Isolation of TAO1, a Protein Kinase That Activates MEKs in Stress-activated Protein Kinase Cascades*

Michele Hutchison‡, Kevin S. Berman‡, and Melanie H. Cobb§

From the Department of Pharmacology, University of Texas Southwestern Medical Center, Dallas, Texas 75235-9041

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Several components of the budding yeast pheromone-response pathway are conserved in mammalian mitogen-activated protein (MAP) kinase pathways. Thus, we used degenerate oligonucleotides derived from the sequence of the Saccharomyces cerevisiae protein kinase Ste20p to amplify related sequences from the rat. One of these sequences was used to clone a rat Ste20p homolog, which we called TAO1 for its one thousand and one amino acids. Northern analysis shows TAO1 is highly expressed in brain, as is a homolog TAO2. Recombinant TAO1 was expressed and purified from Sf9 cells. In vitro, it activated MAP/extracellular signal-regulated protein kinase (ERK) kinases (MEKs) 3, 4, and 6 of the stress-responsive MAP kinase pathways, but not MEK1 or 2 of the classical MAP kinase pathway. TAO1 activated MEK3 but not MEK4 or MEK6 in transfected cells. MEK3 coimmunoprecipitated with TAO1 when they were expressed in 293 cells. In addition, immunoreactive MEK3 endogenous to Sf9 cells copurified with TAO1 produced from a recombinant baculovirus. The activation of and binding to MEK3 by TAO1 implicates TAO1 in the regulation of the p38-containing stress-responsive MAP kinase pathway.

MAP1 kinase pathways have been identified in mammals and in yeast, and each contains a 3-kinase cascade consisting of a MAP kinase or ERK, a MAP/ERK kinase (MEK), and a MEK kinase (MEKK) (1–3). The parallel nature of the yeast and mammalian pathways was first realized when sequences of mammalian ERKs and yeast MAP kinases, KSS1 and FUS3, became available (4–6). Subsequently, information from the yeast pathways has been exploited to identify components and understand relationships in the mammalian cascades. More than a dozen mammalian MAP kinases that lie in several distinct cascades (7, 8) are now known. Fidelity in these pathways is maintained in part by the substrate specificity of the MEK family member. These cascades are differentially responsive to cellular stimuli, including proliferative and survival factors and stress. Several of the mammalian cascades share some regulatory features with yeast systems. The best delineated yeast MAP kinase pathway, activated by mating pheromones, is controlled by a receptor-G protein system and requires at least three protein kinases, Ste20p, Ste11p, and Ste7p, upstream of the MAP kinase Fus3p (9–12).

Ste20p was isolated from Saccharomyces cerevisiae by Ramer and Davis (9) and Leberer and co-workers (11) as a gene whose product functioned downstream of the $\beta y$ subunits of a heterotrimeric G protein but upstream of enzymes in the MAP kinase module (MEKK, MEK, and ERK) of the pheromone-response pathway. Ste11p, the MEKK, may be one of the Ste20p substrates (13); thus, Ste20p-like enzymes may activate MEKKS in mammalian MAP kinase pathways. Ste20p, like its best studied mammalian counterparts, the p21-activated protein kinases (PAKs (14–18)), is thought to be regulated by binding to Cdc42 through a conserved Cdc42/Rac binding region called a CRIB domain (19) although this domain is apparently not required in the pheromone response (20).

We used the sequence of Ste20p to isolate cDNAs encoding homologs from Schizosaccharomyces pombe and mammals (15, 21). Among these was a cDNA encoding a novel Ste20p-related kinase that is highly expressed in brain and does not contain a recognizable CRIB domain. Unlike the PAKs, the newly identified kinase, called TAO1 for its one thousand and one amino acids, phosphorylates and activates MEKs from the stress-responsive MAP kinase cascades. Although its physiological roles have not been determined, TAO1 interacts with MEK3 as deduced by the specific activation of MEK3 in cells by TAO1, the copurification of endogenous MEK3 from Sf9 cells with recombinant TAO1, and the coimmunoprecipitation of MEK3 with TAO1 expressed in 293 cells. These findings suggest that TAO1 may be an important regulator of the p38 MAP kinase pathway.

EXPERIMENTAL PROCEDURES

Isolation of cDNA Clones Encoding TAO1—First-strand cDNA from adult rat brain was used as the template in the first round of PCR with degenerate oligonucleotide primers derived from STE20 sequences 5'-GACGCTGGATCCAA(AG)AT(ACT)GGICA(AG)GGIGC-3' and 5'-GGIGTICOAG/TICTITGGCIAT-3'. The products were used as template in a second round of PCR with nested primers 5'-AA(AG)AGA/GAG(CA)ATIT(C/A)TTAAAT(CT)GA/G(A)AT3'- and 5'-GACGCTGAAATTCCATC/TICTCIGIGG-CCATCCA-3'. The resulting 420-bp product was labeled with [a-32P]dCTP by random-priming and used to probe a genomic library from rat forebrain. The genomic library was prepared using the P.E. Genetics Lambda-ZAPII subcloning system. The cDNA inserts were excised from PstI sites that were introduced into the vector. Plaques of the library from poly(A) RNA were screened with the 420-bp cDNA clone. The positive clones were sequenced by the dideoxy chain-termination method.
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### Table 2

| TAO1 | TAO2 | ccTAO | STE20 | GCK | MLK1 | MST1 | MEKK1 |
|------|------|-------|-------|-----|------|------|-------|
| TA01 | 90   | 65    | 40    | 43  | 32   | 47   | 34    |
| ccTAO| 61   | 39    | 37    | 35  | 30   | 42   | 33    |
| STE20| 40   | 39    | 37    | 35  | 30   | 30   | 27    |
| GCK  | 43   | 42    | 35    | 40  | 29   | 27   | 27    |
| MLK1 | 32   | 30    | 27    | 30  | 29   | 27   | 27    |
| MST1 | 47   | 43    | 42    | 42  | 27   | 28   | 27    |
| MEKK1| 34   | 33    | 27    | 30  | 30   | 30   | 29    |

### Footnotes

1. The Activation of MEKs by TAO1
2. Fig. 1. Nucleotide and protein sequence of TAO1. A, the complete sequence of TAO1. B, the catalytic domains of TAO1, Ste20p, and the C. elegans homolog ccTAO were aligned by eye and the conserved amino acids are in bold. The protein kinase subdomains are indicated by Roman numerals. C, the FASTA program of the Wisconsin GCG package was used to determine the identities within catalytic domains of kinases related to TAO1.

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**In Vitro Kinase Assays**—Kinase assays contained 50 mM HEPES, pH 8, 10 mM MgCl₂, 1 mM dithiothreitol, 0.5 mg/ml myelin basic protein (MBP), 100 µM ATP ([α-32P]ATP, 2–7 cpm/fmol). Reactions were halted with 10 µl of 5X electrophoresis sample buffer followed by boiling, and 20 µl was analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. For linked kinase assays, 50–250 ng of recombinant TAO1 protein was incubated with 50 ng of MEK proteins in 30 µl for 10 min at 30 °C; 5 µl of the reactions were added to second reactions containing K252 ERK2, p38, or the c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) GST-SAPK-β (24, 25) at 10 µg/ml. Phosphoamino acids were determined as described (26). Preparations and characterization of antibodies—Polyclonal antibodies were raised against TAO1 peptides as described (27). The antigen for antisera P820 was TKDAVRELDNLQYRKMKKLL (residues 296 to 315). Antibodies recognizing MEK3, MEK4, and MEK6 were as described (24, 28).

For immunoblot analysis, 50 ng of recombinant TAO1 protein and 100 µg of cell lysate were subjected to SDS-polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes. The membranes were pretreated with 5% non-fat powdered milk in 20 mM Tris-HCl, pH 8, 0.5 mM NaCl, 0.05% Tween 20 for 1 h and then incubated with antisera at 1:500 dilution in the same solution plus 0.25% milk. The membranes were washed with 1:2500 dilution of horseradish peroxidase-conjugated goat-anti-rabbit IgG plus 0.25% milk for 1 h, washed, and visualized by ECL (Amersham Pharmacia Biotech).
RESULTS

Isolation of TAO1 cDNAs—Degenerate oligonucleotide primers designed from the sequence of the S. cerevisiae Ste20p kinase were used in PCR reactions to amplify fragments of related protein kinases from rat cDNAs. One PCR product was used to obtain overlapping cDNAs from a rat forebrain library. The assembled cDNAs predicted an open reading frame of one thousand and one amino acids which we named TAO1 (Fig. 1). The presumed initiator codon begins at base 121 and is preceded by an in-frame stop codon at base 106. The longest 5’ untranslated region was 600 nucleotides. The longest 3’ untranslated region was 1200 nucleotides; however, none contained a poly(A) sequence. In screening the cDNA library for clones to confirm the 5’ end of TAO1, multiple clones representing a second closely related gene, TAO2, were identified.

Amino Acid Sequence of TAO1—TAO1 has a calculated molecular mass of 116 kDa and encodes a serine/threonine protein kinase at its N terminus. In its 700 C-terminal residues, TAO1 contains a possible nucleotide binding site, an acidic domain, and two serine-rich regions. TAO1 does not appear to contain the CRIB domain found in Ste20p or a leucine zipper motif found in the less closely related mixed lineage kinases (MLKs) (29, 30). Of sequences in the data bank, the TAO1 kinase domain has the highest degree of identity, 63%, to a Caenorhabditis elegans putative serine/threonine protein kinase (GenBank™ accession number U32275) (Fig. 1C). The TAO1 catalytic domain is 39% identical to Ste20p and 40% identical to the catalytic domains of the p21-activated kinases PAK1 and PAK2 (14, 16–18). The catalytic domain of TAO1 is only 31% identical to the mixed lineage kinase MLK1, and 33% identical to dual leucine zipper-bearing kinase, also known as MLK2 (29, 31). Among mammalian enzymes, the kinase domain of TAO1 is most closely related to that of germinal center kinase (GCK) and mammalian Ste20p-like kinase 1 (MST1), with 42 and 45% identity, respectively, in the catalytic domains (32–34). Aside from the C. elegans kinase, no other sequences in the data bank are similar to the noncatalytic domains of TAO1.

TAO1 Is Expressed in Brain—The expression of TAO1 was examined in adult rat and human tissues by Northern blot analysis. A probe derived from the catalytic domain of TAO1 hybridized predominately to an mRNA species of approximately 12 kilobases, and less strongly to another of approximately 10 kilobases (Fig. 2A). Of the rat tissues examined, the most mRNA was detected in brain. On prolonged exposure, TAO1 was also detected in heart and lung, but not in skeletal muscle, liver, kidney, testis, epididymis, or spleen. When the same blot was probed with a fragment of the catalytic domain of TAO2, the strongest hybridization signal was also seen in brain. The size of the transcript that hybridized to the TAO2 probe was smaller than for TAO1, at 5 kilobases (Fig. 2B). A probe from the noncatalytic domain of TAO1 was used to assess the mRNA expression pattern in sections of human brain, to minimize possible cross-reaction with the mRNA for TAO2. The strongest hybridization signals with the TAO1...
probe were seen in amygdala, corpus callosum, hippocampus, and substantia nigra, and each of these was stronger than that seen in whole brain (Fig. 2C). Weaker signals were seen in caudate nucleus, subthalamus, nucleus, and thalamus. Analysis of a second human brain Northern blot revealed strong hybridization of the TAO1 probe in cerebellum, putamen, and occipital, frontal, and temporal lobes but much weaker signals in cerebral cortex, medulla, and spinal cord (Fig. 2D).

Expression and Activity of TAO1—To determine whether TAO1 could be detected in mammalian cell lines, lysates were immunoblotted with antisera raised against multiple TAO1 epitopes. None of the five antisera detected TAO1 in lysates of 293, NIH3T3, NG-108, or COS cells (not shown). To confirm that the antisera recognized the protein, recombinant TAO1, TAO1-(1–416), and a kinase-defective mutant TAO1-(D169A) expressed in Sf9 cells were also immunoblotted. The antisera recognized TAO1-(1–416) as a 57-kDa band (Fig. 3B) and TAO1 and TAO1-(D169A) as 140 kDa bands; peptide antigen blocked the immunoblotting signal. HA-TAO1 was also detected in transfected 293 cells as a 140 kDa protein by Western blotting with an antibody directed against the HA epitope (Fig. 3A), as well as anti-TAO1 peptide antibodies (Fig. 3B).

The large size suggests that TAO1 is heavily phosphorylated in cells. Our inability to detect TAO1 in lysates suggests that it is either not expressed or is present only in low amounts in common cell lines.

Both TAO1 and the catalytic fragment TAO1-(1–416) immunoprecipitated from transfected cells phosphorylated MBP in immune complex kinase reactions (not shown). Purified, recombinant TAO1-(1–416) purified from Sf9 cells phosphorylated MBP with a specific activity of 1 μmol min⁻¹ mg⁻¹. Full-length TAO1 has comparable MBP kinase activity, whereas the activity of TAO1-(D169A) is less than 10% of wild-type protein. TAO1 also phosphorylated casein, histone 1, and histone 7 (data not shown). TAO1 and TAO1-(1–416) autophosphorylated extensively on serine and threonine residues (not shown).

TAO1 Activates MEK3, MEK4, and MEK6 in Vitro—The identity between the catalytic domains of TAO1 and MEKK1 is only 33%; however, the identity of the C-terminal half of their catalytic domains is higher, at 42%. This observation, taken together with reports that certain other Ste20p-related kinases activate MEKs directly (35, 36), led us to examine the ability of TAO1 to activate MEK family members.

TAO1-(1–416) was incubated with recombinant MEKs produced in bacteria in the presence of ATP and then aliquots of the reactions were transferred to second reactions. Because of the specificity of MEKs for MAP kinase family members, different substrates were required; ERK2 is activated by MEK1 and MEK2, ERK5 is activated by MEK5, p38 is activated by MEK3 and MEK6, and SAPK-β is activated by MEK4 and MEK7. TAO1-(1–416) phosphorylated and activated MEK3 and enhanced the ability of MEK3 to phosphorylate p38 by approximately 100-fold (Fig. 4, A and D). Phosphoamino acid analysis revealed phosphoserine and phosphothreonine in MEK3 following activation by TAO1. TAO1-(1–416) activated GST-MEK4 5-fold toward p38 and 150-fold toward GST-SAPK-β (Fig. 4, B and D). The difference in fold activation of MEK4 toward the two substrates probably reflects the difference in basal kinase activity of MEK4 toward p38 and SAPK-β in vitro. MEK7 was not tested. TAO1 also increased the ability of GST-MEK6 to phosphorylate p38, by 5-fold (Fig. 4, C and D), despite the high basal activity of MEK6. Recombinant GST-MEK5 was not phosphorylated by TAO1-(1–416) (data not shown). TAO1-(1–416) was also unable to increase the activity of MEK1 or MEK2 toward the substrate K52R ERK2 under the same conditions that TAO1 activates MEK3, MEK4, and MEK6 (Fig. 4D). Full-length TAO1 displayed about 30% of the MEK3-activating ability of TAO1-(1–416). TAO1-(D169A) did not activate any of the MEKs (data not shown).

TAO1 Activates MEK3 in Transfected Cells—To assess the ability of TAO1 to activate MEKs in cells, HA-TAO1 was cotransfected into 293 cells with Myc-MEK3, or Myc-TAO1 was cotransfected with HA-MEK4 or HA-MEK6. In multiple experiments, Myc-MEK3 displayed 3-fold higher activity toward p38 when immunoprecipitated from 293 cells coexpressing TAO1 than from vector-transfected cells (Fig. 5). Kinase-defective TAO1 did not cause any increase in MEK3 activity (not shown). In contrast, coexpression with TAO1 did not increase the activity of immunoprecipitated HA-MEK4 toward GST-SAPK-β, or that of HA-MEK6 toward p38.

Because TAO1 activated MEK3 in vitro and in transfected cells, we wished to determine whether TAO1 could increase the activity of p38 or SAPK-β when coexpressed in 293 cells. Although HA-TAO1, HA-p38, and HA-SAPK-β were expressed...
in transfected 293 cells, after numerous efforts we were unable to express TAO1 together with p38 or SAPK-β. The mechanism for this reduced expression is unknown.

MEK3 Copurifies with TAO1—Although the ability of TAO1 to activate MEK3 was reduced in comparison with that of TAO1-(1–416), several assays showed that the ability of TAO1 to increase phosphorylation of p38 in the linked kinase assays was partly independent of the addition of any MEK. Because TAO1-(1–416) does not phosphorylate p38 directly, we tested TAO1 preparations from Sf9 cells for the presence of associated MEks. TAO1, TAO1-(1–416), and TAO1-(D169A) first purified on nickel-chelate resin were subjected to Western analysis with antisera specific to MEK3, MEK4, and MEK6. MEK3 immunoreactivity was detected in the TAO1 preparation as well as in Sf9 lysates (Fig. 6A). It was more variably present in the TAO1-(D169A) preparation. MEK4 was detected in Sf9 cell lysates (not shown), but not in the TAO1 preparations, whereas MEK6 was detected in neither. This suggests that TAO1 selectively interacts with and regulates MEK3, although both MEK3 and MEK4 are present in Sf9 cells.

To confirm the interaction of TAO1 with MEK3, we tested the ability of TAO1 to coimmunoprecipitate with MEK3 in transfected 293 cells (Fig. 6B). HA-TAO1 was immunoprecipitated with the anti-HA antibody, and the immunoprecipitates were immunoblotted with an anti-Myc polyclonal antibody (Santa Cruz Biotechnology). MEK3 was easily detected in the anti-HA immunoprecipitate if TAO1 was expressed (lanes 2 and 4) but not if TAO1 was omitted (lane 1). MEK3 also coimmunoprecipitated with HA-TAO1-(D169A) (lane 3).

DISCUSSION

We isolated cDNAs from rat encoding the novel Ste20p-related protein. Partial cDNAs encoding a closely related kinase TA02, were also found. TA01 transcripts are highly expressed in brain. Sequences from expressed sequence tag data bases derived from retinal mRNAs reveal the human counterpart for TAO1.

TA01 has MEKK activity. MEKs 3 and 4 are phosphorylated, and their activities are increased as much as 150-fold by TA01 in vitro. MEKs 1, 2, and 5 are not activated by TA01, indicating enzymatic specificity for MEK family members in the stress-response pathways. In transfected cells, TA01 acti-
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MEK3

vates MEK3 modestly, but is without effect on MEK4 or MEK6. The selectivity in transfected cells may arise from the ability of TAO1 to bind MEK3. Significantly, the endogenous MEK3, but apparently not other MEKs, from Sf9 cells copurifies with recombinant TAO1. The isolated catalytic domain of TAO1 does not copurify with MEK3; thus, TAO1 most likely binds to MEK3 through its noncatalytic domain. The interaction of MEK3 and TAO1 was also observed in transfected cells. It is possible that other MEK family members also bind to TAO1 that are not detected with our antibodies. MEK3 is thought to participate in controlling the stress-sensitive p38 MAP kinase cascade (37). Despite our inability to coexpress TAO1 and p38, the cotransfection and the copurification findings suggest that TAO1 may selectively recognize MEK3 and, therefore, may be an important regulator of the p38 kinase in those tissues where it is expressed.

Mammalian relatives of Ste20p are a diverse group and encompass the PAK subfamily (PAK1, 2, and 3) and the MLK subfamily, GCK, and the Nck-interacting kinase, NIK, SOK-1, Krs-1 and -2, and MUK, among many others (14–17, 29–31, 33, 34–38). MUK was isolated in a screen for MEKK isoforms, but has greater identity to MLK. It has been difficult to define roles for these enzymes in the physiological regulation of MAP kinase pathways. Furthermore, in transfected cells several of these enzymes, as first shown with GCK, increase the activity of the JNK/SAPK stress-responsive kinases (35, 46, 47). In the case of NIK and GCK, they may work by binding to MEKK1 (48). However, a subset of these Ste20p-related enzymes also have MEKK activity. For example, MLK2, like TAO1, phosphorylates and potently activates MEKs that lie in the stress-responsive cascades (35, 40). The multiplicity of Ste20p-like kinases suggests that different stimuli that activate the stress-responsive MAP kinase cascades may employ distinct signal transduction mechanisms.

Unlike PKAs, TAO1 does not retain the CRIB domain and does not interact with the small G proteins Cdc42, Rac2, or RhoA in the two hybrid system (data not shown), suggesting a distinct mode of regulation. Efforts to examine regulation of TAO1 have failed to indicate how its activity is controlled. Recombinant TAO1 is highly active when purified from Sf9 cells or upon immunoprecipitation from transfected cells, and, like MEK1, TAO1 is heavily autophosphorylated. Information on regulation of other related kinases is also limited. Krs-1 and Krs-2 are activated by stress such as heat shock, sodium arsenite, and staurosporine, but not by proliferative stimuli (45). Calcium changes the intracellular localization of DLK, although no effects on activity have been demonstrated (31). Understanding the regulation of these Ste20p-related kinases should help to unravel the functions of this group of protein kinases.

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