The p38α MAPK participates in a variety of biological processes. Activation of p38α is mediated by phosphorylation on specific regulatory tyrosine and threonine sites, and the three dual kinases, MAPK kinase 3 (MKK3), MKK4, and MKK6, are known to be the upstream activators of p38α. In addition to activation by upstream kinases, p38α can autoactivate when interacting with transforming growth factor-β-activated protein kinase 1-binding protein 1 (TAB1). Here we used MKK3 and MKK6 double knock-out (MKK3/6 DKO) and MKK4/7 DKO mouse embryonic fibroblast (MEF) cells to examine activation mechanisms of p38α. We confirmed that the MKK3/6 pathway is a primary mechanism for p38α phosphorylation in MEF cells, and we also showed the presence of other p38α activation pathways. We show that TAB1-mediated p38α phosphorylation in MEF cells did not need MKK3/4/6, and it accounted for a small portion of the total p38α phosphorylation that was induced by hyperosmolarity and anisomycin. We observed that a portion of peroxynitrite-induced phospho-p38α is associated with an ~85-kDa disulfide complex in wild-type MEF cells. Peroxynitrite-induced phosphorylation of p38α in the ~85-kDa complex is independent from MKK3/6 because only phospho-p38α not associated with the disulfide complex was diminished in MKK3/6 DKO cells. In addition, our data suggest interference among different pathways because TAB1 had an inhibitory effect on p38α phosphorylation in the peroxynitrite-induced ~85-kDa complex. Mutagenesis analysis of the cysteines in p38α revealed that no disulfide bond forms between p38α and other proteins in the ~85-kDa complex, suggesting it is a p38α binding partner(s) that forms disulfide bonds, which enable it to bind to p38α. Therefore, multiple mechanisms of p38α activation exist that can influence each other, be simultaneously activated by a given stimulus, and/or be selectively used by different stimuli in a cell type-specific manner.

Cellular responses to extracellular stimuli are mediated through intracellular signaling pathways such as the MAPK2 pathways. MAPKs are members of discrete signaling cascades and serve as focal points in response to a variety of extracellular stimuli (1–4). Several distinct groups of MAPKs have been characterized in mammals, and each group is composed of a set of three evolutionarily conserved, sequentially acting kinases: a MAPK, a MAPK kinase (MKK), and an MKK kinase (MAP3K) (5, 6). The MAP3Ks are serine/threonine kinases and are often activated through phosphorylation. MAP3K activation leads to the phosphorylation and activation of an MMK, which then stimulates MAPK activity through dual phosphorylation on threonine and tyrosine residues located in the activation loop of kinase subdomain VIII. Once activated, MAPKs phosphorylate target substrates on serine or threonine residues. It is believed that the substrate selectivity of MAP3K, MKK, and MAPK is conferred by specific interaction motifs located on the kinases and substrates (7, 8). In addition, MAPK cascade specificity is also mediated by scaffolding proteins that organize pathways in specific modules through simultaneous binding of several components (9). Although the kinase cascade is the primary activation mechanism of MAPKs, autoactivation of the MAPK p38α is promoted by its interaction with transforming growth factor-β-activated protein kinase 1 (TAK1)-binding protein 1 (TAB1) or by phosphorylation on Tyr-323 with the tyrosine kinase Zap70 (10–12). To date, however, there is little information regarding whether the multiple mechanisms are simultaneously or individually used by a given stimulus or regarding the contribution of the different pathways.

The p38 MAPK group is composed of p38α, p38β, p38γ, and p38δ (13–15). Among the four members, p38α is the most studied, and it has been implicated in cellular processes such as proliferation, differentiation, apoptosis, senescence, and inflammation (13–15). p38α activation has been observed in response to a variety of extracellular stimuli, including proinflammatory cytokines, bacterial components, and stress (16, 17). MKK3 and MKK6 are the two main MKKs that are known to activate p38α (18, 19). MKK4, an upstream kinase of JNK, can aid in the activation of p38α in cells exposed to various stimuli (18, 20). In addition, p38α can also be activated by interaction with TAB1 (10).

TAB1 was originally identified as an interacting protein of TAK1 (21). The interaction between TAB1 and p38α was later independently found by two research groups (10, 22). A splicing out; TAB1, transforming growth factor-β-activated protein kinase 1-binding protein 1; MAP3K, MKK kinase; TAK1, transforming growth factor-β-activated protein kinase 1; JNK, c-Jun NH2-terminal kinase; TNF, tumor necrosis factor-α; siRNA, small interfering RNA; MKP, MAP kinase phosphatase; WT, wild type; KM, kinase-dead mutant; PBK, PDZ-binding kinase.
variant of TAB1, TAB1β, has also been found to interact only with p38α and not TAK1 (11). In vitro and co-expression experiments have indicated that the interaction of TAB1 and p38α leads to p38α autophosphorylation on the dual phosphorylation sites in the activation loop (10, 11). TAB1-dependent p38α activation appears to play a role in injury response during myocardial ischemia (23), monocyte-derived dendritic cell maturation (24), and peripheral T-cell anergy maintenance (25). On one hand, phosphorylation of TAB1 by p38α has been observed, and negative feedback on TAB1 activation by phosphorylated TAB1 was proposed as a major function of TAB1-p38α interaction (22). On the other hand, a study using MEF cells generated from MKK3/6 DKO mice ruled out the role of TAB1 in tumor necrosis factor-α (TNF)-and UV radiation-induced p38α phosphorylation in MEF cells (20). Therefore, differing opinions exist as to whether MKK-independent p38α phosphorylation occurs.

To better understand the relevance of MKK-independent p38α activation, we examined p38α activation in MKK3/6 DKO and MKK4/7 DKO MEF cells. The MKK independence of TAB1-mediated p38α phosphorylation is supported by our data from MKK3/6 and MKK4/7 DKO MEF cells. We further show that MKK-independent p38α phosphorylation accounts for a small portion of the total p38α phosphorylation induced by some, but not all, of the different ligands in MEF cells. In addition to the role in promoting p38α phosphorylation, we found that TAB1 has an inhibitory effect on peroxynitrite-induced p38α phosphorylation that is associated with a disulfide complex. This p38α phosphorylation is likely to be independent from MKK3/6 because MKK3/6 double knock-out mostly eliminated the phosphorylation of p38α that is not associated with the disulfide complex. Our data indicate that, although the kinase cascade is a major mechanism in p38α activation, multiple mechanisms exist and can be simultaneously activated by a single stimulus, and they may coordinately regulate p38α phosphorylation.

MATERIALS AND METHODS

Reagents—Lipopolysaccharide (Escherichia coli 0111:B4) was purchased from List Biological Laboratories (Campbell, CA), and CpG DNA was purchased from Invivogen (San Diego, CA). Peroxynitrite, thapsigargin, and SB203580 were purchased from Calbiochem. Anisomycin, sorbitol, and anti-FLAG M2 antibodies were purchased from Sigma. Mouse TNF was purchased from R&D Systems (Minneapolis, MN). Antibodies against phospho-p38, phospho-JNK, and phospho-ERK were purchased from Cell Signaling Technology (Beverly, MA), and polyclonal antibodies against p38α, TAB1, and MKK4 were generated from New Zealand White rabbits by stepwise subcutaneous and muscle injections of each of the affinity-purified recombinant proteins.

Vectors and RNA Interference—FLAG-tagged p38α, TAB1, or TAB1β was cloned into the vector pcDNA3. The point mutations in p38α were generated with a QuikChange site-directed mutagenesis kit (Stratagene) and confirmed by DNA sequencing. Oligonucleotides were cloned into the vector pSuper to express short hairpin RNA. The following sequences were targeted: 5’-AGCAGTCTTCTCAACAGCAAG-3’ for siTAB#1 and 5’-AGGCCCTTCTGTGCAAATCTAC-3’ for siTAB#2. Double-stranded RNA targeting the sequence 5’-AATGCGGAGTATGATGTCGAT-3’ of MKK4 was purchased from Dharmaco Inc. (Chicago, IL), and control double-stranded RNA targeting the sequence 5’-ATGATTGGATTGTCAATGGTTCACC-3’ of green fluorescent protein was obtained from Invitrogen.

Cell Culture and Transfection—Wild-type, MKK3/6 DKO, or MKK4/7 DKO MEF cells were maintained in high glucose Dulbecco’s modified Eagle’s medium (Invitrogen) plus 10% fetal bovine serum at 37 °C in a humidified 5% CO2 atmosphere. The transfection of expression vectors was done using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Briefly MEF cells were freshly seeded to 70% confluence 1 day before transfection. 3–5 μg of total DNA were mixed with 5 μl of Lipofectamine 2000 in 200 μl of Opti-MEM (Invitrogen) for 30 min before dropwise addition onto the cells. Analysis of transfected gene expression took place 24–48 h later.

For the selection of TAB1 knockdown MEF cells, cells were incubated in a medium containing 100 μg/ml hygromycin and were further cultured for 2 weeks and then pooled. MEF cells were transfected with siRNA oligonucleotides using Lipofectamine 2000, and then they were incubated for 48 h and subjected to Western blot analysis.

Western Blot Analysis—Cells were washed twice with ice-cold phosphate-buffered saline and were lysed in a lysis buffer containing 50 mM Tris-Cl (pH 7.5), 0.15 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 2 μg/ml aprotinin, and 5 μg/ml pepstatin. After centrifugation at 12,500 rpm for 5 min, the supernatant was collected, and the protein concentration was determined by the Bradford method. Non-reducing cell lysate samples were prepared in lysis buffer without dithiothreitol and were mixed with the SDS-PAGE sample buffer without β-mercaptoethanol. Cell lysates were separated by SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (Amersham Biosciences) and were then immunoblotted with antibodies against FLAG, phospho-p38, phospho-JNK, phospho-ERK, p38α, TAB1, and MKK4.

In Vitro Kinase Assay—In vitro kinase assays were performed using recombinant His-tagged p38α as a kinase and myelin basic protein as a substrate as described previously (16).

RESULTS

TAB1- or TAB1β-mediated Phosphorylation of p38α Occurs in Both Wild-type and MKK3/6 DKO or MKK4/7 DKO MEF Cells—TAB1-mediated p38α phosphorylation in cells can be seen by co-expression of TAB1 and p38α (10). The MKK independence of TAB1-mediated p38α phosphorylation has been established by the observation that TAB1-mediated p38α phosphorylation is not inhibited by dominant negative MKKs, and it is dependent on the intrinsic activity of p38α (10). To determine whether TAB1-mediated p38α phosphorylation in cells is unequivocally independent of MKK3 and MKK6, we co-expressed p38α with TAB1 or TAB1β in MKK3/6 DKO and control wild-type MEF cells. The phosphorylation of p38α was measured by Western blotting with

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Because TAB1-mediated p38α phosphorylation is involved in extra- cellular stimuli-induced p38α activation, we examined p38α phosphorylation in wild-type and MKK3/6 DKO cells in response to different stimuli. As shown in Fig. 2, wild-type MEF cells responded to bacterial lipopolysaccharides and CpG oligonucleotides, to the proinflammatory cytokine TNF, to the endoplasmic reticulum Ca^{2+}-ATPase inhibitor thapsigargin, to the protein synthesis inhibitor anisomycin, to the nitorgen monoxide (NO) producer peroxynitrite, and to hygroscopicity (0.5 M sorbitol). The level of response in p38α phosphorylation varied considerably among the different stimuli. Lipopolysaccharide- and CpG-induced p38α phosphorylation was...
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FIGURE 3. Comparison of anisomycin-, peroxynitrite-, and sorbitol-induced phosphorylation of p38α in wild-type and MKK4/7 DKO MEF cells. Wild-type or MKK4/7 DKO MEF cells were treated with either anisomycin, peroxynitrite, or sorbitol. Cell lysates were prepared and analyzed by immunoblotting with anti-phospho-p38 (\(\ast p\)-p38\(\alpha\)) and anti-FLAG-p38\(\alpha\) antibodies. Two experiments were done with comparable results.

barely detectable, but high levels of p38\(\alpha\) phosphorylation were detected in thapsigargin-, anisomycin-, peroxynitrite-, and hyperosmolarity (sorbitol)-treated cells. The phosphorylation of p38\(\alpha\) was not detected in lipopolysaccharide-, CpG-, TNF-, or thapsigargin-treated MKK3/6 DKO cells, but it was detected in anisomycin-, peroxynitrite-, and sorbitol-treated MKK3/6 DKO cells, albeit the levels of phosphorylation were dramatically reduced. Therefore, in MEF cells, MKK3/6-dependent and MKK3/6-independent p38\(\alpha\) activation occurs in parallel in response to a wide range of stimuli.

Because MKK4 participates in the activation of p38\(\alpha\) under certain conditions, we sought to determine whether MKK4 is involved in anisomycin-, peroxynitrite-, and hyperosmolarity (sorbitol)-induced p38\(\alpha\) phosphorylation using MKK4/7 DKO MEF cells. As shown in Fig. 3, MKK4 deletion had no detectable effect on anisomycin-, peroxynitrite-, and hyperosmolarity (sorbitol)-induced p38\(\alpha\) phosphorylation in MEF cells, indicating that MKK4 is not involved in or only has a little contribution to these stimuli-induced p38\(\alpha\) phosphorylation events.

TAB1 Can Both Positively and Negatively Regulate p38\(\alpha\) Phosphorylation in a Stimulus-dependent Manner—We next examined whether the MKK-independent p38\(\alpha\) phosphorylation shown in Fig. 2 is TAB1-dependent. Because TAB1 knockout cells were not available to us, we used siRNA to knock down TAB1 expression in MEF cells. After testing a number of TAB1-targeting siRNAs, we found two (named siTAB\#1 and siTAB\#2) that were able to effectively knock down TAB1 expression in MEF cells. DNA sequence analysis (Blastn) indicated that siTAB\#1 and siTAB\#2 only target TAB1. We stably expressed siTAB\#1 or siTAB\#2 in MKK3/6 DKO cells and measured TAB1 expression by Western blot analysis. A significant reduction in TAB1 was seen in both siTAB\#1- and siTAB\#2-expressing MKK3/6 DKO cells (Fig. 4A). As shown in Fig. 4B, anisomycin- and hyperosmolarity (sorbitol)-induced p38\(\alpha\) phosphorylation was reduced in cells expressing TAB1-targeting siRNA, suggesting that anisomycin- and hyperosmolarity (sorbitol)-induced MKK-independent p38\(\alpha\) phosphorylation in MEF cells is TAB1-dependent.

To our surprise, peroxynitrite-induced p38\(\alpha\) phosphorylation was not reduced by siTAB\#1 and siTAB\#2 siRNA, but on the contrary, it was significantly enhanced in both cell lines (Fig. 4B). The same results were obtained when pools of independently transfected cells were used in the experiments, indicating that this is unlikely an observation resulting from variation among individual cell clones or different batches of stably transfected cell lines. Therefore, we concluded that TAB1 has an inhibitory effect on peroxynitrite-induced p38\(\alpha\) phosphorylation in MKK3/6 DKO cells.

Previous studies by us and by others have suggested that peroxynitrite-induced p38\(\alpha\) phosphorylation in human embryonic kidney 293 cells and heart cells was mediated in part by TAB1 because SB203580 significantly inhibits peroxynitrite-induced p38\(\alpha\) phosphorylation (10, 23). Unlike peroxynitrite, sorbitol-induced p38\(\alpha\) phosphorylation in human embryonic kidney 293 cells and heart cells cannot be inhibited by SB203580, and anisomycin-induced p38\(\alpha\) phosphorylation is only weekly
inhibited by SB203580. Like the data obtained in human embryonic kidney 293 cells and heart cells, anisomycin-induced MKK-independent p38α phosphorylation in MEF cells was found to be dependent on TAB1 (Fig. 4B). However, although peroxynitrite-induced p38α phosphorylation was not dependent on TAB1 in MKK3/6 DKO MEF cells, sorbitol-induced p38α phosphorylation was (Fig. 4B). To determine whether these differences are due to the different types of cells or the experimental methods used, we used SB203580 to inhibit autophosphorylation-dependent p38α phosphorylation. As shown in Fig. 5, SB203580 partially inhibited anisomycin- and sorbitol-induced p38α phosphorylation, but it did not inhibit peroxynitrite-induced p38α phosphorylation in either wild-type (Fig. 5A) or MKK3/6 DKO MEF cells (Fig. 5B). Therefore, the results obtained through siRNA knockdown of TAB1 and through the inhibition of p38α autophosphorylation are consistent in MEF cells. Based on published data (10, 23) and the data shown in Fig. 5, it can be concluded that it is the cell type that determines whether a TAB1-dependent or -independent mechanism is used by peroxynitrite to induce p38α phosphorylation.

**TAB1 Knockdown in MEF Cells Non-selectively Enhances the Phosphorylation of MAP Kinases in a Ligand-dependent Manner**—Next we sought to determine whether the TAB1 knockdown-mediated enhancement of p38α phosphorylation is specific for p38α. MKK3/6 DKO MEF cells expressing siTAB#1 or siTAB#2 were stimulated with peroxynitrite or sorbitol, and the phosphorylation levels of p38α or JNK1/2 were determined by Western blot analysis using anti-phospho-p38 and anti-phospho-JNK antibodies, respectively. Like the data shown in Fig. 4B, peroxynitrite-induced p38α phosphorylation was found to be much stronger in cells expressing TAB1-targeting siRNA (Fig. 6A). It is important to note that peroxynitrite-induced JNK phosphorylation was also enhanced by TAB1 knockdown (Fig. 6A). In contrast, sorbitol-induced JNK phosphorylation was not affected by TAB1 knockdown. As previously seen in Fig. 4B, sorbitol-induced p38α phosphorylation was inhibited by TAB1 knockdown.

We next examined whether TAB1 knockdown affected p38α phosphorylation in wild-type MEF cells. Consistent with the data obtained with MKK3/6 DKO MEF cells, TAB1 knockdown enhanced peroxynitrite-induced p38α phosphorylation (Fig. 6B). Peroxynitrite-induced ERK phosphorylation was also enhanced by TAB1 knockdown. In a manner consistent with the data shown in Fig. 5A, sorbitol-induced p38α phosphorylation was reduced in TAB1 knockdown cells, and sorbitol-induced ERK phosphorylation was not affected by TAB1 knockdown. Therefore, TAB1 inhibits p38α phosphorylation when cells are stimulated with peroxynitrite but not other stimuli such as hyperosmolarity (sorbitol). Furthermore the enhancement of peroxynitrite-induced phosphorylation by TAB1 knockdown is not restricted to p38α.

A recent report showed that reactive oxygen species sustain JNK activation by inactivating MAP kinase phosphatases (MKPs) (26). The inactivation of MKP-1 by reactive oxygen species is achieved by converting the catalytic cysteine of MKP to sulfenic acid. The increase in oxidized MKP-1 results in a reduction of non-oxidized MKP-1, which can be detected by performing a Western blot using non-reducing and reducing gels. Because peroxynitrite is an NO producer, we examined the peroxynitrite-induced oxidation of MKP-1 in MEF cells and were unable to detect oxidized MKP-1 (data not shown). Also we did not detect a reduction in MKP-1 protein in either sorbitol- or peroxynitrite-treated samples when we reprobed the Western blot membranes shown in Fig. 6A (reprobed results are shown in Fig. 6C). Therefore, modification of MKP-1 is unlikely to be the mechanism used by TAB1 to enhance peroxynitrite-induced p38α phosphorylation.

**The Formation of an ~85-kDa Disulfide Complex Is Involved in Peroxynitrite-induced p38α Phosphorylation in MEF Cells**—In Schizosaccharomyces pombe, the peroxide-induced activation of the p38 homolog Sty1 requires the formation of a peroxide-induced disulfide complex between 2-Cys peroxiredoxin (TpX1) and Sty1 (27). We analyzed whether peroxynitrite induces any disulfide complex that is associated with p38α by Western blot analysis using reducing and non-reducing gels. We found that when peroxyni-
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molecules, between p38α and its binding partner, between p38α binding partners, or even within a molecule in that complex. The membranes shown in Fig. 7, A and B, were stripped and then reprobed with anti-p38α antibodies to show equal sample loading (Fig. 7, C and D). It should be noted that only a very small amount of p38α is associated with the peroxynitrite-induced disulfide complex (Fig. 7A) because anti-p38α antibodies only detected p38α with an apparent molecular mass of ~40 kDa (Fig. 7C). The fact that we could not detect p38α at ~85 kDa with anti-p38α antibodies in peroxynitrite-treated cells is probably because our anti-p38α antibodies were not sensitive enough in comparison with anti-phospho-p38 antibodies. We noticed that the levels of p38α phosphorylation detected by non-reducing and reducing gels were not quite the same. We know that Western blotting with a non-reducing gel is less sensitive and often has a higher background than when using a reducing gel, but we do not know the reason why at this moment.

Peroxynitrite-induced p38α Phosphorylation in MKK3/6 DKO MEF Cells Is Associated with the Disulfide Complex—To address the role of MKKs in p38α phosphorylation in the ~85-kDa complex, we examined peroxynitrite-induced p38α phosphorylation in MKK3/6 DKO cells using non-reducing and reducing gels. We found that peroxynitrite-induced p38α phosphorylation in MKK3/6 DKO cells is mainly associated with the disulfide complex (Fig. 8A, top panel). The role of disulfide was confirmed by analysis using a reducing gel (Fig. 8A, middle panel). Equal sample amount was confirmed by reprobing the membrane with anti-p38α antibodies (Fig. 8A, bottom panel). Because the ~40-kDa phospho-p38α can be seen in the non-reducing gel analysis of wild-type cells (Fig. 7A) but not MKK3/6 DKO MEF cells (Fig. 8A), MKK3 and/or MKK6 must be responsible for the phosphorylation of p38α that is not associated with the disulfide complex in peroxynitrite-treated MEF cells.

To examine the role of MKK4, we used MKK4/7 DKO cells. As shown in Fig. 8B, top panel, phospho-p38α with both high

FIGURE 6. TAB1 knockdown non-selectively enhances peroxynitrite-induced MAP kinase phosphorylation in MEF cells. A, control-, siTAB#1-, or siTAB#2-expressing MKK3/6 DKO MEF cells were treated with or without sorbitol or peroxynitrite for 30 min. Cell lysates were prepared and analyzed by immunoblotting using anti-phospho-p38 (*p-p38α), anti-phospho-JNK (*p-JNK), and anti-p38α antibodies. B, control-, siTAB#1-, or siTAB#2-expressing wild-type MEF cells were treated with or without sorbitol or peroxynitrite for 30 min. Cell lysates were prepared and analyzed by immunoblotting using anti-phospho-p38, anti-phospho-ERK (*p-ERK), and anti-p38α antibodies. C, the samples described in A were analyzed by immunoblotting with anti-MKP-1 antibodies.

FIGURE 7. A small portion of peroxynitrite-induced p38α phosphorylation in MEF cells is associated with a disulfide complex. MEF cells were treated with peroxynitrite for different time periods as indicated. Cell lysates were prepared, resolved using non-reducing (A and C) or reducing (B and D) SDS-PAGE, and then analyzed by immunoblotting using anti-phospho-p38 (*p-p38α) and anti-p38α antibodies.
and low molecular weights was detected in peroxynitrite-treated MKK4/7 DKO cells. The roles of disulfide and equal sample preparation were confirmed by analysis using a reducing gel (Fig. 8B, middle and bottom panels). This data confirmed that MKK3 or MKK6 is critical for the phosphorylation of p38α not associated with the disulfide complex.

**TAB1 Knockdown-enhanced p38α Phosphorylation Is Associated with the Disulfide Complex and Requires Intrinsic p38α Activity**—Because TAB1 knockdown did not inhibit but rather enhanced peroxynitrite-induced p38α phosphorylation (Fig. 4), we examined whether it enhances p38α phosphorylation in the ~85-kDa complex. Because peroxynitrite-induced p38α phosphorylation is associated with the disulfide complex in MKK3/6 DKO cells, we compared the levels of phosphorylation in MKK3/6 DKO cells expressing and not expressing siRNA against TAB1. TAB1 knockdown enhanced peroxynitrite-induced p38α phosphorylation (Fig. 9A). As shown earlier, phospho-p38α was detected as being ~85 and ~40 kDa in non-reducing and reducing gels, respectively (Fig. 9A), indicating an association between phospho-p38α and the disulfide complex.

The phospho-p38α in the ~85-kDa complex was detected in both MKK3/6 DKO and MKK4/7 DKO cells (Fig. 8, A and B), suggesting that MKK3, MKK4, and MKK6 are not necessary for the p38α phosphorylation occurring in the ~85-kDa complex. However, this cannot exclude the possibility that all three contribute to p38α phosphorylation in the ~85-kDa complex and that neither a knock-out of MKK3/6 nor a knock-out of MKK4 is sufficient to block this pathway. To address this possibility, we used siRNA to knock down MKK4 expression in MKK3/6 DKO cells. Because peroxynitrite-induced p38α phosphorylation is stronger in MKK3/6 DKO cells expressing siTAB1#1 or siTAB2#2 than in those expressing neither, we used MKK3/6 DKO cells that stably expressed siTAB1#1 or siTAB1#2 for this experiment. As shown in Fig. 9B, knockdown of MKK4 in MKK3/6 DKO cells did not have a significant effect on peroxynitrite-induced p38α phosphorylation. This suggests that the phosphorylation of p38α in the ~85-kDa complex is MKK-independent. However, a definite conclusion cannot be reached because knockdown of MKK4 cannot be completed. Therefore, we examined whether the phosphorylation of p38α in the ~85-kDa complex depends on the intrinsic activity of p33α. In MEF cells we transiently expressed either FLAG-tagged p38α(WT) or the FLAG-tagged catalytically inactive p38α mutant p38α(KM), and then stimulated the cells with peroxynitrite. Phospho-p38α and FLAG-p38α were analyzed using non-reducing gels (Fig. 9C). The association of phospho-FLAG-p38α with a disulfide complex was found in cells.
expressing FLAG-p38α(WT) but not FLAG-p38α(KM). This suggests that p38α phosphorylation in the ~85-kDa complex requires intrinsic p38α activity (e.g., autophosphorylation). We noticed that ectopic overexpression of FLAG-p38α (either WT or KM) reduced the phosphorylation of endogenous p38α in the ~85-kDa complex; this could have resulted from competition.

The data in Fig. 5 show that the p38 inhibitor SB203580 did not inhibit peroxynitrite-induced p38α phosphorylation, whereas the data in Fig. 9C suggest that this p38α phosphorylation requires intrinsic p38α activity. To address this confusion, we examined whether SB203580 would function differently under reduced and oxidized environments because the disulfide complex should be in an oxidized environment. An in vitro kinase assay with recombinant p38α and myelin basic protein (as a substrate) was used to determine the inhibitory effect of SB203580 on p38α activity in the presence of GSH, GSH and GSSG (1:1), or GSSG, p38α is insensitive to inhibition by SB203580 in an oxidized environment (Fig. 9D). This may explain why peroxynitrite-induced p38α phosphorylation requires its intrinsic kinase activity but is insensitive to SB203580.

Unlike the Tpx1-Sty1 Complex in Yeast, p38α Does Not Associate with Its Partner in the ~85-kDa Complex through Disulfide Bonding—Because yeast Tpx1 forms a disulfide complex with Sty1 in co-expression experiments (27), we sought to determine whether the same complex can be formed in mammalian cells. There are five members of 2-Cys peroxiredoxin in mammals, and we co-expressed peroxiredoxin-1, a counterpart of Tpx1, with p38α. We were unable to detect a disulfide complex formation between peroxiredoxin-1 and p38α both before and after peroxynitrite or H2O2 stimulation (data not shown). We used 1-Cys peroxiredoxin (peroxiredoxin-6) and obtained the same result (data not shown). It is possible that another form of peroxiredoxin is responsible for forming the disulfide complex with p38α in mammals. However, it is also possible that the complex we observed is different from the Tpx1-Sty1 complex in yeast.

Because Cys-35 in Sty1 forms a disulfide bond with Tpx1 in yeast, one way to address this question is to determine whether p38α utilizes Cys-39, which corresponds to Cys-35 in Sty1, to form a disulfide bond in the ~85-kDa complex. Because ectopically overexpressed FLAG-p38α apparently can replace the endogenous p38α in the ~85-kDa complex (Fig. 9C), we overexpressed mutants of p38α to determine the role of cysteine residues. There are four Cys residues in murine (and human) p38α. We mutated each of the four Cys residues to Ser in p38α, expressed the mutants in MKK3/6 DKO cells, and examined whether any of the mutants is not associated with the ~85-kDa complex when treated with peroxynitrite. Mutating Cys-39, Cys-119, Cys-162, or Cys-211 did not eliminate peroxynitrite-induced p38α phosphorylation and the association of p38α with the ~85-kDa complex (Fig. 10A), suggesting two possibilities: either none of the four Cys residues is required for p38α to bind with the ~85-kDa complex or more than one Cys is involved in the binding. We then generated several mutants, including various combinations of double mutants, a triple mutant of Cys-39, Cys-119, and Cys-211, and a quadruple mutant of all four residues. As shown in Fig. 10, B, C, and D, all mutants were capable of being associated with the ~85-kDa complex and phosphorylated in MKK3/6 DKO cells after peroxynitrite treatment. This indicates that the association of p38α with the ~85-kDa complex is not through disulfide bonds. Therefore, the disulfide bond required for p38α to bind to the ~85-kDa complex is most likely formed between p38α binding partners or within a p38α binding partner.

DISCUSSION

The activity of p38α is regulated by cellular mechanisms that control its phosphorylation and dephosphorylation. As for the phosphorylation mechanisms, the trikinase cascade is the major pathway. However, our study using MKK knock-out MEF cells demonstrated that other cellular events are involved in p38α activation. Phosphorylation of p38α can be mediated by interactions with TAB1 and can be modulated by a yet unknown binding partner(s) in a manner dependent upon a disulfide complex. First we show that more than one p38α activation pathway could be activated in the same cell by a single
stimulus, second we show that the selection of p38α activation mechanisms was both cell type- and cell stimulus-dependent, and third we show that different pathways may target differently localized p38α.

Using MKK knock-out MEF cells, we demonstrated that MKK-independent p38α phosphorylation can indeed occur in cells (Fig. 1). TAB1-dependent p38α phosphorylation contributed to a small portion of p38α phosphorylation in MEF cells treated with some stimuli (Figs. 2, 3, and 4). However, this does not exclude the possibility that TAB1-mediated p38α activation plays a key role in certain biological processes. There are reports that TAB1-dependent p38α activation plays an important role in cellular responses to ischemia in the heart (23), the maturation of monocyte-derived dendritic cells (24), the maintenance of peripheral T-cell energy (25), and intracellular parasite-induced interleukin-12 production (28). It is interesting to note that under certain conditions TAB1 also had an inhibitory effect on p38α phosphorylation (Fig. 4). We show that the inhibitory effect of TAB1 on p38α phosphorylation was restricted to p38α in a peroxynitrite-induced ~85-kDa complex (Figs. 7, 8, and 9). Because p38α phosphorylation in the ~85-kDa complex required intrinsic p38α activity (Fig. 9C), the inhibitory effect of TAB1 on this p38α phosphorylation could be a result of competition between different p38α regulation pathways. Because the inhibitory effect of TAB1 on MAP kinase phosphorylation was heavily dependent on stimuli like peroxynitrite, a specific signaling mechanism must be required for TAB1 to elicit this function. Phosphorylation of TAB1 by p38α on Ser-432, Thr-431, and Ser-438 was originally shown by Cheung et al. (22) and was proposed to be a feedback mechanism on TAK1 activation based on the observation that p38α inhibition resulted in increased TAK1 activity. Negative feedback by phosphorylated TAB1 on TAK1 cannot explain why TAB1 knockdown enhances p38α phosphorylation because our experiments were done in cells lacking MKK3 and MKK6, the downstream kinases of TAK1. However, the involvement of other types of negative feedback control, such as phosphatase induction, is still possible.

The TAB1-enhanced p38α phosphorylation in peroxynitrite-treated MEF cells is likely to be independent from MKK (Fig. 8). Unfortunately an siRNA approach cannot definitely exclude the possibility that MKK3/6 and MKK4 can compensate for each other in controlling p38α phosphorylation in the ~85-kDa complex (Fig. 9B). It is also possible that a kinase other than MKK3, MKK4, or MKK6 plays a role, but no additional MKK can be found in either the human or mouse genomes. The protein kinase PDZ-binding kinase (PKB/TOPK) was suggested to be a p38 kinase in a report (29), but because PKB has no effect on JNK or ERK phosphorylation (29), it cannot be a kinase responsible for the TAB1 knockdown-enhanced phosphorylation of p38α, JNK, and ERK in peroxynitrite-treated MEF cells. A study in yeast suggested a model in which peroxide-induced Sty1 activation requires Tpx1-Sty1 disulfide bond formation and Wis1 (MKK)-dependent phosphorylation (27). We show that the ~85-kDa complex in mammalian cells is different as p38α did not form a disulfide bond with its partner in this complex (Fig. 10). In addition, peroxide-induced Sty1 activation is primarily dependent on Tpx-1, whereas only a small portion of peroxynitrite-induced p38α phosphorylation was associated with the ~85-kDa disulfide complex. Furthermore although yeast uses a two-component system to activate the Sty1 kinase cascade, a two-component system has not been found in mammals.

The activation of p38α MAP kinase is involved in a variety of cellular changes (13, 14). Different stimuli may share some common mechanisms, such as the well established activation cascade of p38α by MKK3/6. Different stimuli may also use different mechanisms to activate p38α. Complex formation through non-covalent and disulfide bonds is involved in the regulation of p38α activation under different conditions. To elicit different cellular responses, different pathways may activate p38α in different subcellular locations or protein complexes. The same MKK-mediated p38α activation could occur in different places within cells or within different protein complexes. Cell type is particularly important as we found that different cell types may utilize different mechanisms to mediate MAPK activation by peroxynitrite (10, 23). Taken together, we have shown that p38α activation can be regulated by multiple mechanisms and that different modules of p38α activation exist for different stimuli and different cell types. The presence of multiple p38α activation mechanisms may be important for cells to differentially respond to a wide range of physiological and pathological stimuli with the necessary selectivity and fidelity.

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