Regulation of Stress-responsive Mitogen-activated Protein (MAP) Kinase Pathways by TAO2*

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Previous studies demonstrated that in vitro the protein kinase TAO2 activates MAP/ERK kinases (MEKs) 3, 4, and 6 toward their substrates p38 MAP kinase and c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK). In this study, we examined the ability of TAO2 to activate stress-sensitive MAP kinase pathways in cells and the relationship between activation of TAO2 and potential downstream pathways. Over-expression of TAO2 activated endogenous JNK/SAPK and p38 but not ERK1/2. Cotransfection experiments suggested that TAO2 selectively activates MEK3 and MEK6 but not MEKs 1, 4, or 7. Coimmunoprecipitation demonstrated that endogenous TAO2 specifically associates with MEK3 and MEK6 providing one mechanism for preferential recognition of MEKs upstream of p38. Sorbitol, and to a lesser extent, sodium chloride, Taxol, and nocodazole increased TAO2 activity toward itself and kinase-dead MEKs 3 and 6. Activation of endogenous TAO2 during differentiation of C2C12 myoblasts paralleled activation of p38 but not JNK/SAPK, consistent with the idea that TAO2 is a physiological regulator of p38 under certain circumstances.

TAO1 and TAO2 are closely related protein kinases whose cDNAs were originally isolated from rat based on sequence similarity to the yeast p21-activated protein kinase Ste20p (1, 2). The domain organization and regulation of TAOs is distinct from yeast p21-activated protein kinases. The kinase domain is at the TAO N terminus, and neither TAO1 nor TAO2 contains consensus motifs for activation by small G proteins. Each TAO is greater than a thousand amino acids in length; thus, each contains a regulatory domain of over 700 amino acids. A kinase that is over 90% identical to TAO2 was also identified in a screen for RNAs overexpressed in human prostate carcinoma; in this context it was named PSK for prostate-derived STE20-like kinase (3). A third kinase, named JIK for JNK1 inhibitory kinase, has a similar organization and size and is over 60% identical to TAOs (4). A chicken TAO-like kinase has also been found and is called KFC for kinase from chicken (5).

TAO1 and TAO2 activate stress-sensitive MAP kinase cascades in vitro by phosphorylating the upstream MAP/ERK kinases (MEKs), MEKs 3, 4, 6, and 7 (1, 2). Phosphorylation by TAOs increases their activities toward the downstream MAP kinases, p38 MAP kinase, and the c-Jun N-terminal kinase/stress-activated protein kinases (JNK/SAPKs). These in vitro activities of TAO1 and TAO2 suggest that they function in stress responsive pathways within the cell as MEKK-level kinases. In vitro studies revealed a MEK binding domain just C-terminal to the TAO catalytic domain (2). This domain binds MEK3 and MEK6 with selectivity over other MEK family members; these MEKs are directed toward p38 family members. Both prostate-derived STE20-like kinase and kinase from chicken were reported to activate JNK/SAPKs in cotransfected cells (3, 5). JNK inhibitory kinase, in contrast, was reported to inhibit JNK/SAPK activity (4). Effects of these TAO-related kinases on p38 were not reported.

Numerous MEKK-level kinases have been implicated in regulating the stress-responsive JNK/SAPK and p38 pathways (reviewed in 6, 7). We wished to determine the behavior of TAOs in cells to clarify their roles in regulating these pathways. Thus, TAO2 was coexpressed in cells with various MEKs and MAP kinases, and endogenous MAPKs were immunoprecipitated from TAO2-transfected cells to examine effects of TAO2 expression on their activities. We also developed antibodies that could immunoprecipitate native TAO2 to determine whether TAO2 and MEKs could be communoprecipitated and tested the capacities of various stimuli to activate TAO2. Finally, we examined the relationship between TAO2, p38, and JNK/SAPK activities during differentiation of C2C12 myoblasts.

EXPERIMENTAL PROCEDURES

Plasmids, Mutagenesis, and Proteins—A fragment encoding TAO2 (1–451) was amplified by polymerase chain reaction with a BamHI site and EcoRI site incorporated at its 5′ and 3′ ends, respectively. The BamHI- and EcoRI-digested polymerase chain reaction product was ligated into BamHI- and EcoRI-digested pGEX-KG to create the pGEX-KG-TAO2 (1–451) plasmid. A BamHI/SalI-digested fragment from pGEX-KG-TAO2 (1–451) was ligated to BglII/SalI-digested pCMV5-Myc or BglII/SalI-digested pCMV5-HA to create pCMV5-Myc-TAO2 (1–451) or pCMV5-HA-TAO2 (1–451), respectively. The resulting plasmids were analyzed by EcoRI digestion and confirmed by sequencing. The D169A mutation was introduced into both plasmids with the QuikChange kit (Stratagene) according to the manufacturer’s recommendations. The K82M and K64M mutations were created in the original pNPT7–5-MEK6 and pNPT7–5-MEK3 plasmids (kindly provided by Signal Pharmaceuticals and Kunliang Guan, respectively) also using the QuikChange kit (Stratagene) according to the manufacturer’s recommendations. The K82M and K64M mutations were created in the original pNPT7–5-MEK6 and pNPT7–5-MEK3 plasmids (kindly provided by Signal Pharmaceuticals and Kunliang Guan, respectively) also using the QuikChange kit (Stratagene) according to the manufacturer’s recommendations. The K82M and K64M mutations were created in the original pNPT7–5-MEK6 and pNPT7–5-MEK3 plasmids (kindly provided by Signal Pharmaceuticals and Kunliang Guan, respectively) also using the QuikChange kit (Stratagene) according to the manufacturer’s recommendations. The K82M and K64M mutations were created in the original pNPT7–5-MEK6 and pNPT7–5-MEK3 plasmids (kindly provided by Signal Pharmaceuticals and Kunliang Guan, respectively) also using the QuikChange kit (Stratagene) according to the manufacturer’s recommendations. The K82M and K64M mutations were created in the original pNPT7–5-MEK6 and pNPT7–5-MEK3 plasmids (kindly provided by Signal Pharmaceuticals and Kunliang Guan, respectively) also using the QuikChange kit (Stratagene) according to the manufacturer’s recommendations.
kindly provided by Don Arnett. pCEP4-HA-ERK2, pSRa-HA-JNK1, pSRa-HA-SAPK6, and pCEP4-HA-p38a were as described (9, 10). pCMV5-HA-MEK1, pCMV5-HA-MEK4, pCMV5-Myc-MEK3, pSRa-HA-MEK5, pCMV5-MEK5K, and Ras\\textsuperscript{12,12} were kindly provided by Jeff Frost and Jennifer Swantek and were described elsewhere (11–13). pCS3\\textsuperscript{3}–MT-Myc-MEK7 was provided by Shuichan Xu.

**Cell Culture, Cell Lysates, and Transfections**—293 cells and Neuro2A cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 1% L-glutamine, and 100 units/ml penicillin/streptomycin. ATT20, GC-B6, PC12, and PC12M were cultured under similar conditions. C2C12 cells were purchased from ATCC. To prepare whole cell lysates, cells were washed once with cold phosphate-buffered saline and lysed with Triton X-100. 20 mM β-glycerophosphate, 0.5 mM sodium orthovanadate, 1 mM EDTA, and 1 mM EGTA. Insoluble material was pelleted by centrifugation, and supernatants were removed, snap frozen in liquid nitrogen, and stored at −80°C. For cell fractionation, cells were washed once with phosphate-buffered saline and resuspended in hypotonic buffer (10 mM Hepes, pH 7.6, 1.5 mM MgCl₂, 10 mM NaCl, 1 mM EDTA, and supplemented with protease inhibitors and phosphatase inhibitors as above). Cells were lysed with a Dounce apparatus, and the nuclei were collected by sedimentation at 800 g for 5 min. A particulate fraction was collected by sedimenting the supernatant at 100,000 × g for 30 min. The nuclear pellet was washed with 20 mM Hepes, pH 7.6, 2.5% glycerol, 0.42 mM NaCl, 1.5 mM MgCl₂, 1 mM EDTA, and 1 mM EGTA for 30 min. Both the washed nuclear pellet and particulate fraction were lysed with 1% SDS, 10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 1 mM EGTA, and supplemented with protease inhibitors and phosphatase inhibitors as above. Three μg of various MEK or MAPK constructs were cotransfected with 2 μg of either empty vector control or TA02 constructs in 293 cells grown to 80% confluence on 60-mm dishes using the calcium phosphate method. Cells were serum-starved for 24 h beginning the second day and then harvested. Alternatively, cells were transfected with 5 μg of either empty vector control or TA02 constructs to examine the activities of endogenous MAPKs.

**Antibodies, Immunoblots, and Immunoprecipitation**—A polyclonal antisera (U2253) was raised against the antigenic peptide MPAGGRASSKLDPDVAILFFK (residues 1–21 of TA02 protein) in rabbits. This peptide was conjugated to Limulus hemocyanin (14) and dialedy into phosphate-buffered saline. A total of five boosts were performed. The final bleed of U2253 was used in all subsequent studies involving TA02 antibodies. For immunoblot analysis, recombinant proteins or cell lysates were subjected to SDS-PAGE and then transferred to nitrocellulose membranes. The membranes were blocked with 5% milk in TBST (20 mM Tris, pH 8.0, 500 mM NaCl, and 0.05% Tween20) overnight and then incubated with primary antibody diluted in TBST plus 0.5% milk at a 1:10,000 dilution for 1 h. After three washes with TBST, the membranes were incubated with secondary antibody diluted in TBST plus 0.5% milk for 30 min. Membranes were washed again with TBST three times and then incubated with its substrate Myc-(1–103) by any of the cotransfected TA02, A, HA-JNK1 was transfected into 293 cells with empty vector or with various Myc-tagged TA02 constructs. HA-JNK1 was immunoprecipitated from the cells and assayed with ATF2 as substrate. Top panel, autoradiogram showing phosphorylation of ATF2 by JNK1. Bottom panel, immunoblot of lysates with anti-HA antibody, indicating that equal amounts of JNK1 were expressed. B, experiments as in A with HA-SAPK6. C, experiments as in A with HA-p38. D, experiments as in A with HA-ERK2 except that Myc-(1–103) was utilized as the substrate. One of three similar experiments.

**Differentiation of C2C12 Cells**—Mouse myocyte C2C12 cells were purchased from ATCC. Cells were grown in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum to 80% confluence and then placed in Dulbecco’s modified Eagle’s medium with 2% horse serum for 4 days to allow differentiation essentially as described (16, 17). Half of this medium was replaced with fresh medium daily. During differentiation, cells were treated daily with either dimethyl sulfoxide (Me₂SO) diluent or 15 μM SB203580 in Me₂SO. At the indicated times, cells were harvested in detergent lysis buffer as described, and enzymes were isolated and assayed as described in the figure legend.

**RESULTS**

**Stress-sensitive MAPKs Are Activated by Cotransfected TA02**—To investigate TA02 selectivity in intact cells, cotransfection experiments were performed. In a cotransfection assay, an epitope-tagged MAPK construct was transfected into 293 cells with either the empty vector control or with a differently tagged TA02 construct. The overexpressed MAPK was immunoprecipitated from the cells and assayed using transcription factor substrates (Fig. 1). HA-JNK1, when cotransfected with the TA02 protein, became much more active toward its substrate ATF2 (Fig. 1A). Similarly, HA-SAPK6 was activated by the TA02 constructs toward ATF2 (Fig. 1B). The activity of HA-p38 on its substrate ATF2 was also greatly enhanced by cotransfected TA02(1–320) or TA02(1–451) (Fig. 1C). In contrast to these stress-activated MAPKs, ERK2 was not activated toward its substrate Myc-(1–103) by any of the cotransfected...
TAO2 plasmids (Fig. 1D) further supporting the conclusion that TAO2 is not an upstream activator of the classical ERK pathway.

TAO2-(1–451) appeared to be generally more active than TAO2-(1–320) or TAO2-(1–993). TAO2-(1–993) is probably less active because earlier studies with protein produced in Sf9 cells, suggested that it contains a negative regulatory domain that inhibits its activity (2). Studied on kinase from chicken also indicated an inhibitory effect of the C terminus on its catalytic domain (5). The difference in action between TAO2-(1–320) and TAO2-(1–451) is likely due to the absence and presence, respectively, of the MEK3/6 binding domain. This domain of TAO2 has been localized to residues 314–451 (2). Thus, TAO2-(1–320), the TAO2 minimal catalytic domain, lacks the MEK3/6 binding site and, thus, displays little selectivity among stress-sensitive MEKs.

**TAO2 Activates Endogenous, Stress-sensitive MAPKs—**The above studies demonstrated that JNK/SAPK and p38 can be activated by TAO2 in overexpression systems. Therefore, we examined effects of TAO2 on endogenous MAPKs. Empty vector control, TAO2-(1–451), or kinase-dead TAO2-(1–451)DA were transfected into 293 cells, and endogenous p38, JNK/SAPK, and ERK1/2 were immunoprecipitated from transfected cells and assayed using transfection factor substrates. Both endogenous p38 and endogenous JNK/SAPK were significantly activated by TAO2-(1–451) toward p38 in comparison, activities of MEK3 and MEK6 were enhanced by cotransfected TAO2 (lanes 11 and 13) suggesting that TAO2 selectively activates MEK3 and MEK6 of the p38 pathway in intact cells. A kinase-dead construct, TAO2-(1–451)DA, failed to activate cotransfected MEK6 (lane 14) indicating that TAO2 kinase activity is essential.

**Overexpressed TAO2 Preferentially Phosphorylates MEK3 and MEK6—**-pCMV5-Myc-TAO2-(1–451) and TAO2-(1–451)DA were transfected into 293 cells (Fig. 4A). Overexpressed TAO2 proteins were then immunoprecipitated from the cells through their Myc epitope and assayed using catalytically defective MEKs 3, 4, and 6 as substrates. Kinase reactions were resolved on SDS-PAGE and subjected to autoradiography (Fig. 4B). MEK3KM and MEK6KM were phosphorylated by TAO2-(1–451) to different extents (lanes 1 and 5), whereas MEK4KM, which should migrate right above the autophosphorylated MEK2 band, was barely phosphorylated by TAO2-(1–451)DA indicating that TAO2 kinase activity is essential.

Endogenous TAO2 Forms a Complex with MEK3 and MEK6 in Vivo—A polyclonal TAO2 antiserum was generated against an N-terminal peptide from TAO2. The antibody was preincubated with antigenic peptide to demonstrate that the peptide blocked recognition of the TAO2 band (Fig. 5A). We examined several cell lines of neuronal origin because Northern analysis
indicated TAO2 mRNA is most highly expressed in brain (1). Using the antibody, we found that TAO2 is expressed in numerous cell lines including 293 fibroblasts (Fig. 5B). By fractionation TAO2 is found in both cytosol and particulate fractions of 293 cells but is not detectable in nuclei (Fig. 5C).

Given the apparent importance of the MEK3/6 binding site for TAO2 specificity, we determined if endogenous TAO2 associates with MEK3 in vitro. 293 cells were transfected with Myc-MEK3. Endogenous TAO2 was immunoprecipitated from the cells with the anti-TAO2 antibody and blotted with anti-Myc antibody. Myc-MEK3 was detected in the TAO2 immune complexes (Fig. 6A). To investigate whether coprecipitation can be demonstrated with both endogenous proteins, TAO2 was immunoprecipitated from proliferating 293 cells with the anti-TAO2 antibody, and the precipitates were then blotted with a goat anti-MEK6 antibody. Endogenous MEK6 was found in immune complexes with endogenous TAO2 but not with Raf, the MEKK-level activator of the ERK pathway (Fig. 6B), indicating that this interaction is specific. No such interactions could be observed between TAO2 and MEK4 or MEK7 (data not shown), consistent with results above and in vitro binding studies (2).

**Sorbitol Stimulates the Activity of Endogenous TAO2—** We wished to determine whether TAO2 itself can be activated by stress stimuli that reportedly activate JNK/SAPK or p38. To this end, 293 cells were serum-starved for 24 h and then exposed to osmotic stresses, NaCl, sorbitol, or the microtubule-directed drugs, Taxol and nocodazole. Because TAO2 contains many sites of autophosphorylation, autophosphorylation is an easy method to detect its activity (1, 2). Endogenous TAO2 was immunoprecipitated from the cells and its ability to autophosphorylate was measured. TAO2 autophosphorylation was most highly stimulated by sorbitol and slightly elevated by NaCl, taxol, and nocodazole (Fig. 7A). Endogenous TAO2 immunoprecipitated from untreated or sorbitol-treated cells was then assayed using kinase-dead MEK3 and MEK6 as substrates to confirm that enhanced autophosphorylation is a reflection of enhanced TAO2 activity under these circumstances. Phosphorylation of both MEK3 and MEK6 by TAO2 was enhanced by sorbitol treatment (Fig. 7B). Thus, sorbitol, and to a lesser extent several other cell stresses, increased TAO2 activity.

**Activities of Stress-sensitive Kinases and TAO2 during Differentiation of C2C12 Myoblasts—** C2C12 myoblasts can be induced to differentiate and form myotubes in culture (16–18). p38 is activated during the differentiation process, and its activation is required for differentiation induced by either insulin or growth factor withdrawal. In contrast, JNK/SAPK activities have been reported to change little, and AP-1 activity, which is enhanced by JNK/SAPKs, is suppressed during differentiation consistent with no role of JNK/SAPKs in differentiation (16–18). We wished to determine whether changes in TAO2 activity occurred during differentiation. If so, that would support the idea that activation of endogenous TAO2 parallels activation of p38 but not the JNK/SAPK pathway. C2C12 cells were maintained in differentiation medium with or without the p38 inhibitor SB203580 for 4 days (Fig. 8A). Myotubes were obvious in the day 4 cultures maintained in the absence of SB203580 but were largely absent if cells had been constantly exposed to SB203580. The amount of p38 protein did not change, but, as expected, its activity increased on or before day 2 and remained elevated through day 4 (Fig. 8, B and C). SB203580 suppressed the elevated activity. The amount of TAO2 protein in the cells decreased significantly over the diff-
differentiation time course in a manner largely or entirely insensitive to SB203580 (Fig. 8B). However, TAO2-specific activity increased over the time course of differentiation also in an SB203580-independent manner despite the decrease in its mass (Fig. 8D). The amount of JNK/SAPK protein in the cells increased; nevertheless, its specific activity decreased over the time course (Fig. 8, B and E). In contrast to p38 and TAO2, JNK/SAPK activity in cells measured on day 0 was substantial perhaps due to the effects of serum growth factors. Because JNK/SAPK activity is not required for differentiation, its activation may be suppressed during this process. Thus, TAO2 activity parallels p38 activity but not JNK/SAPK activity. This is consistent with the idea that TAO2 is coupled to p38 but not JNK/SAPKs in cells.
DISCUSSION

Recent molecular cloning studies have revealed the existence of a multitude of MEKK-level kinases in mammalian systems including TAO1 and TAO2 (1, 19–21). This diversity may allow multiple mechanisms for activation of MAP kinase subgroups in a ligand- and cell type-dependent manner. In several cases, MAPK regulation by MEKK-level kinases correlates well with the ability of MEKks to phosphorylate and activate corresponding MEK proteins in MAPK modules. Thus, we previously examined the substrate specificity of TAOs in vitro and, in this report, TAO2 in cell culture systems. In vitro, the purified, recombinant, catalytic domains of TAO1 and TAO2 activated MEKs 3, 4, and 6 toward their substrates, p38 and JNK/SAPK (1, 2). In contrast, when an active TAO2 truncation mutant was cotransfected with various MEK proteins only MEK3 and MEK6 of the p38 pathway were activated. TAO2 truncation mutants activated both cotransfected and endogenous p38 and JNK/SAPKs. By monitoring TAO2, p38, and JNK/SAPK activities during C2C12 differentiation, we were able to show that conditions that lead to activation of endogenous TAO2 are associated with increases in p38 but not JNK/SAPK activity. This sort of evidence supports the conclusion that TAO proteins are primarily coupled to the p38 pathway and have little role in regulating JNK/SAPK.

The specificity for MEKs 3 and 6 may reflect the importance of the MEK binding domain of TAO2. Earlier we identified this TAO domain immediately C-terminal to its kinase domain and found that it recognized an N-terminal motif in MEKs 3 and 6 (1, 2). Thus far, we have not found a direct site for p38 binding on TAO2. Because the JNK/SAPK activators, MEK4 and MEK7, neither bind TAO2 nor are activated by TAO2 in cells, activation of JNK/SAPK by TAO may be due in part to TAO2 overexpression and to the relaxed specificity of MEK6. Indeed, in vitro kinase studies have shown that MEK6 can phosphorylate JNK/SAPK to an appreciable extent, although the specific activity is lower than that of MEK4 toward JNK/SAPK (data not shown).

Enzyme-substrate interactions have received much attention in studying molecular determinants of pathway specificity. Evidence for the importance of these interactions comes from studies of cAMP-dependent protein kinase and the ERK and JNK/SAPK MAPK modules among others (22–25). A docking site for p38 in MEK6 and MEK3b, an alternatively spliced isoform of MEK3, appears to contribute to specificity of p38 signaling (26). The association of TAO with MEK3/6 and p38 indirectly through the MEK in signaling complexes provides compelling evidence for their interrelated functions even in the absence of information regarding their physiological roles. By selectively interacting with and phosphorylating MEK3 and MEK6 in vitro, TAO2 works in spatial proximity to these immediate upstream regulators of p38, thereby facilitating signal transduction from TAO2 to MEK3/6 and subsequently to p38.

Certain MEKks implicated in regulating JNK/SAPK and p38 are reportedly responsive to cellular stresses. For example, MEKK1 can be activated by cross-linking of high affinity IgE receptors or microtubule-directed drugs (15, 27, 28) and TAK1 is sensitive to UV, sorbitol, and Fas receptor ligation (29). To identify stimuli or ligands that regulate TAO2, we generated a high-titer TAO2 antibody that enabled us to immunoprecipitate endogenous TAO2 proteins from cells treated with a wide array of stimuli. Of the more than twenty stimuli tested, most had no detectable effect on TAO2 activity (data not shown). The osmotic stress, sorbitol, as well as some other stresses, activated TAO2 modestly suggesting that TAO2 may indeed be involved in stress-responsive pathways. Kinase-dead TAO2 did not block activation of p38 by sorbitol (data not shown). How- ever, many MEKks are activated by sorbitol, and all may contribute to p38 activation. If this is correct, it will be difficult to identify the individual contributions of these MEKks to sorbitol-induced p38 stimulation. Importantly, we have recently been able to show that kinase-dead TAO2 was effective in blocking activation of p38 by carbachol (30). Thus, the aggregate of our studies link TAO1/2 to a subset of conditions regulating the p38 MAP kinase cascade.

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