We previously reported the cloning of the thousand and one-amino acid protein kinase 1 (TAO1), a rat homolog of the \textit{Saccharomyces cerevisiae} protein kinase sterile 20 protein. Here we report the complete sequence and properties of a related rat protein kinase TAO2. Like TAO1, recombinant TAO2 selectively activated mitogen-activated protein/extracellular signal-regulated kinase kinases (MEKs) 3, 4, and 6 of the stress-responsive mitogen-activated protein kinase pathways \textit{in vitro} and copurified with MEK3 endogenous to SF9 cells. To examine TAO2 interactions with MEKs, the MEK binding domain of TAO2 was localized to an 135-residue sequence just C-terminal to the TAO2 catalytic domain. \textit{In vitro} this MEK binding domain associated with MEKs 3 and 6 but not MEKs 1, 2, or 4. Using chimeric MEK proteins, we found that the MEK N terminus was sufficient for binding to TAO2. Catalytic activity of full-length TAO2 enhanced its binding to MEKs. However, neither the autophosphorylation of the MEK binding domain of TAO2 nor the activity of MEK itself was required for MEK binding. These results suggest that TAO proteins lie in stress-sensitive kinase cascades and define a mechanism by which these kinases may organize downstream targets.

\textbf{Isolation of the Protein Kinase TAO2 and Identification of Its Mitogen-activated Protein Kinase/Extracellular Signal-regulated Kinase Kinase Binding Domain*}

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\textbf{EXPERIMENTAL PROCEDURES}

\textit{Isolation of cDNA Clones Encoding TAO2—A 420-base pair PCR product was obtained as described (8) using oligonucleotides based on the yeast Ste20p sequence. This product was labeled with [\(\alpha\-32P\)]dCTP by random priming and used to probe approximately 1.3 \(\times\) 10\textsuperscript{6} plaques...}
of a random-primed adult rat forebrain cDNA library and approximately 0.6 × 10^6 plaques of an oligo(dT)-primed rat brain cDNA library (both provided by Jim Boulter, UCLA). cDNA clones encoding TAO1 and TAO2 were obtained. Subsequent rounds of screening yielded the full-length TAO2 cDNA, which was assembled into pBluescript from 3 of 6 overlapping cDNA fragments. The amino acid sequence of the nucleotide sequence of the assembled TAO2 cDNA was deposited in GenBankTM with the accession number AF140556.

**Plasmid Construction—**pBluescript-TAO2(1–320), containing the catalytic domain of TAO2, and a catalytically defective mutant pBluescript-TAO2D169A were generated by PCR. Wild-type TAO2, TAO2-(1–320), and TAO2(1–320) were cloned into pSET2B (Invitrogen) to incorporate a MRGSH6 tag and subsequently transferred into the baculoviral shuttle vector pVL1393. Recombinant viruses were selected as described (8). For expression in mammalian cells, the cDNAs encoding these TAO2 proteins were also cloned into pCMV5, which had been modified to place a Myc epitope tag at the N terminus of the encoded protein. A truncated, catalytically defective TAO2 in pSET2B was created by changing lysine 57, in the conserved VAIK motif, to alanine (K57A) by PCR.

For binding assays, fragments of TAO2 were subcloned into pGEX-KG by PCR. TAO2-(314–451) was subsequently transferred into pRSETA utilizing the BamHI and EcoRI restriction sites. Catalytically defective MEK3 was created in pNPT7–5 by changing lysine 64 to methionine (K64M). A MEK1/6 chimera, which contains the N-terminal domain of MEK1 and the C-terminal domain of MEK6, and a MEK6/1 chimera with the reciprocal domains (see Fig. 4B) were transferred into pRSETB or -C, respectively, from the original pGEX-KG-MEK1/6 and MEK6/1 plasmids (generously provided by Lori Christersson) utilizing the BamHI and HindIII restriction sites.

**Expression and Purification of Recombinant Proteins from Sf9 Cells and Bacteria—**Recombinant histidine-tagged TAO2, TAO2-(1–320), and TAO2D169A were expressed and harvested from Sf9 cells as described previously for TAO1 (8). Proteins were adsorbed to Ni2+-nitrilotriacetic acid-agarose (Qiagen) and eluted with a gradient of 20–250 mM imidazole in 0.5 mM dithiothreitol (DTT) and 0.3 M NaCl. His6- TA02D169A was further purified on MonoQ (Amersham Pharmacia Biotech) by elution with 50–450 mM NaCl in 1 mM DTT, 0.2 mM EDTA, 1 mM benzamidine, 10% glycerol, and 20 mM Tris, pH 8. TAO2 was detected by Western blotting with an antibody to the MRGSH6 epitope (Qiagen) and silver staining. GST fusion proteins, His6-tagged TAO2 and TAO2D169A were expressed and harvested from Sf9 cells as described previously (14). The TAO2 protein kinase domain displays 90 and 63% identity with TAO1 and the Caenorhabditis elegans TAO ortholog (CeTAO, accession number U32275), respectively (not shown). TAO2 displays marked similarities to TAO1 and the C. elegans kinase outside the catalytic domain (Fig. 1B).

**Expression and Activity of TAO2—**Truncated, recombinant TAO2-(1–320) purified from Sf9 cells phosphorylated MBP with a specific activity of 0.6 μmol-min⁻¹ mg⁻¹. The full-length protein purified on MonoQ had lower intrinsic activity, about 10% of the truncated enzyme (not shown). Kinase-deficient mutants, His6-TAO2D169A expressed in Sf9 cells and purified on MonoQ or His6- TAO2D169A purified from bacteria, were inactive toward MBP in vitro (Fig. 2B). TAO2 and TAO2-(1–320) expressed in either Sf9 or mammalian cells autophosphorylated extensively on serine and threonine residues (data not shown). TAO2-(1–320) was unable to increase the activity of MEK1 or MEK2 toward their substrate p38 (Fig. 2B). Neither TAO2-(1–320), consistent with its lower activity toward MBP, displayed about 20% of the MEK3-activating ability of TAO2-(1–320), consistent with its lower activity toward MBP. Aliquots of the first stage reactions were transferred to second reactions to measure the phosphorylation of appropriate MAP kinase substrates by the recombinant MEKs (Fig. 2A). TAO2-(1–320) activated MEK3 and MEK6 40- and 20-fold, respectively, toward their substrate p98 (Fig. 2B). TAO2 also increased the ability of MEK4 to phosphorylate its substrate SAPK by 7-fold. TAO2-(1–320) was unable to increase the activity of MEK1 or MEK2 toward their substrate K52R ERK2. Full-length TAO2 displayed about 20% of the MEK3-activating ability of TAO2-(1–320), consistent with its lower activity toward MBP. Neither TAO2 mutants D169A nor K57A activated any of the MEKs (data not shown). TAO2-(1–320) expressed in 293 cells also enhanced the ability of MEK3 and MEK4 to phosphorylate their substrates (not shown).

**TAO2 Interacts with MEK3—**We found that recombinant TAO1 copurified with MEK3 endogenous to Sf9 cells, and overexpressed TAO1 interacted with MEK3 in 293 cells (8). These observations led us to investigate whether TAO2 has similar properties. TAO2 proteins overexpressed in Sf9 cells (Fig. 3A) were purified on nickel resin and immunoblotted for MEK3. As
a control, SF9 cell lysates not expressing TAO2 were processed similarly. MEK3 endogenous to SF9 cells was associated with full-length, wild-type TAO2 (Fig. 3B, lane 2; Fig. 3C, lane 1) but not TAO2D169A (Fig. 3C, lane 2), TAO2-(1–320) (Fig. 3B, lane 3), or beads incubated with lysates from uninfected SF9 cells (Fig. 3B, lane 1; Fig. 3C, lane 3). These results demonstrated that TAO2 binds to MEK3, the interaction is mediated by the noncatalytic region of the protein, and TAO2 catalytic activity enhances MEK3 binding to the full-length protein.

To determine the domain in TAO2 that mediates the interaction with MEK3, the series of fragments that span the noncatalytic domains of TAO2 were expressed as GST fusion proteins and tested for their abilities to bind His6-MEK3 in vitro (Fig. 3D). The MEK3 binding domain was localized to an 135–residue region, residues 314–451, just C-terminal to the TAO2 catalytic domain. This region was further subdivided, but all of the shorter fragments containing residues 395–451 were degraded. TAO2-(314–377), which precedes the polyglutamic acid region, was insufficient for MEK3 binding.

**Fig. 2. TAO2 has MEKK activity.** A, linked kinase assays were used to measure activation of various MEK family members by recombinant TAO2-(1–320) purified from SF9 cells. Phosphorylation of appropriate MAP kinase substrates by the MEK family members in second reactions are shown. B, data represented in A have been quantitated and are plotted as fold activation of MEKs by TAO2-(1–320). One of five similar experiments is shown.

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**TAO2 Binds MEKs 3 and 6 in Vitro but Not MEKs 1, 2, or 4—**To investigate the binding specificity of the TAO2 MEK binding domain, His6-tagged MEK proteins were compared for their abilities to bind His6-MEK3 in vitro (Fig. 3D). The MEK3 binding domain was localized to an 135–residue region, residues 314–451, just C-terminal to the TAO2 catalytic domain. This region was further subdivided, but all of the shorter fragments containing residues 395–451 were degraded. TAO2-(314–377), which precedes the polyglutamic acid region, was insufficient for MEK3 binding.

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consistent with their significant sequence similarity compared with the other MEK family members. Chimeric proteins generated from MEK6 and MEK1 (Fig. 4B) were used to determine the portion of the MEK that binds to the TAO2 domain. His6-MEK1/6 was unable to bind to TAO2-(314–451), whereas GST-MEK6/1 is as efficient as GST-MEK6 in binding to the TAO2 fragment (Fig. 4, C and D).

As noted earlier, catalytically defective TAO2 was deficient in MEK3 binding. To explore the underlying reason, we asked whether autophosphorylation of TAO2 might have an effect on its ability to bind to MEK3. The MEK3 binding fragment of TAO2 was autophosphorylated by the catalytic domain of TAO2 on both serine and threonine residues (Fig. 5, A and B). We, thus, first phosphorylated TAO2-(314–451) with TAO-(1–320) for different lengths of time to determine whether phosphorylation would alter its binding activity. Different concentrations of ATP and Mg2+ were also tested in the binding assay. Little or no effect of the autophosphorylation state or [ATP-Mg2+] on MEK3 binding activity was observed (Fig. 5C). To determine whether MEK3 kinase activity was necessary for binding to TAO2, the binding of kinase-inactive MEK3 (K64M) was tested (Fig. 5D). This defective mutant binds to TAO2 as well as wild-type MEK3, suggesting that MEK3 kinase activity is dispensable for interaction with TAO2.

DISCUSSION

We isolated cDNAs encoding TAO2, a homolog of the previously reported TAO1 (8). We found that TAO2, like TAO1, activated MEKs in the stress-responsive MAP kinase pathways and displayed stable binding to MEK3 endogenous to Sf9 cells. In examining TAO2 expressed in Sf9 cells, we found that the full-length enzyme was significantly less active than the truncated kinase. Thus, the full-length protein was inhibited relative to its truncated forms. Subsequent work indicated that full-length TAO1 is also less active than proteins with C-terminal domain truncations. The inherently higher activity of fragments of TAO1 and -2 suggested that we may have removed an autoinhibitory or pseudosubstrate domain. However, we have not yet identified such a domain, as none of the recombinant fragments from the putative regulatory domain of TAO2 inhibited the activity of its catalytic domain (not shown).

Because TAO2 was purified in a stable complex with MEK3...
endogenous to Sf9 cells, we localized the MEK binding domain to a small, ~135-amino acid fragment, residues 314–451, just C-terminal to the catalytic domain of TA02. The N-terminal half of this fragment, residues 314–377, did not bind to MEK3. Because TA01 and TA02 both bind MEKs but TA01 has no polylactamase stretch, it seems unlikely that these residues participate in MEK binding. Thus, residues from 395 to 451 are most likely required for the stable association with MEKs. These results are consistent with the weak binding of TA01-(1–416) to MEK3 compared with the strong binding displayed by full-length TA01 (8) and suggest that residues 404–446, which are well conserved between TA02 and TA01, contain the MEK binding domain.

Because TA01 and -2 can activate MEK3, 4, and 6 in vitro, we determined the specificity of the MEK binding domain of TA02. We found that TA02 binds to MEK3 and MEK6, but not to MEK4, despite the fact that MEK4 is an in vitro substrate. The N terminus of the MEK is required for this binding, whereas the C terminus is dispensable. This behavior may be a general property of the organization of MAP kinase cascades. The N termini of other MEK family members contain binding domains for proteins in their cascades. MEK1 binds with high affinity to ERK2 through a basic motif N-terminal to its catalytic domain. MEK1 has been proposed to retain ERK2 in the cytoplasm of unstimulated cells through binding to this site (26), and activation of ERK2 may be impaired if this binding domain is absent. MEK4 is reported to require its N-terminal extension to interact with both MEK1, an activator, and its substrates, JNK/SAPKs (17). An inhibitory interaction between MEK4 and JNK/SAPKs has also been mapped to this N-terminal domain. This suggests that the stable association of MEK3 or MEK6 with TAO proteins will link their physiological functions to p38 but not JNK/SAPK pathways by restricting their intracellular targets. Future biochemical studies will focus on determining the functions of the other domains of TA01 and TA02.

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