 proteins were immunoprecipitated. To precipitate two different constructs on the same band, lysates from independent transfections were mixed together prior to immunoprecipitation, which was then carried out as described above. The precipitated proteins were washed twice with kinase buffer (20 mM HEPES (pH 7.6) and 10 mM MgCl\textsubscript{2}). Kinase assays were performed in 50 μl of kinase buffer with 5 μCi of [γ-\textsuperscript{32}P]ATP (3000 Ci/mmol; PerkinElmer Life Sciences) or in 1 μl ATP for 25 min at 30 °C. The kinase reaction was stopped by adding Laemmli sample buffer, and each gel was electrophoresed and processed for kinase assay. Dried gels were exposed to phosphorimaging screens and scanned and analyzed using the Amersham Biosciences Storm system.

**In Vitro Kinase Assay**—For auto- and transphosphorylation studies, Myc-tagged MST1, MST1(D326N), MST1(K587R/D326N), MST1-1(2) was expressed in HEK293 cells. Proteins were isolated 2 h after transfections, and the expression levels of diverse Myc-MST1 protein variants were assessed by Western blotting with anti-Myc antibody. Myc-tagged MST1 proteins were immunoprecipitated. To precipitate two different constructs on the same band, lysates from independent transfections were mixed together prior to immunoprecipitation, which was then carried out as described above. The precipitated proteins were washed twice with kinase buffer (20 mM HEPES (pH 7.6) and 10 mM MgCl\textsubscript{2}). Kinase assays were performed in 50 μl of kinase buffer with 5 μCi of [γ-\textsuperscript{32}P]ATP (3000 Ci/mmol; PerkinElmer Life Sciences) or in 1 μl ATP for 25 min at 30 °C. The kinase reaction was stopped by adding Laemmli sample buffer, and each gel was electrophoresed and processed for kinase assay. Dried gels were exposed to phosphorimaging screens and scanned and analyzed using the Amersham Biosciences Storm system.

**Cloning and Expression of MST1**—MST1 was cloned from mouse osteolast-like cells by reverse transcription-PCR using specific primers for the mouse sequence and is referred to throughout as MST1(WT). A nucleotide missense mutation (Lys instead of Glu at residue 198), by comparison with the published mouse and human sequences, was corrected by site-
directed mutagenesis (K198E). Constructs described hereafter were expressed with an N-terminal Myc tag, unless otherwise noted. The general features of MST1 and amino acid residues targeted in this study are depicted schematically in Fig. 1.

Kinase Activity and Cytoskeletal Effects—NIH-3T3 cells were transfected with full-length MST1 or cleaved MST1-(1–326), visualized in Fig. 2 (A and B) by immunoblot and in-gel kinase analyses, respectively. The in-gel kinase activity of the MST1(WT) construct was substantial (Fig. 2B) and was not further increased by staurosporine treatment of transfected NIH-3T3 or COS-1 cells (data not shown). Overexpression of active MST1(WT) caused cell rounding and eventual detachment of NIH-3T3 cells. This was recorded as a reduction in the number of adherent cells 24 h after transfection (Fig. 3, A and B) and by the increase in the activity of the wild-type kinase in the floating cell population (Fig. 3C).

To investigate the contribution of MST1 cleavage and kinase activity to the effects of the exogenous constructs on morphology, we expressed kinase-dead (MST1(K59R)), truncated (MST1-(1–326)), and caspase-insensitive (MST1(D326N)) mutant kinases (Fig. 2, A and B) in NIH-3T3 cells. Consistent with a previous report (13), kinase activity was required for MST1 induction of cell rounding and detachment (Fig. 3, A and B). Interestingly, the morphological phenotype and cell detachment generated by the full-length caspase-resistant (MST1(D326N)) and truncated (MST1-(1–326)) constructs were indistinguishable from those elicited by the MST1(WT) kinase. MST1 kinase activity thus seems to be both necessary and sufficient to produce morphological changes, in contrast to chromatin condensation, which requires MST1 caspase cleavage or expression of the truncated species (26).

To further investigate the link between MST1 activity, caspase activity, and cell detachment, we separately collected and analyzed adherent and detached cells. In terms of total protein, there were ~3-fold more floating cells when transfected with MST1(WT) or MST1(D326N) than when transfected with vector as measured 24 h post-transfection. Caspase activities were measured directly or by PARP cleavage. Detachment of cells correlated with induction of apoptosis, as assessed by generation of the 89-kDa PARP fragment (Fig. 3C, lower panel), which was cleaved in all floating cell populations transfected with vector, MST1(WT), or MST1(D326N). This correlated with fluorometric measurements of caspase-3 and caspase-9 activities, which were increased by >90-fold in floating versus adherent cells (activity/mg of protein). Total caspase-3 activity in floating cells expressing MST1(WT) and MST1(D326N) was increased by up to 6.5- and 4.2-fold, respectively, compared with the vector control. This reflected both an increase in the number of detached cells and an up to 2-fold average increase in caspase activity per floating cell.

MST1 activity was determined by in-gel kinase assays at the same 24-h time point (Fig. 3C). To more accurately assess kinase activity per cell, lanes were loaded with equivalent amounts of protein. The activities of full-length MST1(WT) and MST1(D326N) were increased by ~20-fold in floating versus adherent cells. The activity of the 36-kDa MST1(WT) caspase cleavage product was mostly found in the floating cell population and at levels equal to the full-length kinase. In adherent cells, the 36-kDa caspase-cleaved MST1(WT) kinase showed about one-fourth of the activity of the full-length kinase. Because MST1(D326N) was caspase-resistant, no 36-kDa kinase activity was observed, and the full-length activity was approximately equal to that of the combined full-length and caspase-cleaved MST1(WT) kinases. Together, these data suggest that expression of active MST1 leads to concomitant cell detachment and caspase activation. Caspase cleavage of MST1 in and of itself is not required for either process.

Regulation of MST1 Kinase Activity by Phosphorylation at Thr183 within the Activation Loop—Alignment of MST1 subdomain VIII with other members of the Ste20 family and MKK6 kinases (Fig. 4A) shows conservation in MST1 of phosphoregulated residues. Thr177 aligns with the phosphoacceptor Ser189/Ser207 residues in MKK3 and MKK6, respectively; and Thr183 aligns with the phosphorylated Thr423 residue in PAK1. Thr175 and Thr187 are also conserved among the Ste20 family members, although phosphorylation at these sites has not been described.

We used two approaches to investigate putative phosphorylation at these residues and their relationship to MST1 kinase function. (a) Using site-directed mutagenesis, we replaced all four putative phosphorylatable residues with alanine (T175A, T177A, T183A, and T187A) or glutamate (T175E, T177E, T183E, and T187E) and assessed catalytic activities (Fig. 4B). (b) We used phospho-specific antibodies to probe for putative phosphorylation at Thr177 (antibody Ab1) and Thr183 (antibody Ab2) (Fig. 4C), similar to a previous approach assessing phosphorylation at Thr423 of PAK1 (23).

Mutation of Thr183 and Thr187 altered the catalytic activity of MST1 (Fig. 4B, lower panel). The T183A mutation reduced the estimated activity to 1–3% of that of MST1(WT). This substitution was somewhat more effective than the analogous T423A mutation in PAK1, which maintains 12% of the wild-type kinase activity (23). Even more substantial was the complete loss of catalytic activity observed with the T187A mutation. Consistent with a role for Thr183 phosphorylation in MST1 kinase activation, the T183E mutant was partially active, with glutamate substituting for a putative phosphorylated threonine. However, the T187E mutant remained completely inactive, which is more analogous to the effects of similar substitutions in MAPKs such as ERK2 (24). As in the case of the K59R mutant, lack of kinase activity following expression of T183A (Fig. 3, A and B) and T187A or T187E (data not shown) resulted in little or no morphological rounding and detachment of NIH-3T3 cells. Meanwhile, expression of the mostly active T183E mutant caused morphological rounding and detachment that were comparable to those caused by the wild-type kinase (Fig. 3, A and B). Mutations of Thr175 and Thr177 did not alter catalytic activity (see T175A, T175E, T177A, and T177E in Fig. 4B, lower panel), and morphological cell rounding was comparable to that obtained with the MST1(WT) kinase (data not shown).

Immunological Detection of MST1 Thr177 and Thr183 Phospho-
— A phosphorylation-dependent MST1 band shift was recently reported for the cleaved MST1 kinase (13), although the responsible residue(s) were not identified. Likely candidates are identified here because mutations of Thr177 and Thr183 altered the electrophoretic mobility of the kinase. The T177A mutant showed reduced mobility of both the full-length and caspase-cleaved kinases (Fig. 4B). This was evident in independent constructs generated in different mutagenesis experiments. Meanwhile, the partially active T183E mutant generated two bands at 36 kDa, with the slower migrating band corresponding to that of T177A. We therefore more carefully examined the MST1(WT) kinase, also revealing a 36-kDa major faster migrating species and a minor slower migrating band. Interestingly, T177E migrated exclusively as the faster species, without evidence of any slower migrating band. This suggests that changes in electrophoretic mobility might correlate with phosphorylation at Thr177.

To examine phosphorylation in this domain, immunoblots were probed with phospho-specific antibodies that could detect putative phosphorylation at Thr177 and Thr183 (Fig. 4C). Antibody Ab1 (Thr177) and antibody Ab2 (Thr183) bound to MST1(WT), suggesting that these residues were indeed phosphorylated. In the K59R mutant, recognition by these antibodies was greatly diminished, suggesting that intrinsic kinase activity might be required for phosphorylation to occur at these sites. As expected, the T175A mutation did not alter recogni-
Mapping of MST1 Kinase Sites of Phosphorylation

**Intermolecular Autophosphorylation of the MST1 Activation Loop**—As noted above, phosphorylation at Thr<sup>177</sup> and Thr<sup>183</sup> within the activation loop was diminished when the inactive K59R kinase was expressed, suggesting possible autophosphorylation at these sites. However, the T183A and T187A mutants are inactive, thus reducing the probability of activation via intramolecular autophosphorylation. On the other hand, most cells do express endogenous MST1, which has high basal activity and might serve to activate newly synthesized MST1. We therefore investigated putative (MST-MST) intermolecular autophosphorylation. To achieve this, we used active kinase constructs to phosphorylate inactive MST1(K59R) in vitro. This inactive mutant cannot autophosphorylate, although its putative phosphorylation sites remain intact. The relative low level of phosphorylation at Thr<sup>183</sup> in MST1(K59R), possibly mediated by endogenous MST kinase, leaves a window for in vitro measurement of transphosphorylation.

Active and inactive kinases were separately expressed in COS-1 cells as truncated (MST1(1–326)) and caspase-resistant (MST1(D326N) and MST1(K59R/D326N)) forms. These could be separated electrophoretically after in vitro kinase reactions were completed. Lysates from separately expressed MST1 constructs were combined to simultaneously immunoprecipitate active and inactive constructs on the same beads. The full-length active kinase (MST1(D326N)) was combined with the truncated inactive kinase (MST1(K59R)-(1–326)), and the full-length inactive kinase (MST1(K59R/D326N)) was combined with the truncated active kinase (MST1(1–326)). As a negative control, we combined both inactive kinases: full-length MST1(K59R/D326N) and...
In vitro kinase assays were performed on the beads as described under "Materials and Methods." Active MST1 constructs effectively autophosphorylated either intra- or intermolecularly when $[\gamma^{32}P]ATP$ was included in the kinase reaction, as measured by phosphorimaging (Fig. 5A). Intermolecular transphosphorylation of full-length and truncated kinase-inactive MST1(K59R) constructs was also observed (compare bands within the same box in Fig. 5A). This occurred when inactive constructs were immunoprecipitated along with the active kinase. As expected, the combination of two inactive constructs resulted in lack of substantial phosphorylation of either species.

To further investigate which residues are targeted by intermolecular autophosphorylation, phospho-specific antibodies recognizing Thr$^{177}$, Thr$^{183}$, and Thr$^{387}$ were used as probes (Fig. 5B). Kinase reactions were performed in the absence of radioactive tracer, and phosphorylation was detected by Western blotting. As expected, some basal phosphorylation of the inactive constructs was observed (second lane of each panel in Fig. 5B), putatively induced by endogenous active MST1. When truncated inactive MST1 was phosphorylated by the full-length active construct, phosphorylation at Thr$^{177}$ was increased by 17-fold, and phosphorylation at Thr$^{183}$ by 4.4-fold. The full-length kinase was also phosphorylated by the truncated active kinase. This increased phosphorylation at Thr$^{177}$ by 4.1-fold and at Thr$^{183}$ by 1.5-fold. The smaller magnitude of increase in phosphorylation at these sites of the full-length K59R mutant reflected both the higher base-line...
phosphorylation in COS-1 cells and the higher base-line phosphorylation of the full-length versus truncated K59R mutant in these cells. The latter effect may be mediated though dimerization with endogenous MST kinase, which cannot be achieved by the truncated mutant.

We also examined phosphorylation at Thr\textsuperscript{183}, which is part of an (R/K)XX(X/S/T) motif targeted by several kinases. A phospho-specific antibody recognizing this motif (phospho-Ab3) strongly recognized MST1(WT) (Fig. 5B), but only weakly recognized the catalytically inactive K59R mutant. The targeted residue (Thr\textsuperscript{183}) was identified by site-directed mutagenesis, with T387A eliminating almost all recognition by this antibody (data not shown). In vitro phosphorylation of the full-length K59R mutant at Thr\textsuperscript{287}, detected by phospho-Ab3, was increased by 7.3-fold by coprecipitation with the wild-type truncated kinase (Fig. 5B). The truncated MST1-(1–326) kinase, which lacks Thr\textsuperscript{287}, was very weakly detected by phospho-Ab3, but only after in vitro kinase reaction. The residue responsible for this reactivity was identified as Thr\textsuperscript{183}, which is part of an (R/K)XX(X/S/T) motif (KRNT). Phospho-Ab3 has known partial cross-reactivity with this motif, and the T183A or T183E mutation abrogated its binding to the truncated 36-kDa MST1 kinase (data not shown).

MST1-MST1 trans-autophosphorylation at the critical residue Thr\textsuperscript{183} was further investigated by cotransfection of N-terminally Myc-tagged and C-terminally V5-tagged constructs into the same cells. Myc-tagged kinases were subsequently immunoprecipitated with anti-Myc antibody after boiling lysates. Immunoprecipitates were probed with anti-Myc (Fig. 5C, upper panel) and anti-V5 (middle panel) antibodies by Western blot analyses, which showed that only Myc-tagged constructs were immunoprecipitated by these means. Blots were then probed for phosphorylation at Thr\textsuperscript{183} using phospho-Ab2. In comparison with expression of Myc-MST1(K59R) alone, coexpression with MST1(WT)-V5 resulted in a 3.7-fold increase in specific phosphorylation (normalized to relative expression levels) at Thr\textsuperscript{183}. When coexpressed with inactive MST1(T183A)-V5, a 30% decrease in phosphorylation was observed versus expression of MST1(K59R) alone.

Together, these data suggest that MST1 is autoactivated by intermolecular transphosphorylation of the activation loop of MST1 at phosphoacceptor sites Thr\textsuperscript{177} and Thr\textsuperscript{183} as well as Thr\textsuperscript{287}. Despite its location within the inhibitory domain, mutation of Thr\textsuperscript{387} to alanine was without apparent effect (data not shown).

MST1 Activates the SAPK/JNK Pathway in Detached Cells—It has been reported that p38 and JNK kinases can be activated by MST1 when either kinase is overexpressed simultaneously with Mst and with either MEK6 or MEK7, respectively (4). No elevation of endogenous p38 and JNK pathways was observed by overexpression of MST1 alone (16). In the present study, we observed that overexpression of MST1 in NIH-3T3 cells caused notable detachment of transfected cells. Furthermore, active MST1 was found mostly in the floating population (Fig. 3C). We reasoned that most downstream signaling activity might also reside within the floating cell population. Whereas MST1(WT) induced cell detachment, the T183A mutant did not (Fig. 2). Nonetheless, a small proportion of the T183A-transfected population, comparable to that of vector control-transfected cells, was nonspecifically detached. We therefore examined separately the floating and adherent cells transfected with MST1(WT) and the T183A mutant (Fig. 6). To obtain a comparable number of floating cells, we transfected 4-fold more NIH-3T3 cells with the T183A mutant than with MST1(WT). Analyses were then performed using equivalent (microgram) amounts of cell lysate derived from the floating or adherent cells from each type of transfection.

Consistent with the observation that active MST1 was found mostly in detached cells (Figs. 3 and 6A, upper panels), phosphorylation at Thr\textsuperscript{183} and Thr\textsuperscript{177} was detected only in the floating cell population by immunoblot analyses (Fig. 6A, lower panels). Although the full-length T183A kinase was not enriched in either population, there was substantial accumulation of the caspase-cleaved kinase in the floating cells. Thus, although T183A, like the vector control, did not induce cell detachment, the activated caspases detected in this small population of cells (Fig. 3) effectively cleaved the inactive MST1 mutant. A minor 36-kDa band of kinase activity was also detected by in-gel kinase assay. Due to the very low number of floating cells after T183A transfection, this level of detection was not observed when lysates were derived from mixed populations of floating and adherent cells (Figs. 2B and 4B). Because active MST1 (wild-type and active mutants) detached substantially more cells, the activity of the expressed kinase was readily detected in lysates derived from combined adherent and floating cells.
Myc-tagged MST1 caspase cleavage product are indicated. Lower panels). Myc-tagged full-length MST1 and the antibody (B) or in-gel kinase assays using myelin basic protein as a substrate (B). MST1(D326N), and substitutions of the phosphoacceptor site Ser 327 with alanine (S327A) and glutamic acid (S327E) were expressed in MCF7 or WTC3 cells for 18 h. Caspase activity was then induced by TNF/CHX treatment for 6 h and repressed by cotreatment (DEVD), -8 (IETD), and -9 (LEHD) activities were measured fluorometrically. Values are given as relative light units (RLU).

p38 seems to depend on the detachment of cells rather than on signaling cascade in NIH-3T3 and COS-1 cells. Activation of p38 seems to depend on the detachment of cells rather than on downstream kinases p38, JNK, and p38 activation may be the indirect consequence of this action. Because similar downstream activation events take place upon expression of truncated and caspase-resistant mutants, this appears to be a shared function of both kinase species.

Effects of Ser327 Phosphorylation on MST1 Cleavage in Caspase-3-positive and Caspase-3-negative Cells—In cells expressing active MST1, in particular the floating cell population, there is substantial cleavage of the kinase to form the MST1(1–326) species. Caspase-3, -6, -7, and -9 were previously shown to cleave MST1 at the consensus sequence DEMD326 in vitro (10, 13).

We next examined the floating cells for activities of the downstream kinases p38 and JNK (Fig. 6B). Consistent with the original hypothesis, analyses using phospho-specific antibodies showed that these kinases were activated only in detached cells; however, the responses differed for each downstream kinase. For p38, phosphorylation levels were comparable in cells expressing MST1(WT), inactive MST1(T183A), or even the vector control (data not shown), suggesting that detachment, and not MST1 activity, drives activation of the kinase. However, because functional MST1 causes cell detachment, p38 activation is a consequence of MST kinase activity. Detection of active p46 and p54 JNK isoforms using phosphospecific antibody also showed that activity was limited to the floating cell population (Fig. 6B). However, in contrast to p38, the massive increase in JNK phosphorylation was detected only in cells expressing active MST1. We also examined phosphorylation of the JNK activator MKK4 at its activation loop. Despite the failure to detect substantial levels of active JNK in the T183A transfectants, a modest level of active MKK4 was detected in these cells. However, this was markedly increased in the floating cells expressing MST1(WT). In separate analyses of adherent cells, activation of MKK4 and JNK was also detected, albeit more weakly. However, the p38 response noted above was not detected. In related analyses, the MST1(D326N) and truncated MST1(1–326) mutants elicited a similar response to that of the wild-type kinase, whereas the response induced by the vector controls resembled that induced by MST1(T183A) (data not shown). Similar results were obtained using COS-1 cells for transfection experiments.

Together, these data suggest that MST1 activates the JNK signaling cascade in NIH-3T3 and COS-1 cells. Activation of p38 seems to depend on the detachment of cells rather than on MST1 kinase activity itself. However, because MST1 causes detachment of transfected cells, p38 activation may be the indirect consequence of this action. Because similar downstream activation events take place upon expression of truncated and caspase-resistant mutants, this appears to be a shared function of both kinase species.

The massive increase in JNK phosphorylation was detected only in cells expressing active MST1. We also examined phosphorylation of the JNK activator MKK4 at its activation loop. Despite the failure to detect substantial levels of active JNK in the T183A transfectants, a modest level of active MKK4 was detected in these cells. However, this was markedly increased in the floating cells expressing MST1(WT). In separate analyses of adherent cells, activation of MKK4 and JNK was also detected, albeit more weakly. However, the p38 response noted above was not detected. In related analyses, the MST1(D326N) and truncated MST1(1–326) mutants elicited a similar response to that of the wild-type kinase, whereas the response induced by the vector controls resembled that induced by MST1(T183A) (data not shown). Similar results were obtained using COS-1 cells for transfection experiments.

Together, these data suggest that MST1 activates the JNK signaling cascade in NIH-3T3 and COS-1 cells. Activation of p38 seems to depend on the detachment of cells rather than on MST1 kinase activity itself. However, because MST1 causes detachment of transfected cells, p38 activation may be the indirect consequence of this action. Because similar downstream activation events take place upon expression of truncated and caspase-resistant mutants, this appears to be a shared function of both kinase species.

Effects of Ser327 Phosphorylation on MST1 Cleavage in Caspase-3-positive and Caspase-3-negative Cells—In cells expressing active MST1, in particular the floating cell population, there is substantial cleavage of the kinase to form the MST1(1–326) species. Caspase-3, -6, -7, and -9 were previously shown to cleave MST1 at the consensus sequence DEMD326 in vitro (10, 13).

To further examine the mechanisms controlling caspase cleavage of MST1 in living cells, we used MCF7 cells, which lack caspase-3 (25), and WTC3/MCF7 cells, which stably express caspase-3. Despite the lack of caspase-3, MCF7 cells undergo apoptosis in response to death factors (26). To measure caspase-3, -6, and -7 activities, we measured cleavage of a DEVD peptide (Fig. 7A). In enzymatic assays of MCF7 cells, DEVDase activity was observed, despite the absence of caspase-3. Activity was stimulated 20-fold by TNF/CHX treatment in MCF7 and WTC3/MCF7 cells. In comparison with TNF/CHX-treated MCF7 cells, WTC3/MCF7 cells had about half of the total caspase activity in the uninduced (control) state and ~10-fold higher activity upon TNF/CHX treatment. Under induced conditions, PARP and MST1(WT) cleavage was observed in a caspase-3-independent manner (Fig. 7B). As expected, cleavage of both substrates was increased by TNF/CHX treatment, and this was completely inhibited by the general caspase inhibitor benzylxycarbonyl-Val-Ala-Asp fluoromethyl ketone (Z-VAD) 50 μM).
Autophosphorylation at Ser\textsuperscript{327} in MST1 has previously been implicated in suppression of caspase cleavage \textit{in vitro} (10). To further examine the role of Ser\textsuperscript{327} phosphorylation in cleavage by caspases in intact cells, we studied the modulation of caspase cleavage using mouse MST1(S327A) and MST1(S327E) in caspase-3–positive (WTC3/MCF7) and caspase-3–negative (MCF7) backgrounds (Fig. 7C). Generation of MST1(S327A) caspase fragment-(1–326) (expressed as percent of total expressed MST1 activity) was essentially identical to that of MST1(WT) and was measured as 4.9% (control) and 12.4% (TNF/CHX-treated) in MCF7 cells and increased to 25% (control) and 48% (TNF/CHX-treated) in WTC3 cells. This suggests that autophosphorylation at Ser\textsuperscript{327} in MST1(WT) is substantially below saturation or that phosphorylation at this site does not confer caspase resistance \textit{in vivo}. However, consistent with autophosphorylation–induced resistance to caspase-6, -7, and/or -9, MST1(S327E) was left completely uncleaved (<0.1%) in MCF7 cells under all conditions, comparable to the D326N substitution. Unexpectedly, cleavage of the S327E mutant was restored in caspase-3–expressing WTC3/MCF7 cells to 11% (control) and 24% (TNF/CHX-treated) (Fig. 7C, right panels). This proteolysis was directly correlated with authentic caspase-3 expression. Specifically, DEVDase activity was evident in apoptotic MCF7 cells (TNF/CHX-treated) (Fig. 7A), but no cleavage of MST1(S327E) was detected (Fig. 7C, left panels). In contrast, despite lower levels of DEVDase activity in WTC3 cells (control), MST1(S327E) was cleaved. Thus, in the living cell, MST1(WT) autophosphorylation at Ser\textsuperscript{327} may preferentially block cleavage in the absence of authentic caspase-3 activity.

\section*{Discussion}

Overexpression of full-length wild-type or truncated active MST1 was previously shown to cause morphological rounding and cell detachment (13). Until now, it was unknown whether MST1 cleavage is a prerequisite for these phenotypes. The present study has defined a shared role for the uncleaved and cleaved kinases in controlling cell morphology. Furthermore, we have defined a role for threonine residues in the activation loop of subdomain VIII (Thr\textsuperscript{186} and Thr\textsuperscript{187}) in the control of catalytic activity. MST1 itself has been identified as an activator via trans-autophosphorylation of subdomain VIII. Nearby, phosphorylation at Thr\textsuperscript{177} influences electrophoretic mobility, possibly due to a conformational change. Finally, we have further defined the role of Ser\textsuperscript{327} phosphorylation in suppressing caspase cleavage.

Agents known to activate MST1 are limited to stress- and apoptosis-inducing chemicals (9). Besides the apoptotic role of caspases, virtually nothing is known about physiological MST1 regulators. Hence, two different studies have demonstrated differing alterations to MST1 kinase by dephosphorylation \textit{in vitro} (5, 10), implicating unidentified Ser/Thr residues in phosphoregulation of kinase activity. To begin, we considered that for many phosphoregulated kinases, activities are frequently regulated via a short region within subdomain VIII called the “activation loop.” In PAK1, trans- or autophosphorylation at Thr\textsuperscript{422} in the C-terminal kinase domain plays a central role in modulating kinase activity (23). Phosphorylation of PAK1 at Thr\textsuperscript{422} renders it insensitive to its inhibitory module, and a T423A substitution reduced kinase activation by 88% (23). Notably, this left intact substantial basal activity, suggesting constitutive activity or additional regulation through other means. Site-directed mutagenesis of MST1 Thr\textsuperscript{186} to alanine demonstrated its crucial role in kinase activation, eliminating catalytic activity to 1–3%. Furthermore, the T183E substitution, mimicking a phosphorylated residue, substantially restored MST1 kinase activity. This and the demonstration of phosphorylation at this site via phosphorylation state-specific antibodies suggest Thr\textsuperscript{183} as a major site of regulation.

Recently, a phosphorylation state-specific antibody directed against Thr\textsuperscript{402} of PAK2, analogous to Thr\textsuperscript{423} of PAK1 (27), was found to cross-react with endogenous α-PAK (PAK1) and MST kinases in osmotically or chemically stressed neutrophils (28). We utilized this sequence conservation to prove phosphorylation at Thr\textsuperscript{183} of MST1 in the wild-type kinase. While this manuscript was in preparation, Lee and Yonehara (29) described a role for Thr\textsuperscript{183} phosphorylation in nucleocytoplasmic shuttling of MST1 dimers whereby Thr\textsuperscript{183} phosphorylation favors nuclear import of MST1. However, there was no indication as to whether or not a T183A mutation alters catalytic activity. As noted above, we show here that phosphorylation or substitution with an acidic residue at this site is critical for activity. In addition, we identified Thr\textsuperscript{187} as essential for MST1 activity, although activity was not supported by an acidic substitution (T187E).

Two other sites within the MST1 activation loop, Thr\textsuperscript{175} and Thr\textsuperscript{177}, were also considered as candidates for phosphoregulation, but neither was found to be critical for kinase activity. It is notable, however, that the caspase-cleaved wild-type and T177E kinases migrated almost exclusively as a faster species during electrophoresis, whereas T177A showed exclusively reduced mobility. This implicates autophosphorylation at this site in the mobility shift.

We further identified Thr\textsuperscript{387} located within the inhibitory domain (30) as a putative site for autophosphorylation. However, substitutions at Thr\textsuperscript{387} did not appreciably alter kinase activity or morphological rounding and cell detachment (data not shown).

Intra- and intermolecular autophosphorylation at this and other sites seem possible, although previous work suggested Ser\textsuperscript{327} as the primary site for MST1 autophosphorylation \textit{in vitro} because an S327A mutation reduced autophosphorylation by ~90% (10). In consideration of our data, it is likely that phosphorylation at Ser\textsuperscript{327} is well below saturation in the living cell, whereas other phosphoacceptor sites identified here are saturated. As such, Ser\textsuperscript{327} would show the greatest responses \textit{in vitro}. In the present study, \textit{in vitro} transphosphorylation was more easily detected using inactive MST1(K59R) as a substrate. Pre-existing (but reduced) phosphorylation in K59R was detected in the basal state, likely attributable to the intermolecular activities of endogenous MST kinases. Because this resulted in subsaturating levels of phosphorylation at Thr\textsuperscript{177}, Thr\textsuperscript{183}, and Thr\textsuperscript{387}, this left a sizable window for detection of transphosphorylation \textit{in vitro}. By combining K59R with catalytically active MST1, we indeed found substantial increases in phosphorylation at Thr\textsuperscript{177}, Thr\textsuperscript{183}, and Thr\textsuperscript{387} using the phospho-specific antibodies. This indicated that MST1 is capable of intermolecular phosphorylation \textit{in vitro} at all of these sites, and this was further supported in a cell-based assay showing increased phosphorylation at Thr\textsuperscript{183} by cotransfection of catalytically active MST1 (Fig. 5C).

Although intramolecular phosphorylation at all identified sites is still possible, it is unlikely for Thr\textsuperscript{183} (or Thr\textsuperscript{187}) because an alanine substitution at this site, which mimics the unphosphorylated state, abrogated kinase inactivity. We propose that intermolecular phosphorylation at Thr\textsuperscript{183} (and possibly Thr\textsuperscript{187}), mediated by dimerization with resident endogenous MST kinase, provides the means for activation of the newly synthesized kinase. It is notable that the base-line phosphorylation at Thr\textsuperscript{177} and Thr\textsuperscript{183} in the full-length inactive kinase was higher than that in the truncated kinase, which is incapable of dimerization with endogenous MST kinases (Fig. 5B,
second lanes). As expected, due to lower base-line phosphorylation, the in vitro phosphorylation at Thr\textsuperscript{177} and Thr\textsuperscript{183} was increased to 4- and 3-fold higher levels, respectively, than in the full-length kinase.

The fifth phosphoacceptor site of MST1 (Ser\textsuperscript{327}) controls an apoptotic feature of MST1 both in vitro (10) and in intact cells (this study). As a result of caspase cleavage, MST1 can change its cellular localization (13) and accumulate in the nucleus, this study. As a result of caspase cleavage, MST1 can change in vitro caspase-9 (31), which itself can cleave MST1. It is important to consider that caspase-3 itself may not mediate DEVDase activity (attributable to caspase-6 and caspase-7), showing less overall DEVDase activity in the uninduced or TNF/CHX treatment. In contrast, caspase-3-expressing cells, DEVDase activity (attributable to caspase-6 and caspase-7), than with the overall level of caspase activity. Indeed, MCF7 cells lacking caspase-3 exhibited substantial “caspase-3-like” DEVDase activity (attributable to caspase-6 and caspase-7), yet showed no cleavage of the SS27E mutant at all even after TNF/CHX treatment. In contrast, caspase-3-expressing cells, showing less overall DEVDase activity in the uninduced or control state, exhibited substantial cleavage of MST1(S327E).

It is important to consider that caspase-3 itself may not mediate MST1 cleavage in these cells. Caspase-3 can activate caspase-9 (31), which itself can cleave MST1 in vitro (10). Moreover, LEHDase (caspase-9) activity was elevated in TNF-treated WTC3/MCF7 cells expressing caspase-3.

We have observed that kinase-dead MST1 mutants, which are incapable of autophosphorylation at Ser\textsuperscript{327} or any other site, were poorly cleaved in the basal state, but cleaved robustly upon TNF/CHX treatment (data not shown). This suggests that control of cleavage lies outside of the kinase itself. MST1 is believed to stimulate caspase activity directly (4) or to potentiate caspase activation induced by exogenous stimuli (13). In our hands, MST1 induced caspase activation, although only in the detached cells expressing MST1. It is important to note that the caspase-insensitive D326N mutant maintained its ability to induce morphological rounding and detachment. Therefore, caspase cleavage in and of itself is not a prerequisite for eliciting this response. That the detached cells expressing active MST1 exhibited ~2-fold greater caspase activity than similar vector controls suggests some level of direct caspase activation. The most substantial effects were generated through the capacity of active MST1 to increase the number of detached cells and thus increase the total caspase activity within the larger cell population. Thus, caspase activation appears to be a combined consequence of direct action, cell detachment (analogous to anoikis), and, possibly, downstream signaling.

This and previous studies (4) have suggested that activated MST1 can, independent of its cleavage by caspases, signal downstream to JNK and p38 kinases. In NIH-3T3 and COS-1 cells, MKK4 and JNK phosphorylation was induced, and this was strongly correlated with the activity of various MST1 mutants. Activation of MKK7, previously reported (4), was not assessed in the present study. When observed, p38 activation correlated with cell detachment and not with MST1 expression or activity. Thus, in this system, JNK activation appears to be a direct consequence of MST1 kinase activity, whereas p38 activation seems to be an indirect response to changes in cell adhesion.

In summary, our results demonstrate, for the first time, that two residues (Thr\textsuperscript{183} and Thr\textsuperscript{187}) within the MST1 activation loop are essential for kinase activation. Phosphorylation at Thr\textsuperscript{183} (and possibly Thr\textsuperscript{187}) is achieved by intermolecular autophosphorylation within the MST1 dimer. These residues are highly conserved within all members of the MST kinase family and SOK1, suggesting possible similar requirements for phosphoactivation within this group of protein kinases. Once activated, MST1 elicits cell detachment and JNK activation independent of caspase cleavage. MST1 phosphoregulation is even further extended to modulate its own caspase cleavage at Ser\textsuperscript{327}, however, only where and when caspase-3 is absent.

REFERENCES
1. Dan, I., Watanabe, N. M., and Kusumi, A. (2001) *Trends Cell Biol.* 11, 220–230
2. Hagemann, C., and Blank, J. L. (2001) *Cell. Signal.* 13, 863–875
3. Wu, C., Whiteway, M., Thomas, D. Y., and Leberer, E. (1995) *J. Biol. Chem.* 270, 15984–15992
4. Graves, J. D., Gotch, V., Draves, K. E., Ambrose, D. H., Han, D. K., Wright, M., Chernoff, J., Clark, E. A., and Krebs, E. G. (1998) *EMBO J.* 17, 2224–2234
5. Creasy, C. L., and Chernoff, J. (1995) *J. Biol. Chem.* 270, 21695–21700
6. Creasy, C. L., and Chernoff, J. (1995) *Genes (Amst.)* 167, 303–308
7. Qian, Z., Lin, C., Espinosa, R., LeBeau, M., and Rosner, M. R. (2001) *J. Biol. Chem.* 276, 22439–22445
8. Schinkmann, K., and Blenis, J. (1997) *J. Biol. Chem.* 272, 28695–28703
9. Taylor, J. R., Wang, H. C., and Erikson, R. L. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93, 10099–10104
10. Graves, J. D., Draves, K. E., Gotch, V., Krebs, E. G., and Clark, E. A. (2001) *J. Biol. Chem.* 276, 14909–14915
11. Rudel, T., and Bokoch, G. M. (1997) *Science* 276, 1571–1574
12. Sells, M. A., Kraus, U. G., Bagrodia, S., Ambrose, D. M., Bokoch, G. M., and Chernoff, J. (1997) *Curr. Biol.* 7, 202–210
13. Lee, K. K., Ohyama, T., Yajima, N., Tsubuki, S., and Yonehara, S. (2001) *J. Biol. Chem.* 276, 19276–19285
14. Ura, S., Masuyama, N., Graves, J. D., and Gotch, V. (2001) *Genes Cells* 6, 519–520
15. Ura, S., Masuyama, N., Graves, J. D., and Gotch, V. (2001) *Proc. Natl. Acad. Sci. U. S. A.* 98, 10148–10153
16. Lee, K. K., Murakawa, M., Nishida, E., Tsubuki, S., Kawashima, S., Sakamaki, K., and Yonehara, S. (1998) *Oncogene* 16, 3029–3037
17. Reszka, A. A., Halasy-Nagy, J., Rodan, G. A. (1999) *J. Biol. Chem.* 274, 34967–34973
18. Wagner, M., Kakeya, H., and Osada, H. (1999) *Oncogene* 18, 5211–5220
19. Kakeya, H., Onose, R., and Osada, H. (1999) *Ann. N. Y. Acad. Sci.* 886, 273–275
20. Gatti, A., Huang, Z., Tuazon, P. T., and Traugh, J. A. (1999) *J. Biol. Chem.* 274, 8022–8026
21. Walter, B. N., Huang, Z., Jakobi, R., Tuazon, P. T., Almenri, E. S., Litwack, G., and Traugh, J. A. (1999) *J. Biol. Chem.* 274, 28733–28739
22. Reszka, A. A., Halasy-Nagy, J., and Rodan, G. A. (2001) *J. Biol. Chem.* 276, 20293–20302
23. Zenke, F. T., King, C. C., Bohl B. P., and Bokoch, G. M. (1999) *J. Biol. Chem.* 274, 23055–23057
24. Zhang, J., Zhang, F., Ebert, D., Cobb, M. H., and Goldsmith, E. R. (1995) *Structure* 3, 299–307
25. Liang, Y., Yan, C., and Schor, N. F. (2001) *Oncogene* 20, 6570–6578
26. Measner, U. K., Peredo-Fernandez, C., Manderscheid, M., and Pfeilschifter, J. (2001) *Br. J. Pharmacol.* 133, 467–476
27. Jakobi, R., Huang, Z., Walter, B. N., Tuazon, P. T., and Traugh, J. A. (2000) *Eur. J. Biochem.* 267, 4414–4423
28. Lian, J. P., Toker, A., and Badwey, J. A. (2001) *J. Immunol.* 166, 6349–6357
29. Lee, K. K., and Yonehara, S. (2002) *J. Biol. Chem.* 277, 12351–12358
30. Creasy, C. L., Ambrose, D. M., and Chernoff, J. (1996) *J. Biol. Chem.* 271, 21049–21053
31. Fujita, E., Egashira, J., Urase, K., Kuida, K., and Momoi, T. (2001) *Cell Death Differ.* 8, 335–344
