TAB-1 Modulates Intracellular Localization of p38 MAP Kinase and Downstream Signaling*

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Stress-activated mitogen-activated protein (MAP) kinase p38 mediates stress signaling in mammalian cells via threonine and tyrosine phosphorylation in its conserved TGY motif by upstream MAP kinase kinases (MKKs). In addition, p38 MAP kinase can also be activated by an MKK-independent mechanism involving TAB-1 (TAK-1-binding protein)-mediated autophosphorylation. Although TAB-1-mediated p38 activation has been implicated in ischemic heart, the biological consequences and downstream signaling of TAB-1-mediated p38 activation in cardiomyocytes is largely unknown. We show here that TAB-1 expression leads to a significant induction of p38 autophosphorylation and consequent kinase activation in cultured neonatal cardiomyocytes. In contrast to MKK3-induced p38 kinase downstream effects, TAB-1-induced p38 kinase activation does not induce expression of pro-inflammatory genes, cardiac marker gene expression, or changes in cellular morphology. Rather, TAB-1 binds to p38 and prevents p38 nuclear localization. Furthermore, TAB-1 disrupts p38 interaction with MKK3 and redirects p38 localization in the cytosol. Consequently, TAB-1 expression antagonizes the downstream activity of p38 kinase induced by MKK3 and attenuates interleukin-1β-induced inflammatory gene induction in cardiomyocytes. These data suggest that TAB-1 can mediate MKK-independent p38 kinase activation while negatively modulating MKK-dependent p38 function.

Our study not only redefines the functional role of TAB-1 in p38 kinase-mediated signaling pathways but also provides the first evidence that intracellular localization of p38 kinase and complex interaction dictates its downstream effects. These results suggest a previously unknown mechanism for stress-MAP kinase regulation in mammalian cells.

Stress-activated protein kinase p38 is a member of a highly conserved subfamily of mitogen-activated protein (MAP)2 kinases involved in a wide variety of stress responses in organisms ranging from yeast to mammals (1–4). Activation of the p38 pathway is achieved by a cascade of phosphorylation events involving proximal upstream MAP kinase kinases, such as MKK3 and 6, and further upstream MAP kinase kinase kinases, such as TAK1 and ASK1 (3). Phosphorylation of both threonine and tyrosine at a conserved TGY motif leads to p38 kinase activation. This phosphorylation is therefore used widely as a biochemical marker for p38 activation status (5, 6). Phosphorylated (activated) p38 phosphorylates a large number of transcription factors that include NFAT, CHOP, p53, ATF-2, and MEF-2 (3, 4) to regulate gene expression.

Through downstream kinases, such as MAP kinase-activated protein kinase (MAPKAP)-2/3 (MK2, MK3), MNK, and MKS1, p38 activity is also responsible for the phosphorylation of additional downstream targets that include cytosolic PLA2, heat shock proteins, and histone3/HMG-14 (3, 4).

p38 activation plays a critical role in the regulation of pro-inflammatory genes, including TNFa (7, 8) and COX-2 (9–12) in mammalian cells. The onset of heart failure is tightly associated with an elevated inflammatory response, both in human failing hearts arising from a wide variety of etiologies and in several animal models of heart failure (13, 14). In addition, p38 activation is also observed in heart in a variety of pathological conditions, including mechanical stimulation, neural-hormonal stimulation, and cardiac ischemia injury (15–17). Therefore, it has been speculated that p38 activation and subsequent inflammatory induction contribute to pathological changes in the process of heart failure (18). Our recent studies also suggest a direct contribution of p38 activity to inflammatory induction, cardiac dysfunction, and pathological remodeling in heart (19, 20).

In addition to MKK-dependent p38 kinase activation, an alternative pathway leading to p38 activation involving TAB-1 (TAK-1-binding protein) was recently identified (21). Although TAB-1 was originally found to interact with and activate an upstream MAP kinase kinase kinase, TAK-1, Ge et al. (21) showed that TAB-1 can also directly bind to p38 and promote SB203580-sensitive, but MKK-independent, p38 autophosphorylation. Recently, Tanno et al. (22) also implicated TAB-1 in p38 activation in ischemic mouse hearts with homozygous MKK3 null alleles, whereas Li et al. (23) showed an increase in TAB-1 recruitment by p38 in ischemia heart in response to activated AMP-activated protein kinase. Furthermore, TAB-1 expression induces p38α activation leading to IL-10 induction and ERK and IL-2 inhibition in anergic T-cells (24). Although TAB-1 is sufficient to activate p38 kinase in vitro (21) and is involved in p38 activation in cardiomyocytes and T-cells, other recent studies also suggest that TAB-1 might function as a negative feedback regulator between p38 and TAK-1 (25, 26). Although increasing evidence suggests that TAB-1 participates in p38 signaling, the downstream effects of TAB-1-mediated p38 activity in stress signaling and gene regulation are unclear, particularly in comparison with MKK-induced p38 function.

In this report, we demonstrate that TAB-1 expression in cultured neo-
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natal cardiac myocytes is sufficient to induce p38 activity via autophosphorylation. However, TAB-1-mediated p38 activity does not lead to the classical downstream effects of p38 induced by MKK3 activation. In contrast, TAB-1 expression attenuates MKK3-induced downstream signaling, at least in part via competitive binding with p38, removal of p38 from the MKK3 signaling complex, and translocation of p38 to alternative intracellular compartments. Consequently, TAB-1 negatively modulates IL-1β-induced (MKK3/p38-mediated) inflammatory gene expression in cardiomyocytes. In contrast, knockdown of TAB-1 expression in wild-type MEF cells augments TNFα-induced COX-2 expression. These data clearly suggest that TAB-1 not only induces p38 kinase activation but also has an important role in modulating downstream effects, at least in part by altering p38 intracellular localization and complex interaction. Thus, this study reveals a novel mechanism of stress-MAP kinase regulation and demonstrates that downstream effects of p38 activation can be modulated by different upstream activators to yield distinct intracellular localization and complex interaction.

MATERIALS AND METHODS

Plasmids and Adenoviral Constructs—Plasmids encoding TAB-1 (21), TAB-1β (27) and TAB-1 (1–373) (21) were subcloned into the pShuttle-CMV vectors of the Aedas adenovirus system (Stratagene). A full-length TAB-1 CDNA with a DsRed tag was subcloned by PCR into the pShuttle-CMV vector. The C-terminal truncation mutant TAB-1 (333–504) with 3×FLAG tag was generated by PCR-mediated mutagenesis and subcloned into a modified pShuttle-CMV vector. A nuclear localization signal sequence coding for GPKKKRKVG was fused by PCR to the C termini of TAB-1 and TAB-1 (333–504). Subsequently, recombinant adenoviruses were prepared as described (28). Other adenoviral expressing vectors, including AdvHA-MKK3bE, AdvGFP-P38α, AdvFLAG-dnp38α, AdvLacZ, and AdvGFP, were constructed as described previously (28).

Cell Culture—Neonatal ventricular cardiomyocytes from 1–2-day-old Sprague-Dawley rats were isolated using a Percoll gradient method as described previously (28). Cardiomyocytes were plated overnight in Dulbecco’s modified Eagle’s medium/medium 199 (4:1) supplemented with 10% horse serum, 5% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10 mM glutamine. Subsequently, cardiomyocytes were infected with adenoviruses at MOI between 10 and 100 (optimized by protein expression level of the transgene) and incubated for 48 h in serum free medium supplemented with 1% ITS (BD Biosciences). p38-specific inhibitor SB203580 (Calbiochem) or IL-1β (BD Biosciences) was added into the culture medium, either 2 or 12 h after adenovirus infection, as indicated in specific experiments. HEK293, Cos-1, and HeLa cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Wild-type MEF cells (C57Bl/6 background) were maintained in high-glucose Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum. MEF cells were seeded 1 day before transfection, and 3–5 μg of DNA was mixed with Lipofectamine 2000 (Invitrogen) in 200 μl of Opti-MEM (Invitrogen) for 30 min before the addition to the cells. After 24–48 h, the cells were incubated in a medium containing 100 μg/ml of hygromycin and further cultured for 2 weeks and pooled.

Immunoblotting and Immunoprecipitation—The cells were washed twice with ice-cold PBS and harvested in lysis buffer composed of 50 mM TrisCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 1 mM β-glycerophosphate, 2.5 mM sodium pyrophosphate 1 mM Na3VO4, 20 mM NaF, 1 mM phenylmethysulfonyl fluoride, and a tablet mixture of protease inhibitors (Roche Applied Science). Total cell lysates were subjected to SDS-PAGE on 4–12% gradient gels and immunoblotted with antibodies against GFP (Clontech), HA tag, TAB-1, TNFα (Santa Cruz), FLAG (Sigma), COX-2 (Cayman), GAPDH (Chemicon), p38, phospho-p38, MAPKAP2, phospho-MAPKAP2, and phospho-HSP27 (Cell Signaling), respectively. For immunoprecipitation, total cell extracts prepared as described above were incubated with anti-FLAG M2 beads (Sigma) overnight with gentle rocking at 4 °C. The beads were then extensively washed five times with lysis buffer and once with Tris-buffered saline (50 mM TrisCl (pH 7.4), 150 mM NaCl) and then eluted with 100 μg/ml FLAG peptide in Tris-buffered saline. Eluted proteins were analyzed SDS-PAGE and immunoblotting.

Real Time Quantitative Reverse Transcription-PCR—Total RNA was isolated from cultured neonatal cardiac myocytes 48 h after adenoviruses infection using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. Five μg of RNA was used to reverse transcribe the first strand cDNA using Superscript first strand synthesis kit (Invitrogen). Then cDNA transcripts were quantified by iCycler iQ real time PCR detection system (Bio-Rad) using iQ SYBR Green Supermix (Bio-Rad). Each reaction was performed in duplicate, and the values were averaged to calculate the relative expression level. The specific primers for quantitative PCR were: COX-2, 5'-CCAGATGCTATCTTTGGGGA-3' (sense) and 5'-CGCCTTTTGATTAGCTGAGG-G-3' (antisense); ANF, 5'-CTGATGGATTTCAAGAACCTGCT-3' (sense) and 5'-CTCTGGGCTCCAATCTCTGC-3' (antisense); and GAPDH, 5'-TCTTGCACCAACAGTCTTAG-3' (sense) and 5'-GATGACCTTGGCCACAAGTCTG-3' (antisense).

Immunostaining and Fluorescence Microscopy—Cardiomyocytes and Cos-1 cells were cultured on 12-mm coverslips coated with 10 μg/ml Laminin (Invitrogen). 48 h after adenoviral infection, the cells were washed with PBS, fixed for 5 min with 4% paraformaldehyde, permeabilized for 5 min with 0.2% Triton X-100, and then blocked in PBS for 1 h with 3% bovine serum albumin and 5% donkey serum. The cells were then stained with primary antibodies and secondary antibodies diluted in 3% bovine serum albumin and 1% donkey serum in PBS for 2 h, respectively. The primary antibodies used were rabbit anti-anF antibody (Peninsula Laboratory) (1:1000), rabbit anti-HA polyclonal antibody (Santa Cruz) (1:1000), and mouse anti-FLAG M2 monoclonal antibody (Sigma) (1:5000). Secondary antibodies include Alexa568-conjugated donkey anti-rabbit IgG, Alexa568-conjugated donkey anti-mouse IgG, and Alexa640-conjugated donkey anti-rabbit IgG (Molecular Probes). F-actin was probed with fluorescein isothiocyanate-donkey conjugated phalloidin (Molecular Probes). Fluorescence images were obtained with a laser scanning confocal microscope (Olympus Fluoview) and analyzed with MetaMorph (Universal Imaging Corp.) and AutoDeblur (AutoQuant). The co-localization and the proximity of proteins were analyzed using custom made software described in Fig. 7.

RESULTS

TAB-1 Induces p38 Activity via Autophosphorylation in Cultured Rat Myocytes—Rat neonatal ventricular cardiomyocytes (RNVC) have very low level expression of endogenous TAB-1, based on Western blotting. They have an abundant level of p38α protein, mostly in inactive form under basal condition (Fig. 1). These cells present a good model system to investigate the functional effect of TAB-1 on p38 kinase signaling. We expressed human wild-type, full-length TAB-1 in rat neonatal cardiomyocytes via a recombinant adenovirus vector. TAB-1 expression leads to significant activation of the p38 kinase, as determined either by anti-phospho-p38 immunoblotting or by ATF-2 phosphorylation activity of p38 immunocomplexes (Fig. 1). p38 phosphorylation is partially blocked by p38 kinase-selective inhibitor SB203580 (Fig. 1A), in good
agreement with previous observations suggesting that TAB-1-mediated p38 activation involves autophosphorylation (21). A splicing variant of TAB-1 (TAB-1/α/H9252) containing both the p38-binding domain and the protein phosphatase 2C (PP2C)-like domain but lacking the TAK-1-binding domain (27) also activates p38 kinase as reported (21). All of these results suggest that TAB-1 induces bona fide p38 kinase activation in a SB203580-sensitive manner via autophosphorylation.

**TAB-1 Does Not Induce p38 Downstream Signaling Events Associated with MKK3-p38 Activation**—Expression of MKK3bE, an activated mutant of the p38 upstream activating kinase, in RNVC cells results in significant activation of p38 kinase activity as determined by phospho-p38-specific Western blot (Fig. 2A) (28). As a consequence, TNFα and COX-2 expression are induced at both the protein and mRNA levels (Fig. 2, A and B). In addition, cardiac ANF gene expression is induced, and myofilament organization is enhanced as part of the p38-mediated stress response as reported previously (28) (Fig. 2, C and D). In contrast, TAB-1 expression in RNVC cells does not activate any of these well-established p38 downstream responses or target gene induction in cardiomyocytes, despite the fact that comparable levels of p38 activation by MKK3bE and TAB-1 are achieved (Fig. 2).

**FIGURE 1.** TAB-1 activates p38 kinase in cultured cardiomyocytes. A, total cell extracts were analyzed by immunoblotting for phospho-p38, p38, TAB-1, and TAB-1/β present in cultured RNVC 48 h after infection with adenovirus vectors expressing LacZ, TAB-1, or TAB-1/β. For some cultures (lanes 1), 1 μM SB203580 was added into the medium as indicated 2 h after infection. B, p38 kinase activity was determined by incubating recombinant ATF2 with phospho-p38 immunoprecipitated from cardiomyocytes expressing either LacZ or TAB-1. Equal loading was confirmed by immunoblotting of ATF2 protein. MW(kd), molecular mass (in kDa).

**FIGURE 2.** TAB-1-mediated p38 activity does not lead to expected downstream effects in cardiomyocytes. A, the phosphorylation status of p38, as well as the expression levels of MKK3bE, TAB-1, total p38a, COX-2, and pro-TNFα were measured by immunoblotting of extracts from RNVC infected with AdvLacZ, AdvMKK3bE, or AdvTAB-1. Antibodies are described under “Materials and Methods.” B, 48 h after infection with AdvLacZ, AdvMKK3bE, or AdvTAB-1, total RNA was extracted from cardiomyocytes and used for real time reverse transcription-PCR analysis to determine the amounts of COX-2 mRNA. The results represent the mean values of three experiments with standard deviation. #, p < 0.01, AdvMKK3bE versus AdvLacZ or AdvTAB-1 samples, Student’s t test. C, representative immunofluorescent images for ANF (detected with a polyclonal anti-ANF antibody, red) and filament-actin (stained with fluorescein isothiocyanate-conjugated phallolidin, green) in RNVC infected with adenoviral vectors expressing LacZ, MKK3bE, and TAB-1 as indicated. D, the relative mRNA levels of ANF, measured by quantitative real time reverse transcription-PCR, in cultures infected with adenoviral vectors expressing LacZ, MKK3bE, and TAB-1. The results are the mean values from three experiments. #, p < 0.01 AdvMKK3bE versus AdvLacZ- or AdvTAB-1-infected cells, Student’s t test. MW(kd), molecular mass (in kDa).
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**FIGURE 3.** TAB-1 antagonizes MKK3-mediated p38 downstream signaling. A, immunoblot to detect phosphorylated p38, MK2, and HSP27, as well as MKK3bE, total p38, total MK2, and COX-2. RNVC cell lysates were prepared after infection with adenoviruses expressing LacZ or MKK3bE and treated with or without 10 µM SB203580, respectively. B, immunoblot to detect TAB-1, MKK3bE, p38, and MK2 and the phosphorylated forms of p38, MK2, and HSP27 in whole cell lysates prepared from RNVC infected with adenoviruses expressing GFP (MOI 100), TAB-1 (MOI 20 and 50), or MKK3bE (MOI 5). C, real-time reverse transcription-PCR analysis of COX-2 mRNA levels in RNVC infected with adenoviruses expressing GFP, TAB-1, and MKK3bE as described for B. **A**, **B**, and **C** as described for B. **A**, **B**, and **C** as described for B. Student’s t test. MW(kd), molecular mass (in kDa).

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TAB-1 Antagonizes MKK3-mediated p38 Downstream Signals—We further investigated the impact of TAB-1 expression on downstream signaling of p38 kinase in RNVC cells. As shown in Fig. 3A, MKK3bE expression induces phosphorylation of downstream kinase MAPKAPK-2 (MK2) and HSP27 as expected and SB203580 (10 µM) significantly reduces MK2 and HSP27 phosphorylation without affecting the total p38 phosphorylation level (Fig. 3A). In contrast, TAB-1 expression does not result in any significant MK2 or HSP27 phosphorylation (Fig. 3B), suggesting that TAB-1-mediated p38 activation does not induce previously characterized downstream signaling. Furthermore, co-expression of TAB-1 with MKK3bE significantly reduces MK2 and HSP27 phosphorylation, without reducing the total level of phosphorylated p38 (Fig. 3B). TAB-1 also attenuates MKK3bE-induced COX-2 expression (Fig. 3C), further supporting the notion that TAB-1 antagonizes MKK3-dependent p38 downstream activity.

We also investigated whether TAB-1 binding to p38 was required for the observed antagonistic activity toward MKK3-induced downstream signaling. When co-expressed in RNVC cells, the full-length TAB-1 and C-terminal fragment (333–504) of TAB-1 can be readily detected in immunocomplexes with p38e following immunoprecipitation using anti-p38 antibodies (Fig. 4A). In contrast, no binding activity can be detected with the TAB-1 N-terminal fragment (residues 1–373), which contains a PP-2C-like domain, confirming the previous finding (21) that the binding motif for p38 is located in the C-terminal domain of TAB-1. Expression of the TAB-1 C-terminal (residues 333–504) fragment shows no ability to induce p38 phosphorylation but does retain potent inhibitory activity for MKK3bE-induced phosphorylation of MK2 and HSP27 (Fig. 4B). In contrast, expression of the TAB-1 N-terminal (1–373) fragment has neither activity for p38 phosphorylation nor any impact on downstream signaling in response to activated MKK3bE (Fig. 4B). These results suggest that, although both N- and C-terminal domains of TAB-1 are required to activate p38 kinase activity, only the C-terminal TAB-1 containing p38-binding domain is both necessary and sufficient to antagonize MKK3-mediated p38 downstream signaling.

**TAB-1 Binds to p38 and Excludes p38 from the Nucleus—**To determine why the TAB-1/p38 interaction down-regulates MKK3-induced downstream signaling, we investigated the effect of TAB-1 on the intracellular localization of p38 kinase. For these experiments, we used a p38-GFP fusion protein as a reporter. In RNVC cells, p38-GFP and HA-MKK3bE are located both in nuclei and in specific reticular patterned structures in the cytosol (Fig. 5A, panels a and b). In contrast, a TAB-1-RFP fusion protein is detectable exclusively in the cytoplasm but not in the nucleus (Fig. 5A, panel c). Co-expression of HA-MKK3bE and p38-GFP demonstrates extensive overlapping of their intracellular distribution in both cytosol and nuclear compartments (Fig. 5A, panels d–f). In contrast, co-expression of TAB-1-RFP with p38-GFP excludes TAB-1-RFP from the nucleus and retains the kinase only in the cytosol (Fig. 5A, panels g–i). This result suggests that TAB-1 is a cytosol localized protein and that TAB-1 expression can alter the intracellular location of p38.

To further demonstrate that TAB-1 can directly modulate p38 intracellular distribution, we generated nucleus-targeted mutants of TAB-1 (TAB-1-NLS-FLAG) and the TAB-1(333–504) C-terminal fragment (TAB-1(333–504)-NLS-FLAG) by adding a nuclear localization signal at the C-terminal of the coding regions (Fig. 5B). The nucleus-targeted TAB-1 and the TAB-1 C-terminal fragment retain p38 binding activity in cells, as determined from co-immunoprecipitation assays (Fig. 5C) and are effectively targeted to nuclei in cardiomyocytes (Fig. 5D, panels b and c). Co-expression of p38e-GFP with either nucleus-targeted TAB-1 or the nucleus-targeted TAB-1 C-terminal fragment results in nearly complete co-localization of p38 and TAB-1 in the nucleus (Fig. 5D, panels d–i). Despite their ability to target p38 into nuclei, both proteins fail to induce p38 downstream signaling, as measured by MK2 phosphorylation HSP27 phosphorylation or COX-2 expression (Fig. 5E). These data demonstrate clearly that (i) the wild-type TAB-1 protein is exclusively localized in the

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**TABLE 2.** Summary of western blot analysis for TAB-1, MKK3bE, p38, and MK2 and the phosphorylated forms of p38, MK2, and HSP27 in whole cell lysates prepared from RNVC infected with adenoviruses expressing GFP (MOI 100), TAB-1 (MOI 20 and 50), or MKK3bE (MOI 5). C-terminal fragment activity for p38 phosphorylation is sufficient to antagonize MKK3-mediated downstream signaling.

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**TABLE 3.** Reverse transcription-PCR analysis of COX-2 mRNA levels in RNVC infected with adenoviruses expressing GFP, TAB-1, and MKK3bE as described for B. COX-2 mRNA levels were determined by real-time reverse transcription-PCR analysis. COX-2 mRNA levels were significantly reduced in RNVC cells infected with adenoviruses expressing TAB-1, MKK3bE, or TAB-1 and MKK3bE compared to control cells infected with adenoviruses expressing GFP. COX-2 mRNA levels were further reduced in RNVC cells infected with adenoviruses expressing TAB-1 and MKK3bE compared to control cells infected with adenoviruses expressing MKK3bE. These results suggest that TAB-1 antagonizes MKK3-mediated downstream signaling, as measured by COX-2 expression.
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TAB-1 Modulates p38 Intracellular Distribution and the Interaction of p38 with MKK3—The above results suggest that TAB-1 not only modulates p38 intracellular localization but also changes the MKK-mediated downstream effects of p38. To investigate the underlying mechanism, we analyzed the effects of TAB-1 expression on the protein-protein interaction between p38 and its upstream activating kinase MKK3. Co-immunoprecipitation analysis demonstrates that TAB-1 expression significantly reduces p38 binding to MKK3 in COS-1 cells (Fig. 6). Supporting this finding, intracellular localization of MKK3bE expression significantly reduces p38 binding to MKK3 in COS-1 cells (Fig. 6). Co-immunoprecipitation analysis demonstrates that TAB-1 expression significantly reduces p38 binding to MKK3 in COS-1 cells (Fig. 6).

To further investigate the functional role of TAB-1 in cytokine-mediated signaling, we studied mouse embryonic fibroblast in which significant endogenous TAB-1 is present (Fig. 9A). Two MEF cell lines are established using different small interfering RNAs against endogenous TAB-1 mRNA. As shown in Fig. 9A, TAB-1 protein expression is significantly lowered in both small interfering RNA-treated MEF cell lines. TNFa-induced COX-2 expression is augmented in TAB-1 knockdown MEF cells, providing evidence that TAB-1 plays a role in regulating COX-2 expression in response to TNFa stimulation.
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**FIGURE 5.** Intracellular localization of p38α is regulated by TAB-1 and MKK3E. A, immunofluorescent confocal images were acquired from RNVC expressing GFP-p38a (panel a, green), HA-MKK3E (panel b, red), and TAB-1-RFP (panel c, red), showing their respective intracellular localization patterns. Panels d–f are images from RNVC co-expressing GFP-p38 and HA-MKK3E, visualized for GFP (panel d) or HA (panel e) or merged (panel f). Panels g–i are images from RNVC co-expressing GFP-p38 and TAB-1-RFP visualized for GFP (panel g), RFP (panel h), or merged (panel i). B, schematic showing the structure of TAB-1 and the TAB-1(333–504) N-terminal truncation mutant modified to carry a nuclear localization signal. A nuclei localization sequence and a 3×FLAG tag were fused to the C terminus of TAB-1 and TAB-1(333–504) by PCR as described under “Materials and Methods.” C, in vivo binding of nuclear targeted TAB-1 mutants with endogenous p38. TAB-NLS-FLAG and TAB-1(333–504)-NLS-FLAG were transiently expressed in HeLa cells. Immunoprecipitation from the total cell extracts with M2 anti-FLAG antibody was followed by immunoblot with anti-FLAG or anti-p38 antibodies as indicated. D, immunofluorescent confocal images of GFP-p38a (panel a), TAB-1-NLS-FLAG (panel b) and TAB-1(333–504)-NLS-FLAG (panel c) were shown in RNVC after Adv-mediated expression. Images of nuclei-targeted TAB-1 mutants (panels e and h) and GFP-p38α (panels d and g) were also visualized individually or merged (panels f and i) in RNVC co-expressing GFP-p38α with either TAB-1-NLS-FLAG (panels d–i) or TAB-1(333–504)-NLS-FLAG (panels g–i). E, immunoblot of protein samples from cells expressing TAB-1-NLS-FLAG, TAB-1(333–504)-NLS-FLAG or MKK3E (as labeled on top) using antibodies for MKK3, TAB-1, p-HSP27, MAPKAP2, and COX-2 as indicated. MW (kDa), molecular mass (in kDa).

MEF cells compared with the control cells (Fig. 9B). Similar results are observed using anisomycin as an alternative p38 activator (data not shown). Therefore, both gain-of-function and loss-of-function studies support a potentially important role for TAB-1 as a signaling modulator in inflammatory response.

**DISCUSSION**

Stress-activated MAP kinase p38 is a highly conserved signaling molecule responsible for a variety of stress responses in different mammalian cells. Like other MAP kinase family members, the p38 kinase catalytic activity is activated by upstream MAP kinase kinases, including MKK3, MKK6, and, with lesser specificity, by MKK4 (3, 6). p38 activation is implicated in many critical cellular functions, including gene regulation, apoptosis, energy metabolism, cellular differentiation, and proliferation, presumably via an array of downstream target molecules (4). However, it is unclear how these diverse downstream signaling pathways are regulated in response to a variety of different stimuli that activate p38 kinase activity. Our data suggest that TAB-1 is not only a MKK3-independent activator of p38 MAP kinase but is also a potent modulator of p38 intracellular localization and p38 signal complex formation in the cytosol. By competing with MKK3 for p38 binding, TAB-1 antagonizes MKK3-mediated activation of downstream kinases and, consequently, inflammatory gene induction. Our observations demonstrate that the differential intracellular localization of activated p38 MAP kinase can be dictated by two different upstream molecules, MKK3 versus TAB-1, and result in dramatically different downstream consequences. Thus, the modulating effect of TAB-1 may offer a potentially important molecular mechanism contributing to the fine tuning and the functional diversity of p38 pathways in mammalian cells.

Our study redefines the role of TAB-1 in p38 regulation. TAB-1β, a splicing variant of TAB-1 lacking the TAK-1-binding motif (27), has the same capacity as full-length TAB-1 to activate p38 kinase activity in cardiomyocytes. Truncated N- and C-terminal portions of TAB-1 that lack either the p38-binding domain or PP2C-like domain do not activate p38 kinase activity. Therefore, the p38 MAP kinase-binding motif and the PP2C-like domain of TAB-1 are both required for p38 activation in cardiomyocytes as reported (21). In contrast, the C-terminal portion of TAB-1(333–504) containing the p38-binding motif is sufficient to retain p38 in the cytosol, dictates the intracellular distribution of p38, and inhibits MKK3-mediated signaling. In the cytosol, TAB-1 and MKK3 direct p38 into different intracellular compartments with distinct localizations. Therefore, TAB-1 can function both as an activator
and a tethering factor for p38 kinase. Activation of p38 by TAB-1 requires both p38 binding and PP2c-like domain of TAB-1, whereas tethering requires only the C-terminal domain containing the p38-binding motif. The potent inhibitory function of TAB-1 (333–504) for MKK3-mediated signaling indicates clearly that TAB-1/p38 interaction is both necessary and sufficient to modulate p38 downstream signaling. It seems likely that TAB-1-mediated changes in p38 kinase cellular localization contribute to the previously reported negative feedback function for TAB-1 in p38 signaling (25). A significant number of scaffold proteins have been identified for MAP kinase signaling cascades, all of which possess the hallmark of interacting with multiple components of the kinase signaling complex (29). Some of them, including KSR, MP1, JIP-1, and JIP2, can target MAP kinase complexes to specific subcellular locations, such as plasma membrane, late endosome, or kinesin cargo (transport vehicles) (30–33). However, unlike TAB-1, none of them has an intrinsic activation function toward the targeted protein kinases.

In addition, local signaling complex interaction appears to be critical to achieve spatio-temporal regulation of other protein kinases, including cAMP-dependent protein kinase, protein kinase C, and tyrosine kinases, as revealed by genetic fluorescent probes (34–38). Indeed, nuclear localized TAB-1 fails to activate p38 downstream signaling, suggesting that TAB-1 and MKK form different signaling complexes for p38. TAB-1 appears to be a unique signaling molecule that functions both as an upstream MKK-independent activator of p38 activity and as a scaffold protein that modulates the intracellular localization and signal complex interaction of the activated p38 kinase.

TAB-1 gene inactivation leads to an embryonic lethal phenotype with impaired TGF-β signaling and cardiovascular defects (39), suggesting a
vital role of TAB-1 in cardiovascular development and function. However, the specific role of TAB-1 in p38-mediated function versus TGF-β/TAK-1 signaling in heart or other cell systems is unknown. Tanno et al. (22) demonstrated that TAB-1 binding to p38 was induced in ischemic hearts but not in TNFα-treated hearts, suggesting that different mechanisms are involved in p38 activation under different stress conditions. Li et al. (23) also recently reported some interesting observations on AMP-activated protein kinase-mediated p38 activation that involves TAB-1. The data from this study indicate that these different mechanisms of p38 activation would lead to distinct downstream signaling events. Although a negative modulating role of TAB-1 for MKK3-mediated signaling has been clearly demonstrated in our study, the selective downstream effect of TAB-1-mediated p38 activity in the cytosol remains unclear and requires further studies. Given the diverse roles of p38 in various cellular functions, it is conceivable that TAB-1-mediated p38 activity is responsible for a specific subset of these activ-

FIGURE 7. TAB-1 disrupts p38α-MKK3 complex in cells. TAB-1 disrupts p38α and MKK3 complex in cytosol. COS-1 cells were infected with GFP-p38α and HA-MKK3bE without (panels a–f) and with TAB-1-RFP (panels g–l). p38α and MKK3bE were visualized by GFP and anti-HA labeling, respectively. The right panels are the corresponding overlays of the left and center panels. Panels d–f and j–l are magnified regions of the squares in panels a–c and g–i, respectively. The images were restored with three-dimensional deconvolution, and the degree of association between p38α and MKK3bE was quantified in the absence (panels m and n) and presence (panels o and p) of TAB-1. For the quantification of protein proximity index of p38α versus MKK3 and vice versa, cell planes excluding the nucleus were divided in 2 × 2-µm squares by a grid, and the squares of p38α and MKK3 images with values larger than 0.5 of the total image intensity were selected. The correlation coefficient and its statistical significance (P) of each square were calculated after thresholding to 0.25 of its maximum intensity. The protein proximity index was calculated from (number of squares with positive correlation coefficient and P < 0.05)/(total number squares). Panels m–p show distribution histograms of square correlation coefficient (panels m and o) and P SIGN VALUE (Ref. 40) correlation coefficient plots (panels n and p) in the absence (panels m and n) and presence of TAB-1 (panels o and p). In these bar graphs (panels m and o), red areas indicate significant positive correlation coefficient. Note the reduction of the red area by TAB-1, which is quantified in panel q. Panel q shows quantitative values of protein proximity index (mean ± S.E., n = 6) for p38α versus MKK3 (green) and MKK3 versus p38α (red) without TAB-1 (no pattern) or with TAB-1 (diagonal line pattern). From 10 to 20 planes were analyzed for each cell and total of six cells were analyzed from each treatment group.
GAPDH, or phospho-p38 were analyzed by immunoblotting as indicated. MEFl cells (TAB-1 knockdown augments TNF (with anti-GAPDH antibody as labeled using protein samples from wild-type MEF cells 1-kockdown in wild-type MEF cells. pSuper vector expressing siTAB-1 was transfected in MARCH 3, 2006 • VOLUME 281 • NUMBER 9 rat neonatal myocytes and core support from Cardiovascular Research Laboratories.

FIGURE 8. TAB-1 attenuates IL-1β-induced p38 downstream signaling and COX-2 expression. RNVC were infected with AdvGFP (100 MOI) or AdvTAB1(333–504)-FLAG (50 MOI) for 48 h. The cells were analyzed by immunoblot for GAPDH, TAB-1, total and phospho-p38, phospho-MK2, and COX-2 as indicated. MW(kd), molecular mass (in kDa).

FIGURE 9. Endogenous TAB-1 attenuates TNFα-induced COX-2 expression. A. TAB-1 knockdown in wild-type mouse MEF cells. pSuper vector expressing siTAB-1 was transfected in wild-type mouse MEF cells. The sequences targeting TAB-1 are 5′-AGCAGTCTCTTCACACAGCAAG-3′ for siTAB-1 and 5′-AGGCCCTCTGTGCAATCTAC-3′ for siTAB-2. TAB-1 expression was analyzed by immunoblotting using anti-TAB-1 antibody and reprobed with anti-GAPDH antibody as labeled using protein samples from wild-type MEF cells (lane C) and MEF cells treated with siTAB-1 (lane 1) or siTAB-2 (lane 2), respectively. B. TAB-1 knockdown augments TNFα induced COX-2 expression in MEF cells. Both TAB-1 small interfering RNA (siRNA)-treated MEF cell lines (lanes 1 and 2) and control wild-type MEF cells (lanes C) were stimulated by medium or TNF for 6 h, and protein levels of COX-2, GAPDH, or phospho-p38 were analyzed by immunoblotting as indicated.

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