Mitogen-activated protein kinases (MAPKs) are highly conserved mediators of signal transduction that are present in all eukaryotes and play essential roles in regulating cell differentiation, cell proliferation, and cell death. The core of the cascade consists of three sequentially acting protein kinases: a MAPK, a MAPK kinase, and a MAPK kinase kinase (1). In yeast, there are five MAPK cascades that have pivotal roles in cellular signaling pathways. After recovery in SOC medium (GIBCO BRL) at 37 °C for 3 h, cells (Stratagene), which are deficient in three of the primary DNA repair pathways. After recovery in SOC medium (GIBCO BRL) at 37 °C
for 1 h, the culture was diluted to 10 ml of SOC for further incubation overnight. The next day, 200 μl of the culture was diluted with a fresh 10 ml of SOC and grown overnight, while the remaining culture was used for plasmid DNA preparation. The second overnight culture was further diluted for another overnight incubation and plasmid DNA preparation. A plasmid overnight culture was also used for PCR analysis of plasmid DNA. These three plasmid DNA preparations were pooled together as the mutated PEG202-c-Raf library, which was transformed into yeast EGY48 along with PJG-MEK in order to screen for a mutated PEG202-c-Raf protein that has a high affinity for MEK. A mutant construct was isolated (PEG202-Raf-M), and we confirmed that the mutant Raf-M had an enhanced affinity for MEK by retransforming the PEG202-c-Raf-M construct back into yeast EGY48 with PJG-4-5-MEK and testing its interaction with MEK. Sequencing the c-Raf insertion in PEG202 revealed a mutation at residue Ser420 that introduced a new stop codon at this site, resulting in a truncated C-terminal Raf protein.

Yeast Two-hybrid Screen—The LexA yeast two-hybrid system was kindly provided by Dr. Roger Brent. A human fetal library, made from a 22-week-old human fetal frontal cortex, was used to search for proteins interacting with the kinase domain of mutant PEG202-Raf-M (see above). Two clones showing strong interaction with Raf were obtained. Routine yeast work and yeast transformation were performed as described (36).

PCR Analysis of cDNA Libraries—DNA from two human fetal brain libraries, a human testes library, and multiple human tissue cDNA panels from CLONTECH were analyzed by polymerase chain reaction (PCR) to identify proteins that interact with Raf. A modified Raf bait plasmid, we obtained two cDNA clones containing the predicted amino acid sequences of MST4a, isolated by a yeast two-hybrid screen, and the full-length MST4a generated by PCR. Subdomains characteristic of protein kinases are indicated by Roman numerals. The kinase subdomains IX, X, and XI are underlined. Sequences in common between MST4 and MST4a are shaded.

immune complex was washed three times with lysis buffer and two times with kinase buffer (20 mM Hepes, pH 7.4, 10 mM MgCl₂, 1 mM MnCl₂, 1 mM dithiothreitol, 0.2 mM sodium vanadate, 10 mM p-nitrophenyl phosphate). 5 μg of MgB₃ substrate was used per reaction in kinase buffer containing 5 μCi of [γ-³²P]ATP. The kinase reaction was incubated for 25 min at 30 °C and then boiled with sample buffer for 5 min. The reaction products were separated on 12% SDS-polyacrylamide gels and transferred to a nitrocellulose membrane. Western blot analysis was performed as described previously (37). For MAPK assays, either HA-ERK2, HA-JNK, or HA-p38 was expressed in cells along with FLAG-MST4 expression vectors and/or Myc-Raf-1 expression vectors as indicated and immunoprecipitated with anti-HA antibodies as above. Kinase activities were monitored by immunoblotting with the appropriate anti-phospho-ßTyr antibodies (New England Biolabs, Boston).

Fluorescence in Situ Chromosomal Hybridization—Human metaphase cells were prepared from phytohemagglutinin-stimulated peripheral blood lymphocytes. The MST4 probe was a cDNA probe containing full-length MST4a. Fluorescence in situ hybridization was performed as described previously (38). Biotin-labeled probes were prepared by nick translation using Bio-16-dUTP (Enzo Diagnostics). Hybridization was detected with fluorescein-conjugated avidin (Vector Laboratories), and chromosomes were identified by staining with 4,6-diamidino-2-phenylindole dihydrochloride.

RESULTS

Molecular Cloning of MST4 and MST4a, New Members of the Mammalian Ste20-like Kinase Family—A human fetal brain library was employed in a LexA-based yeast two-hybrid screen to identify proteins that interact with Raf. A modified Raf bait was created by mutagenizing the yeast plasmid PEG202 containing the catalytic domain of Raf and selecting for enhanced interaction with MEK1 (see “Materials and Methods”). Using the modified Raf bait plasmid, we obtained two cDNA clones that coded for the same previously uncharacterized cDNA. Sequence analysis showed that both clones encoded a polypeptide of 354 amino acids, which was later termed MST4a (Fig. 1).

Eukaryotic protein kinases have a common catalytic core structure in their kinase domain, which typically contains 11

FIG. 1. Sequence comparison of MST4 and MST4a. Shown are the predicted amino acid sequences of MST4a, isolated by a yeast two-hybrid screen, and the full-length MST4a generated by PCR. Subdomains characteristic of protein kinases are indicated by Roman numerals. The kinase subdomains IX, X, and XI are underlined. Sequences in common between MST4 and MST4a are shaded.
PCR from different cDNA libraries. Left panel, the PCR products were amplified from placenta and testis Marathon ready cDNA and fetal brain cDNA. Right panel, PCR products were amplified from CLONTECH multiple human tissue cDNA panels. Samples were subjected to electrophoresis in a 1.2% agarose gel as described under "Materials and Methods."

conserved subdomains (39). Comparison of the MST4a protein sequence with that of MST family members revealed that MST4a lacked part of the kinase domain. In order to see if MST4a might be a splicing variant of a full-length MST cDNA, the kinase subdomains I–XI in MST4 cDNAs from three separate libraries were examined by PCR analysis. As shown in Fig. 2 (left panel), two PCR products with different sizes were amplified from a fetal human brain (CLONTECH) cDNA library that was different from the human brain cDNA library used in the original yeast two-hybrid screen as well as from a testes and a placenta library. Sequence analysis indicated that the smaller PCR product had the same sequence as the kinase domain of MST4a, and the larger PCR product had an additional cDNA insert. Subcloning the full-length fragments into an expression vector and subsequent sequencing revealed that the larger PCR product corresponded to the full-length MST cDNA containing the missing IX, X, and XI kinase domains (MST4, Fig. 1). The deduced protein sequence of MST4, consisting of 416 amino acids (Fig. 3A), has a kinase domain at the N terminus and a regulatory domain at the C terminus. Comparison of the protein sequence of MST4 with other enzyme sequences (Fig. 3B) indicated that the kinase domain is most closely related to the catalytic domains of Mammalian Ste20-like kinases MST1 (51% identity), MST2 (54% identity), MST3 (65% identity), and SOK1 (63% identity). The C-terminal regulatory domain of MST4 is most similar to the C-terminal domains of MST3 and SOK1.

Most of the genomic DNA sequence of human MST4 has been mapped. Interestingly, there is a putative 186-base pair exon corresponding to kinase subdomains IX–XI that, if spliced out of the MST4 mRNA transcript, could account for the sequence found in the truncated MST4a variant. These results suggest that MST4a is likely to be an alternatively spliced form of MST4. When we did a more comprehensive analysis of the tissue distribution of MST4 and MST4a by PCR, we were able to detect both forms in cDNAs from a variety of other adult and fetal human tissues (CLONTECH) including kidney, lung, liver, placenta, pancreas, intestine, skeletal muscle, heart, and pancreas as well as fetal thymus, spleen, muscle, liver, and kidney (Fig. 2, right panel). In most tissues, the major expressed form is MST4, but it appears that MST4a is more highly expressed in the brain.

Tissue Distribution and Chromosomal Localization of MST4—A human multiple tissue Northern blot was used to identify the expression pattern of MST4. Only one mRNA band with a size of 3.6 kilobases was detected. The results showed that MST4 is ubiquitously expressed, with strong expression in placenta, weak expression in skeletal muscle and pancreas, and moderate expression in brain, heart, lung, liver, muscle, and kidney. It was not possible to determine the difference in expression of MST4 versus MST4a by this approach, since the difference in size between the two mRNAs is too small to be detected by Northern analysis (Fig. 4). To map the MST4 gene, we performed fluorescence in situ hybridization using a biotin-labeled MST4 probe on normal human metaphase chromosomes. Hybridization of the MST4 cDNA probe resulted in specific labeling only of the X chromosome (Fig. 5). Labeling of Xq25–27 was observed on four (eight cells), three (16 cells), or two (one cell) chromatids of the X chromosome homologues in 25 cells examined from mitogen-stimulated lymphocytes isolated from a healthy female. Of 82 signals observed, one signal (1.2%) was located at Xq25, 67 signals (82%) were located at Xq26, and 14 signals (17%) were located at Xq27. No background signals were observed at other chromosomal sites. We also observed a specific signal at Xq26 in an additional hybridization experiment using this probe (data not shown). These results indicate that the MST4 gene is localized to Xq26. This result was later verified by examination of the relative location of the MST4 gene in the X chromosome genomic map.

Catalytic Activity of MST4 and MST4a—In order to determine whether MST4 or its variant is an active kinase, the N-terminal domains of MST4 and MST4a were tagged with a FLAG epitope, and the cDNAs were expressed in 293 cells using a cytomegalovirus promoter (Fig. 6A). To control for nonspecific kinase activity, a kinase-inactive mutant of MST4, MST4-K53R, was also transiently transfected into 293 cells. Following transfection, 293 cells were lysed, and the cell lysates were subjected to immunoprecipitation with anti-FLAG antibody (Fig. 6B). The immunoprecipitates were then assayed for in vitro kinase assay using myelin basic protein as a substrate. The results indicate that MST4 is an active kinase, whereas MST4a did not exhibit kinase activity (Fig. 6B). Interestingly, the kinase activity of MST4 isolated from cells grown in serum-free or serum-containing medium was comparable, indicating that MST4 possesses a high basal kinase activity (Fig. 6C). Under the same conditions, a phosphoprotein with the same size as MST4 (46 kDa) was also detected in an in vitro kinase reaction when immunoprecipitates containing MST4 but not kinase-inactive MST4 (MST4-K53R) were used, suggesting that MST4 also functions as an autophosphorylating kinase (Fig. 6C).

Since MST4a was isolated by association with the Raf kinase domain in a yeast two-hybrid system, we determined whether MST4 or MST4a associate with c-Raf-1 in cells. Therefore, COS cells were co-transfected with expression vectors for Myc-Raf-1 and FLAG-MST4 or FLAG-MST4a. Raf was then immunoprecipitated with an anti-Myc antibody and analyzed for MST4 association by immunoblotting with an anti-FLAG antibody. Conversely, MST4 or MST4a was immunoprecipitated with an anti-FLAG antibody and analyzed for Raf association with an anti-Myc antibody. As shown in Fig. 6D, no association of c-Raf-1 with MST4 or MST4a was observed. We also determined whether co-expression of Myc-Raf-1 with FLAG-MST4 altered MST4 kinase activity. Consistent with the co-immunoprecipitation results, no effect of Raf-1 on MST4 kinase activity was detected (data not shown). These results suggest that MST4 is not stably associated with or modulated by c-Raf-1. C-terminal Regulatory Domain of MST4 Enhances but Is Not Essential for Its Kinase Activity—Removal of the C-terminal
regulatory domain of MST1 and MST2 by caspases results in a significant increase in MST1 or MST2 kinase activity (35, 40). To address the function of the C-terminal tail of MST4, a cDNA encoding the N-terminal kinase domain of MST4 but lacking the C terminus of the protein (FLAG-MST4-NT) was transfected into COS cells. For comparison, COS cells were also transfected with expression vectors for FLAG-MST4 or FLAG-MST4-K53R. Following immunoprecipitation with anti-FLAG antibodies, the MST4 proteins were assayed for kinase activity using MBP as a substrate. As shown in Fig. 7, FLAG-MST4-NT has reduced kinase activity compared with MST4, indicating that the C-terminal domain acts to enhance MST4 activity. Interestingly, MST4-NT is no longer autophosphorylated, suggesting that the site of autophosphorylation may be in the C-terminal tail of the protein (Fig. 7).

MST4 Does Not Activate ERK2 in 293 Cells—MST4 is isolated by interaction with the Raf kinase domain and is most similar to mammalian Ste20-like kinases. A, cDNA and predicted protein sequence of the full-length MST4a. B, sequence comparison between MST4 and other members of the MST family. The predicted amino acid sequence of MST4 was compared with that of the mammalian Ste20-like kinases MST1, MST2, MST3, and SOK1 by Geneworks. Amino acids conserved in all protein are shaded.

Mammalian Ste20-like kinases. A, cDNA and predicted protein sequence of the full-length MST4. B, sequence comparison between MST4 and other members of the MST family. The predicted amino acid sequence of MST4 was compared with that of the mammalian Ste20-like kinases MST1, MST2, MST3, and SOK1 by Geneworks. Amino acids conserved in all protein are shaded.
closely related to MST3, an enzyme that has been reported by one group to potentiate the Raf/MEK/ERK signaling cascade. To determine whether overexpression of MST4 might also activate ERK1 or ERK2, vectors expressing MST4, MST4-K53R or control vector were co-transfected with HA-ERK2 into 293 cells. The tagged ERK2 was immunoprecipitated from the lysates of transfected cells using an anti-HA antibody, and its kinase activity was assayed by Western blotting with anti-phospho-pTExpY MAPK antibody. A sample from each lysate was assayed directly by Western blotting with anti-FLAG antibody to confirm the expression of MST4 (data not shown). As shown in Fig. 8, overexpression of MST4 does not significantly affect the activation of ERK2 in cells grown either in serum or under serum-free conditions. Furthermore, we have also conducted similar experiments involving co-expression of MST4 (or MST4a or MST4-K53R), ERK2, and Myc-Raf-1 to determine whether MST4 can potentiate or inhibit Raf activation of ERK. Again, we could not detect any effect of MST4 on Raf-mediated ERK activity (data not shown), consistent with the lack of association with c-Raf-1 in cells. Thus, despite the interaction of MST4a with the kinase domain of Raf in the yeast two-hybrid system, we have no evidence to date that MST4 either stimulates or potentiates ERK activity in 293 cells.

Similar experiments were done to determine whether MST4 activates or enhances JNK or p38, the other members of the MAPK superfamily. Thus, FLAG-MST4, the kinase-dead mutant FLAG-MST4, or the control vector were expressed along with HA-JNK in 293 cells.
293 cells were co-transfected with either control vector or HA-ERK and either control vector, FLAG-MST4, or FLAG-MST4-K53R. Cells were grown in serum-containing medium for 24 h and then incubated in serum-free medium for another 24 h. The cells were then left untreated (+Ser) or stimulated with 20% serum (+Ser). Following treatment, cell lysates were immunoprecipitated with anti-HA antibody, resolved by SDS-polyacrylamide gel electrophoresis (10%), and immunoblotted with anti-ERK antibody. The blot was then stripped and reprobed with anti-phospho-pTEpY-ERK antibody.

stimulated with sorbitol, and then JNK was immunoprecipitated from the cell lysates with anti-HA antibody. As illustrated in Fig. 9, JNK was stimulated by sorbitol, and neither MST4 nor the kinase-dead MST4 mutant significantly activated or suppressed JNK activity. In a parallel experiment, 293 cells were transfected with expression vectors for FLAG-MST4, the kinase-dead mutant FLAG-MST4, or the control vector along with HA-p38. After further incubation of the cells or stimulation with sorbitol, p38 was immunoprecipitated from the cell lysates with anti-HA antibody. Like the results obtained for ERK and JNK, no effect of MST4 or the kinase-dead MST4 mutant on either p38 stimulation, potentiation, or inhibition was observed (Fig. 10).

DISCUSSION

A novel member of mammalian Ste20-like kinase family, MST4, was cloned and characterized. According to the primary sequence, MST4 belongs to the germinal center kinase subfamily because it has an N-terminal kinase domain and a C-terminal regulatory domain. MST4 is most highly related to MST3 (32), with 68% identity, and SOK (28), with 63% identity within the kinase domain. An inactive mutant of MST4, termed MST4a, was isolated by interaction with the Raf catalytic domain in a yeast two-hybrid screen. Although Ste20-related kinases activate the MAPK cascade in organisms ranging from yeast to mammals, MST4 does not appear to activate or potentiate the ERK, JNK, or p38 MAPK in 293 cells.

Analysis of the genomic sequence indicates that MST4a is an alternatively spliced isoform of MST4. MST4a is missing a 186-base pair exon encoding kinase subdomains IX, X, and XI. Kinase subdomain IX has been shown to be important for the kinase activity of protein kinase Ca, and it has been suggested that domains X and XI function to stabilize the kinase-bound substrate (39). Consistent with this possibility, the expressed MST4a kinase does not exhibit any kinase activity toward MBP, a potent substrate of MST4 as well as other members of the MST family. Since MST4a was independently cloned from two different brain cDNA libraries at reasonably high expression levels and MST4a transcripts can be translated into protein, it is likely that MST4a represents an alternative isoform of MST4 in the brain as well as other tissues.

The lack of kinase activity in MST4a suggests that it may function as a decoy or dominant-negative kinase, representing an alternative mechanism for regulating MAPK signaling cascades. Several other kinases and receptors appear to have similar splice variants. A 3’ exon encoding 10 amino acids corresponding to subdomains IX and X of MEK5 can be spliced out during transcription of MEK5 (41), and an alternatively spliced exon also has been found in the comparable region of JNK/stress-activated protein kinase. (42). Unlike MST4a, the MEK5 isoform that lacks the exon encoding subdomains IX and X has equivalent in vitro protein kinase activity to the full-length MEK5. Thus, it is possible that a large deletion of subdomains IX–XI, like the spliced exon in MST4a, could lead to loss of activity, while a smaller deletion of subdomains IX–X, like the spliced exon in MEK5, still enables the kinase to interact with the substrate. Recently, a novel Ste20 kinase from chickens was cloned (KFC) that is also expressed as a splice variant (43). The short form of KFC has a 69-amino acid deletion outside of the kinase domain and has no impact on cell growth, but the longer version confers a growth advantage to the cell. Thus, the alternative splicing of exons may provide another mechanism for diversifying the function of protein kinases.

There are other examples of nonfunctional, decoy proteins that have important physiological functions. Recently, a novel phosphoserine/threonine-binding protein (STYX), related to dual specificity protein-tyrosine phosphatase, was shown to contain a naturally occurring Gly instead of Cys, the residue that is required and conserved in dual specificity protein-tyrosine phosphatase catalytic loops. The naturally occurring STYX protein does not have phosphatase activity toward
Tyr(P)-containing substrates; however, the substitution of Gly to Cys in recombinant STYX protein confers phosphatase activity, indicating that STYX is a naturally occurring “dominant negative” phosphotyrosine/serine/threonine-binding protein (44). Another example is the TNF receptor superfamily that has two main subgroups of receptors. The first group (DR) possesses the death domain, which couples the receptor to caspase cascade, whereas the second group consists of a death receptor (DR), which is structurally related to DR but lacks the death domain and functions as an inhibitor (45). Taken together, these studies suggest that MST4a could be a naturally occurring dominant-negative kinase that can act as an inhibitor of the signaling pathway regulated by MST4.

Although other MSTs have been implicated as mediators of apoptosis, it appears unlikely that MST4 plays a similar role. Interestingly, MST4 has a sequence (DESDS) that is very similar to MST1 and MST2 (DELDS) at the junction of the N-terminal catalytic and C-terminal domains. However, unlike MST1 and MST2 (35, 40), MST4 was not cleaved in vivo or in vitro by caspase 3 (data not shown). Loss of the C-terminal regulatory domain in MST1 and MST2 results in an activation of the catalytic activity, consistent with a positive feedback role in apoptosis. In contrast, the C-terminal tail of MST4 has an activating regulatory role, and loss of this domain by caspase cleavage would result in decreased kinase activity. Thus, even if MST4 were a target of cleavage by other caspases, the truncated enzyme would not function as a direct downstream mediator of caspase-initiated cell death. Further studies will be required to elucidate its true physiological role.

The role of MST4 in the activation or regulation of MAPK is also unclear at this time. Unlike other MSTs, MST4 was not sufficient by itself to activate ERKs, JNKs, or p38 or to potentiate or inhibit serum or sorbitol stimulation of these enzymes in 293 cells. However, it is possible that MST4 can modulate MAPK signaling in a tissue- or cell-specific manner. Our isolation of 293 cells. However, it is possible that MST4 can modulate MAPK signaling in a tissue- or cell-specific manner. Our isolation of 293 cells. However, it is possible that MST4 can modulate MAPK signaling in a tissue- or cell-specific manner. Our isolation of 293 cells. However, it is possible that MST4 can modulate MAPK signaling in a tissue- or cell-specific manner. Our isolation of 293 cells.
Cloning and Characterization of MST4, a Novel Ste20-like Kinase
Zhijian Qian, Clark Lin, Rafael Espinosa, Michelle LeBeau and Marsha Rich Rosner

J. Biol. Chem. 2001, 276:22439-22445.
doi: 10.1074/jbc.M009323200 originally published online April 16, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M009323200

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

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

This article cites 45 references, 23 of which can be accessed free at
http://www.jbc.org/content/276/25/22439.full.html#ref-list-1