Cloning and Characterization of a Human Protein Kinase with Homology to Ste20*

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Caretha L. Creasy‡ and Jonathan Chernoff
From the Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111

A human protein kinase (termed MST1) has been cloned and characterized. The MST1 catalytic domain is most homologous to Ste20 and other Ste20-like kinases (62–65% similar). MST1 is expressed ubiquitously, and the MST1 protein is present in all human cell lines examined. Biochemical characterization of MST1 catalytic activity demonstrates that it is a serine/threonine kinase, and that it can phosphorylate an exogenous substrate as well as itself in an in vitro kinase assay. Further characterization of the protein indicates MST1 activity increases approximately 3-4-fold upon treatment with PP2A, suggesting that MST1 is negatively regulated by phosphorylation. MST1 activity decreases approximately 2-fold upon treatment with epidermal growth factor; however, overexpression of MST1 does not affect extracellular signal-regulated kinase-1 and -2 activation. MST1 is unaffected by heat shock or high osmolarity, indicating that it is not involved in the stress-activated or high osmolarity glycerol signal transduction pathways. Thus MST1, although homologous to a member of a yeast MAPK cascade, is not involved in the regulation of a known mammalian MAPK pathway and potentially regulates a novel signaling cascade.

Regulation of cell growth and differentiation utilizes a complex mechanism of signaling involving the catalytic activities of protein kinases and phosphatases. The mechanisms used in signal transduction are well conserved in all eukaryotes. In mammalian cells external stimuli acting on both growth factor receptors and some G protein-coupled receptors signal through a kinase cascade resulting in the activation of members of the mitogen-activated protein kinase (MAPK) family, ERK1 and ERK2 (1–3). ERK1/2 phosphorylate a variety of substrates including transcription factors and other kinases (4–6). Activation of ERK1/2 requires phosphorylation of both tyrosine and threonine residues which is mediated by a dual specific kinase termed MEK (7, 8). MEK activation may represent a convergence point for signaling since it is known that both Raf and MEKK phosphorylate MEK (9–11). Studies involving pheromone signaling in both budding (Saccharomyces cerevisiae) and fission (Schizosaccharomyces pombe) yeast have revealed that similar signal transduction mechanisms operate in these evolutionarily divergent organisms (12). Additional signal transduction cascades have since been identified in S. cerevisiae and more recently in mammalian cells. These include pathways responsible for cell wall biosynthesis, hydropertonic sensing, and spore formation in S. cerevisiae and those activated by stress and high osmolarity in mammalian cells (13–23). Structural homologies exist between the mammalian and yeast pathways at the level of MAPK, MEK, and MEKK (11). Complementation studies in the aferomone response pathway with mammalian homologs indicate that the proteins are also somewhat functionally related (24, 25).

In S. cerevisiae a serinethreonine kinase termed Ste20 acts upstream of the MEKK, Ste11, and downstream of the pheromone-linked G protein in the mating pathway (26, 27). The recent identification of rat and human homologs to Ste20, termed p65pask, indicates that an additional level of conservation exists among signal transduction cascades from distantly related species (28). However, the identification of a Ste20 homolog in S. cerevisiae, termed Sp4, which is involved in the spore formation pathway, suggests that conservation at this level in the cascade may be more complex (18). Sp4, while similar to both Ste20 and p65pask throughout the catalytic domain, does not share any significant homologies outside this domain.

We have cloned and characterized a human protein kinase with a similar degree of homology to Ste20, p65pask, Sp4, and a human protein kinase termed GC kinase (29). Because of its homology to Ste20 we have termed this protein MST1 (Mammalian Sterile Twenty-like). Northern analysis indicates that MST1 is ubiquitously expressed. Biochemical characterization of MST1 indicates that it is a serinethreonine kinase and that it may be negatively regulated by phosphorylation. MST1 does not function in the MAPK signal transduction cascade nor is its activity increased in response to growth factors, heat shock or high osmolarity suggesting that it is involved in an as yet unidentified signal transduction pathway.

EXPERIMENTAL PROCEDURES

cDNA Cloning and Sequencing—Degenerate oligonucleotide primers, 5'-CGGATTCGCGGARHTGNGCNGTAAARGC-3' and 5'-CGGATTCVYTYNNACYTNGGSGSCTTARAA-3' encoding the sense and antisense strands of amino acids, GE(I/L/M)MAKGV and FWMA/S/T/R/G)PVE(W/M)I/VD/E/K/N, flanked by restrictions sites for BamHI and EcoRI, respectively, were used to amplify DNA from a human lymphocyte cDNA library (30) using Vent polymerase (New England Biolabs). An aliquot of the reaction was used for a second
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round of amplification using the same 5′ oligonucleotide and 5′-CG-GAATTCRTTNGCNCYTNTCNRCKRTG-3′, which encodes the antisense strand of H(RS/DVM/IK)KGAN. A polymerase chain reaction was carried out using an Idaho technologies thermal cycler (an initial denaturation at 94 °C for 1 min, 30 amplification cycles of 1 s at 94 °C, 5 s at 35 °C, 15 s at 65 °C, followed by 1 min at 74 °C). A fragment of approximately 340 bp was obtained and used to screen 5 × 105 plaques from the same cDNA library using standard methods (31). Those clones containing the largest inserts were restricted and both strands sequenced using Sequenase 2.0 (U.S. Biological Corp.). Amino acid sequence comparisons were made using the University of Wisconsin Genetics Computer Groups programs, Pileup and Prettyploy (plurality = 3.5) (36).

Plasmids—The plasmids, pJ3M-MST1, pJ3H-MST1, or pcMV5MST1, were used in transient transfections where indicated. Oligonucleotide primers, 5′-CCGGATCCCGCAGCGGAGGCTACG-3′ and 5′-GAATTCCTCGAGGCCACG-3′ (linker sequence) were used to amplify the MST1 cDNA. The amplified fragment was digested with BamH I and EcoR I (at bp 1329, a site 3′ to the stop codon at 1483 bp) and subcloned into pJ3M or pJ3H, SV40-based vectors containing a myc-epitope tag or hemagglutinin epitope tag (HA) 5′ to the BamHI site (32). To construct pcMV5-MMST1, Myc-1 was removed from a HindIII-XhoI fragment and subcloned into the HindIII-SalI site of pcMV5 (33). The plasmid pJ3H-ERK contains ERK1 cloned as a BamH I-EcoR I fragment into pJ3H (32).

Northern Blot Analysis—An adult human multitu-tissue Northern blot (Clontech) was hybridized with a random primed (Stratagene) 32P-labeled MST1 probe generated from either a region encoding the catalytic domain (nucleotides 178–481) or the 3′-untranslated region (nucleotides 1529–1930). Hybridization was carried out at 65 °C essentially as described elsewhere (31). The integrity of the mRNA was confirmed via hybridization to a 32P-labeled actin probe.

Cell Culture—Cell lines were grown in the appropriate growth media (AG876, RPMI 1640, 10% fetal bovine serum; NIH3T3, DMEM, 10% calf serum; HeLa, COS, 293, A431, and Rat1A, DMEM, 10% fetal bovine serum) containing 50 units/ml penicillin, 50 μg/ml streptomycin, and 100 μg/ml mycillin where indicated. Cells were grown to confluence, washed twice in serum-free growth medium, and subcloned into 6-well dishes (Costar) in 3 ml of 62.5 mM glycerol phosphate, 1 mM MgCl2, 0.1 mM EGTA, 50% glycerol, or buffer alone was added. The reaction mixtures were incubated at 30°C for 1 h followed by three washes in Nondenaturating SDS sample buffer. The beads were then resuspended in SDS sample buffer and boiled for 5 min.

Western Blot Analysis—Proteins resolved on a 10% SDS-polyacrylamide gel (35) were transferred to a PVDF membrane in transfer buffer (125 mM Tris, 96 mM glycine, 20% methanol) and placed at 100 V for 1 h. The membrane was blocked overnight in 5% nonfat milk (50 ml of 10% nonfat milk, 5 ml of 10% BSA, 0.05% Tween-20) and incubated with the 32P ATP (33). The blots were washed three times in 1× TBS-T20 for 5 min each, goat-α-rabbit conjugated alkaline phosphatase antibodies (1:5000) (Sigma) or goat-α mouse conjugated horseradish peroxidase antibodies (1:4000) (Bio-Rad) were added. Immunoprecipitated complexes were washed three times in 1× TBS-T20 for 5 min each, goat-α-rabbit conjugated alkaline phosphatase antibodies (1:5000) (Sigma) or goat-α mouse conjugated horseradish peroxidase antibodies (1:4000) (Bio-Rad) were added. Immunoprecipitated complexes were washed three times in 1× TBS-T20 for 5 min each.

Fig. 1. Nucleotide and predicted amino acid sequence of MST1. The proposed initiation codon is in bold. The region amplified using degenerate oligonucleotides is underlined. Amino acids (in one-letter code) and nucleotides are numbered at the left and right, respectively.
Amino Acid Sequence Analysis—The MST1 cDNA encodes a 487-amino acid protein with an expected molecular mass of 55,721 Da. The amino-terminal half of the protein contains the 11 subdomains characteristic of a serine/threonine kinase (Fig. 2) (42). The carboxyl-terminal half of the protein lacks any notable sequence motifs; however, this portion of the protein is quite acidic (pI of 4.4). The MST1 kinase domain is most similar to Ste20, p65pak, Sps1, and GC kinase (64, 64, 62, and 65%, respectively). While Ste20 expressed at high levels is able to complement a ste20 null allele, MST1 expressed from the same promoter does not complement, indicating that these genes are not functionally homologous (data not shown). Unlike Ste20 and p65pak, MST1, Sps1, and GC kinase (64, 64, 62, and 65%, respectively). While Ste20 expressed at high levels is able to complement a ste20 null allele, MST1 expressed from the same promoter does not complement, indicating that these genes are not functionally homologous (data not shown). Unlike Ste20 and p65pak, MST1, Sps1, and GC kinase do not contain recognizable Cdc42/Rac1 binding elements. Overall the organization of MST1 is more similar to that of Sps1 and GC kinase which have a putative regulatory domain at the carboxyl terminus; however, there does not appear to be any striking similarities within this domain.

Expression of MST1—The expression of MST1 was examined in a variety of adult human tissues. A probe specific to the kinase domain (encompassing nucleotides 178–481) identified a transcript at approximately 7 kb in all tissues and another at approximately 3.4 kb which is very abundant in kidney, placenta, and skeletal muscle tissues (Fig. 3). However, a probe specific to the 3′-untranslated region (nucleotides 1529–1930) hybridized to only the larger 7-kb transcript (data not shown). The smaller transcript is most likely either a homolog to MST1 or a splice variant of the larger transcript.

Western Blot Analysis—Consistent with the mRNA expression pattern, a polyclonal antiserum (m1–45) directed against the carboxyl terminus of MST1 (amino acids 276–487) identified a 56-kDa polypeptide in all human cell lines examined (AG876, A431, 293, and HeLa) and also in monkey cells (COS) (Fig. 4A). A less prominent, slightly smaller polypeptide was also identified in AG876, 293, and A431 cells. In addition, the 56-kDa polypeptide could be immunoprecipitated from COS

Fig. 2. A comparison of the MST1 catalytic domain to Ste20, p65pak, Sps1, and GC kinase. A, the 11 subdomains conserved in protein kinases are indicated in roman numerals above the sequences. The numbers at the right indicate amino acids. B, schematic shows the percent amino acid similarity between MST1 and related kinases. The percentages were obtained by comparing the catalytic domains of each deduced amino acid sequence.
cells using the same antibody. However, MST1 was not detected in either rat cell line (Rat1A or PC12) and was detected at very low levels in mouse cells (NIH3T3). When used to transfect COS cells, Myc-epitope tagged MST1 (Myc-MST1) was detected as a slightly slower migrating band than endogenous MST1 (Fig. 4A). This increase in molecular mass is consistent with the size of the Myc-epitope.

**MST1 Is a Serine/Threonine Kinase—** Endogenous MST1 was immunoprecipitated from COS cells (lane 9) and immunoprecipitated Myc-epitope tagged MST1 (lanes 11) from transiently transfected COS cells (lane 10). Immunoblotting with the anti-MST1 antibody (m1–45) and subsequent detection was performed as described under “Experimental Procedures.” B, in vitro kinase assays. Endogenous MST1 was immunoprecipitated from COS cells with m1-45 (lane 1). As a negative control an equal amount of protein was immunoprecipitated with preimmune serum (lane 2). Myc-MST1 was immunoprecipitated from transiently transfected COS cells using the anti-myc antibody, 9E10 (lane 3). As a negative control COS cells were mock transfected with the vector alone (lane 4). Immunoprecipitations were incubated in kinase buffer containing [γ-32P]ATP as described under “Experimental Procedures.” C, phosphoamino acid analysis of in vitro phosphorylated MST1 (B, auto) and myelin basic protein (B, MBP). Phosphoamino acids were resolved in one dimension using a pH 2.5 buffer (31). Positions of unlabeled phosphoamino acids are indicated below the autoradiograph.

**MST1 Does Not Activate the ERK1/2 MAPK Pathway—** To determine if MST1 functions to activate the ERK1/2 pathway, COS cells were transiently transfected with Myc-MST1. Upon stimulation of starved COS cells with EGF, endogenous ERK1 is upshifted and kinase activity increases 8-fold; however, no upshift or increase in ERK1 kinase activity occurred when MST1 was overexpressed nor did overexpression of MST1 block activation of ERK1 by EGF (Fig. 5A).
Western blot analysis of Myc-MST1 transfected COS cells with anti-Myc antibodies shows a protein migrating at 57 kDa consistent with the size of tagged MST1; however, a smaller protein (approximately 40 kDa) is also recognized that has significantly greater kinase activity than the p57 form (Fig. 5, protein (approximately 40 kDa) is also recognized that has with anti-Myc antibodies shows a protein migrating at 57 kDa cated immunoprecipitates using anti-HA antibodies.

described under "Experimental Procedures." PP2A treated immunoprecipitates were separated in an SDS-polyacrylamide gel containing 0.25 mg/ml MBP followed by an in gel kinase assay as described under "Experimental Procedures." B, Western blot of indicated immunoprecipitates using anti-HA antibodies.

To determine if the phosphorylation state of MST1 affected kinase activity, immunoprecipitated HA-MST1 was treated with calf serum, lysophosphatidic acid, 12-O-tetradecanoylphorbol-13-acetate, AlF₄⁻, heat shock, or high salt; however, while positive controls were activated, MST1 activity was not affected (data not shown).

PP2A Treatment Stimulates MST1 Kinase Activity—To determine if the phosphorylation state of MST1 affected its kinase activity, immunoprecipitated HA-MST1 was treated with the protein serine/threonine phosphatase, PP2A, and subjected to an in gel kinase assay with MBP as the substrate. Immunoprecipitated HA-ERK1 with and without EGF stimulation is shown as a control. While stimulated ERK1 activity decreased substantially upon treatment with PP2A, MST1 activity increased ~3-4-fold indicating that the enzyme may be negatively regulated by serine/threonine phosphorylation (Fig. 6). We do not believe the increase in activity is due to the removal of phosphate from potential autophosphorylation sites since in the absence of MBP an increase in kinase activity is not seen following treatment with PP2A (data not shown).

We have cloned and characterized a human protein kinase with considerable similarity to Ste20 and other Ste20-like kinases. Biochemical characterization of this kinase indicates that it is a prominent renaturable kinase in COS cells. We have tentatively named this kinase MST1 pending an assignment of a biological function. MST1 is expressed at approximately equal levels in all tissues. The MST1 protein is present in all human and monkey cell lines that we examined; however, it could not be detected in either rat cell line (Rat1A and PC12) and only at low levels in a mouse cell line (NIH3T3). We do not believe this is due to the inability of the MST1 polyclonal antiserum to recognize rodent MST1 since MST1 can be immunoprecipitated from NIH3T3 cells using the same antiserum (data not shown), rather MST1 is present at low levels in these lines.

An in vitro kinase assay using immunoprecipitated MST1 demonstrated that it can phosphorylate itself and an exogenous substrate, and phosphoamino acid analysis indicated that MST1 is a serine/threonine kinase. While MST1 kinase activity is readily detectable in an in vitro and in gel kinase assay, we do not believe MST1 is in its most active state for two reasons. First, treatment of MST1 immunoprecipitated from starved COS cells with PP2A results in an approximate 3-4-fold increase in kinase activity. We do not see any difference in MST1 activity throughout the cell cycle (data not shown); therefore, we do not believe that inhibition of activity is specific to quiescence. This result suggests that MST1 is held in a partially inactive state through serine/threonine phosphorylation. Second, a carboxyl-truncated form of MST1, arising apparently from proteolysis, is much more active than full-length MST1 (Fig. 5). This latter result indicates that the carboxyl terminus has an inhibitory role.

While the catalytic domains of MST1 and Ste20 are 64% similar, it is unable to complement a ste20 null allele in S. cerevisiae using a quantitative mating assay. Also, overexpression of MST1 does not activate ERK1/2 in COS cells. The inability of several growth factors, heat shock, or high osmolarity to increase MST1 activity indicates that MST1 is not involved in the MAPK or the HOG signal transduction cascades. The 2-3-fold decrease in MST1 activity following EGF stimulation, while modest, may indicate that MST1 is involved in a pathway which is affected by growth factors acting on tyrosine kinase receptors that is distinct from the MAPK pathway. However, because MST1 does not appear to be in its most active state, it is difficult to determine if a decrease in this low level of activity is biologically significant. The assignment of MST1 to a particular signal transduction cascade awaits identification of effectors of MST1 kinase activity and the target(s) of its catalytic activity.

At this time we do not understand the significance of the homologies between MST1, Ste20, and the other Ste20-like kinases. The kinase domains of all of these proteins are quite conserved and represent what is becoming a growing family of Ste20-like kinases. The high degree of similarity between Ste20 and p65ak, including the conservation of the Cdc42/Rac1 binding element, suggests that these proteins perform similar functions and may represent a distinct subfamily, while MST1, GC kinase, and SPS1 may be a part of another subfamily. Identification of substrates for these Ste20-like kinases may help determine the importance of these similarities.

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Fig. 6. PP2A treatment stimulates MST1 kinase activity. COS cells were transiently transfected with vector alone, pJ3H-ERK1 or pJ3H-MST1, starved for 20 h, followed by treatment with EGF (400 ng/ml) for 10 min. After immunoprecipitation each sample with divided equally in two and treated with PP2A (0.5 unit) or mock treated with buffer alone as described under "Experimental Procedures." A, PP2A treated immunoprecipitates were separated in an SDS-polyacrylamide gel containing 0.25 mg/ml MBP followed by an in gel kinase assay with MBP as the substrate. Immuno-
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