MEKK-1, a Component of the Stress (Stress-activated Protein Kinase/c-Jun N-terminal Kinase) Pathway, Can Selectively Activate Smad2-mediated Transcriptional Activation in Endothelial Cells*

(Received for publication, October 28, 1998, and in revised form, December 31, 1998)

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Smad proteins are essential components of the intracellular signaling pathways utilized by members of the transforming growth factor-β (TGF-β) superfamily of growth factors. Certain Smad proteins (e.g. Smad1, -2, and -3) can act as regulated transcriptional activators, a process that involves phosphorylation of these proteins by activated TGF-β superfamily receptors. We demonstrate that the intracellular kinase mitogen-activated protein kinase kinase kinase-1 (MEKK-1), an upstream activator of the stress-activated protein kinase/c-Jun N-terminal kinase pathway, can participate in Smad2-dependent transcriptional events in cultured endothelial cells. A constitutively active form of MEKK-1 but not mitogen-activated protein kinase kinase-1 (MEK-1) or TGF-β-activated kinase-1, two distinct intracellular kinases, can specifically activate a Gal4-Smad2 fusion protein, and this effect correlates with an increase in the phosphorylation state of the Smad2 protein. These effects do not require the presence of the C-terminal SSXS motif of Smad2 that is the site of TGF-β type 1 receptor-mediated phosphorylation. Activation of Smad2 by active MEKK-1 results in enhanced Smad2-Smad4 interactions, nuclear localization of Smad2 and Smad4, and the stimulation of Smad protein-transcriptional coactivator interactions in endothelial cells. Overexpression of Smad7 can inhibit the MEKK-1-mediated stimulation of Smad2 transcriptional activity. A physiological level of fluid shear stress, a known activator of endogenous MEKK-1 activity in endothelial cells, can stimulate Smad2-mediated transcriptional activity. These data demonstrate a novel mechanism for activation of Smad protein-mediated signaling in endothelial cells and suggest that Smad2 may act as an integrator of diverse stimuli in these cells.

The transforming growth factor-β (TGF-β)1 superfamily of growth factors and cytokines is involved in a wide variety of physiological and pathophysiological processes in the cardiovascular system, and the signaling mechanisms utilized by this class of effectors are rapidly being elucidated. The discovery of Smad proteins and the demonstration that they can mediate many of the transcriptional effects of these growth factors has been an important advance (1–3). The central roles of these proteins in mediating TGF-β responses in cells is highlighted by the demonstration that mutations in Smads 2, 3, and 4 have been causally linked to specific malignancies, and disruption of the Smad2 and Smad4 genes in mice results in early embryonic lethality (4, 5). As the detailed molecular mechanisms of Smad protein signaling have emerged a number of functional interactions between these proteins and other signaling pathways have been reported. For instance, recent work has demonstrated that the classic mitogen-activated protein kinase pathway (MAPK/ERK) can negatively regulate bone morphogenetic protein/Smad1-dependent transcriptional responses. This appears to occur as a result of the phosphorylation of Smad1 by a member of the Erk kinases and subsequent translocation of Smad1 out of the nucleus (6). In contrast to this inhibitory effect, the stress-activated protein kinase pathway (i.e. the SAPK/JNK pathway) has been implicated as a positive regulator of certain Smad-dependent effects. Previous data from several groups have documented the importance of AP-1 and related transcriptional effectors in some TGF-β-mediated transcriptional responses, and a recent report demonstrated a direct functional interaction between Smad3 and c-Fos/c-Jun in mediating TGF-β-dependent transcription (7, 8).

Recently, signals derived from growth factor receptors containing tyrosine kinase activities were shown to be capable of modulating Smad-dependent effects. This was suggested to occur as a result of activation of a kinase downstream of MEK-1, an upstream activator of the classic MAPK/ERK kinase pathway, resulting in the phosphorylation of Smad2 (9). In addition, a variety of other kinases have been implicated in TGF-β signaling, such as TAK-1 and TAB, although their precise roles have not been elucidated (10–12). Taken together, these data indicate that multiple signal transduction cascades may modulate Smad signaling in cells and begin to provide possible mechanisms by which signals derived from non-TGF-β family members may impact TGF-β superfamily responses.

MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; SAPK, stress-activated protein kinase; JNK c-Jun N-terminal kinase; AP-1, activating protein-1; TAK-1, TGF-β-activated kinase-1; BARE, bovine aortic endothelial cells; NF-κB, nuclear factor-kappa B; MEK-1, MAPK kinase-1; MEK-1, MEK kinase-1; CBP, CREB-binding protein; CRE, cyclic AMP response element; LSS, laminar shear stress; TjIRI, TGF-β type I receptor.

* This work was supported in part by National Institutes of Health Grants P50-HL6985 and R37-HL51150 and an Unrestricted Award for Cardiovascular Research from the Bristol-Myers Squibb Pharmaceutical Research Institute (to M. A. G.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Supported by a fellowship for medical students from The Stanley J. Sarnoff Endowment for Cardiovascular Science.

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1 The abbreviations used are: TGF-β, transforming growth factor-β;
Furthermore, these data suggest that Smad proteins may be involved in the transduction of diverse signals in cells. In this report we demonstrate that MEKK-1, a MAPK kinase kinase that is an upstream activator of the SAPK/JNK pathway, is capable of selectively activating Smad2-dependent transcriptional activity independently of TGF-β in cultured endothelial cells. These data demonstrate a functional interaction between the SAPK/JNK and Smad signaling pathways and suggest that Smad protein signaling may be modulated by MEKK-1 or related kinases in endothelial cells.

**EXPERIMENTAL PROCEDURES**

**Cells, Reagents, and Constructs—**Primary bovine aortic endothelial cells (BAEC) were isolated as described and cultured in Dulbecco's modified Eagle's media supplemented with 10% heat-inactivated bovine calf serum, 2 mM l-glutamine, 250 units/ml penicillin G, and 250 μg/ml streptomycin. These were utilized at passages 3–12. COS-7 cells were maintained in the same medium. The pST promoter, Smad protein expression constructs, Smad-GAL4VP16 fusion constructs, and CBP fusion constructs have been described previously (14). The plasmogen activator inhibitor-1 promoter (P800) was provided by David Loskutoff, dominant negative MEKK-1 by Roger Davis, and activated TAK-1 by Shuji Sako. The active MEKK-1, MEK-1, and protein kinase A expression plasmids were obtained from Stratagene. The activated MEKK-1 construct consists of amino acids 380–672 of MEKK-1. The activated MEK-1 construct consists of the 2218/E222 derivative that has had amino acids 32–51 deleted. The c-Jun and Elk1 fusion proteins consist of amino acids 1–223 of c-Jun and 307–427 of Elk1 fused to the Gal4 DNA binding domain, respectively. The CRE, AP-1, and NF-κB-dependent promoters consist of four tandem CRE sites (AGGCTGACGTCAGAG), seven tandem AP-1 sites (TGACTAAT), or four tandem NFκB sites (TGGGAGATTCGCCG) cloned upstream of a minimal TATA box and luciferase reporter gene. The Smad2 mutants pMStat2α2P and pMStat2α2P have been described previously (14). For the labeling experiments, a Flag epitope replaced the action of MEKK-1 as well as Smad proteins. In addition, a kinase that has been implicated in TGF-β and activase is capable of activating components of the SAPK/JNK pathway, but its precise role is unknown (12). In preliminary titration experiments in cultured bovine aortic endothelial cells (BAEC), a ratio of active kinase to reporter gene plasmid of 1 to 100 was found to be sufficient to reproducibly activate transcription, and under these conditions only MEKK-1 was reproducibly capable of activating the TGF-β-responsive promoters P800 and pSTP. Comparable levels of active MEK-1 or active or wild type TAK-1 were unable to reproducibly activate these promoters, although active TAK-1 could stimulate the P800 promoter when expressed at very high levels (data not shown). Fig. 1 demonstrates the response of the pSTP and P800 promoters to coexpression of active MEKK-1, active MEK-1, or a constitutively active TGF-β type-1 receptor (actTβRI). Both promoters are induced significantly by the active TGF-β1 receptor and active MEKK-1 but not by active MEK-1. These kinases are expressed in combination with the activated TGF-β receptor, MEKK-1 appears to stimulate the activity of both promoters in a manner additive to the TGF-β receptor, whereas MEK-1 has little effect. To explore the mechanisms of these effects in more detail, the pSTP promoter was stimulated with either active TpRI or active MEK-1, and the effects of coexpression of a dominant negative form of MEKK-1 or Smad7, an inhibitory Smad protein, was examined. As shown in Fig. 1C, the induction of pSTP by the active TGF-β receptor was partially inhibited by the dominant negative MEKK-1 and was potently inhibited by coexpression of Smad7. Similarly, the induction of pSTP by active MEK-1 was inhibited by coexpression of both dominant negative MEKK-1 and Smad7. Fig. 1E is a series of Western blots demonstrating the expression levels of the activated kinases utilized in these experiments. At the level of expression plasmids used (5 ng) we reproducibly observed a level of activated MEKK-1 or MEK-1 protein approximately 3–5 times that of the corresponding endogenous protein, and the relative levels of the two overexpressed active kinases (MEKK-1 and MEK-1) appeared comparable. Taken together, these results suggest that maximal transcriptional induction of the P800 and pSTP promoters by the active TGF-β receptor requires the action of MEKK-1 as well as Smad proteins. In addition, the observation that Smad7 can block active MEKK-1-medi-
ated stimulation of these promoters suggests that Smad proteins are playing a role in the MEKK-1-mediated transcriptional stimulation.

To investigate this hypothesis directly, we utilized a Gal4-based transcriptional system. We have previously shown that this system faithfully recapitulates ligand (TGF-β)-induced, Smad-dependent activation of transcription in cultured endothelial cells (14). Bovine aortic endothelial cells (BAEC) were cotransfected with constructs encoding fusion proteins between the Gal4 DNA binding domain and either full-length or C-terminal MH2 domains of Smad2, -4, -6, and -7 together with a Gal4-dependent luciferase reporter gene and constitutively activated forms of MEKK-1 or MEK-1. As shown in Fig. 2, MEKK-1 markedly stimulates Smad2-dependent transcription in this system. The low basal level of luciferase activity observed in the absence of MEKK-1 probably reflects a small amount of endogenous TGF-β activation in the endothelial culture system. Smad4 demonstrates a reproducibly lower level of basal transcriptional activity in this system and this is not enhanced in the presence of MEKK-1. Gal4 constructs containing only the C-terminal MH2 domain (along with the linker region) of these proteins (pM2C and pM4C) demonstrated significantly increased basal levels of transcriptional activity and were also markedly stimulated by MEKK-1. This observation is consistent with previous data demonstrating that the transcriptional activation domain is contained within the C-terminal half of the Smad protein (MH2 domain) and indicates that MEKK-1 can stimulate Smad2- and Smad4-dependent transcription in the absence of the inhibitory MH1 domain. In contrast to Smad2 and Smad4, neither Smad6 nor Smad7 demonstrated any significant basal or MEKK-1-induced transcriptional activity in endothelial cells. Moreover, overexpression of a constitutively activated form of MEK-1 does not stimulate the transcriptional activity of any Smad construct tested in this system (Fig. 2). Fig. 2B demonstrates that the MEKK-1-mediated stimulation of GAL4-Smad2 (pM2)-mediated transcription is inhibited by coexpression of either the dominant negative MEKK-1 or Smad7. Taken together, these results indicate that MEKK-1 appears capable of selectively stimulating Smad2-dependent transcriptional activity in BAEC and that Smad7 can interfere with this process.

MEKK-1 has been reported to modulate the activity of a number of signal transduction pathways in addition to the SAPK/JNK pathway such as NF-κB/IκB and p53-mediated events (19–21). To examine the selectivity of our endothelial cell system, we performed a number of controls to assess the
specificity of the observed MEKK-1-mediated stimulation. Conditioned media from endothelial cells expressing active MEKK-1 consistently failed to stimulate p3TP or GAL4-Smad2-mediated transcription, and inclusion of a neutralizing antibody to soluble TGF-β in the media of the transfection studies (in quantities sufficient to inhibit the activity of exogenously added active TGF-β1 at 1.0 ng/ml) did not inhibit MEKK-1-mediated stimulation of Smad-dependent transcription (data not shown). These results indicate that MEKK-1 is not inducing the expression of TGF-β which can then act in a paracrine manner.

To ensure that MEKK-1 activation of Smad2 and Smad4 was not the result of promiscuous signaling or indiscriminate stimulation of the general transcriptional apparatus in this overexpression system, we examined the response of a series of well characterized control promoters and transcriptional effectors. As shown in Fig. 3, both constitutively activated kinases (MEKK-1 and MEK-1) demonstrated a selective pattern of promoter activation when expressed at these levels in BAEC (see Fig. 1E). Active MEKK-1 potently stimulated a coexpressed AP-1-dependent promoter, whereas an NF-κB-dependent promoter was also stimulated but to much lesser degree. Active MEKK-1 was unable to significantly stimulate a CRE-dependent promoter, as compared with a constitutively activated form of protein kinase A, a known activator of CRE-dependent transcription (Fig. 3). Thus, expression of active MEKK-1 appears to be eliciting the expected pattern of promoter specificity in this endothelial cell context. To examine further the selectivity of transcription factor activation in our system, we examined the ability of active MEKK-1 and MEK-1 to stimulate the activity of fusion constructs between the GAL4 DNA binding domain and the activation domains of the transcription factors c-Jun, Elk-1, and VP-16 (pMJun, pMElk-1, and pMVP16, respectively). Active MEKK-1 markedly stimulated the transcriptional activity of pMJun but, at best, was only a modest activator of pMElk-1 in our system (Fig. 3). Conversely, active MEK-1 overexpression potently activated pMElk-1 but not pMJun, consistent with the role of MEK-1 as an activator of the classic MAPK (ERK) signal transduction pathway. Neither of these kinases had a significant effect on the high basal transcriptional activity of the GAL4-VP16 fusion protein. Taken together, these data demonstrate that when carefully expressed at relatively low levels in cultured endothelial cells, constitutively activated forms of both MEKK-1 and MEK-1 consistently demonstrate a pattern of promoter and transcription factor activation that is selective, and under these conditions, only MEKK-1 appears capable of significantly stimulating Smad2-dependent transcription.

MEKK-1 Transcriptional Stimulation Correlates with a Change in the Phosphorylation State of Smad2—Considerable experimental evidence supports a model of Smad signaling that involves phosphorylation of certain Smad proteins (e.g. Smad2) on a series of C-terminal serines (termed the SSXS motif) by the kinase present in TGF-β family type 1 receptors (22, 23). To investigate the role of this C-terminal SSXS motif of Smad2 in the transcriptional response to active MEKK-1 described above, we generated two mutant Gal4-Smad2 fusion constructs (pM2*C and pM2*C*) that lack these serine residues. We have previously shown that the full-length Smad2 mutant (pM2*C) is unresponsive to TGF-β in BAEC, whereas the truncated Smad2 mutant (pM2*C*) retains considerable transcriptional activation ability (14). When these constructs were cotransfected into BAEC together with a constitutively activated form of MEKK-1, the full-length mutant Smad2 was not stimulated (Fig. 4). In contrast, pM2*C possessed a significant basal level of transcriptional activity that was markedly enhanced by MEKK-1 (Fig. 4). MEK-1 overexpression resulted in no significant stimulation of either Smad2 mutant (Fig. 4).

To determine if MEKK-1 stimulation of Smad2 transcriptional activity also correlated with a change in its phosphorylation state, we performed a series of in vivo labeling experiments. Cells were transfected with epitope-tagged Smad2 expression constructs along with constitutively activated forms of the TGF-β type I receptor (TβRI, MEKK-1, or MEK-1) and metabolically labeled in the presence of [32P]orthophosphate. As shown in Fig. 4, Smad2 demonstrated a basal level of phosphorylation when overexpressed. In the presence of either soluble TGF-β or active TβRI, the phosphorylation state of Smad2 increases although the soluble TGF-β ligand stimulus consistently results in a lower level of Smad2 phosphorylation in this system. This is in agreement with previous data describing the role of the TGF-β type I receptor kinase in the phosphorylation of Smad2 (22). In the presence of MEKK-1, the phosphorylation state of Smad2 significantly increases as compared with either TβRI or the untreated control, whereas active MEK-1 only modestly increased Smad2 phosphorylation.

To determine if the MEKK-1-mediated increase in the phosphorylation state of Smad2 occurs on the SSXS motif known to be the substrate for the TGF-β type I receptor, we examined the phosphorylation of the Smad2 mutant in which the SSXS motif has been deleted (Smad2*P). As with wild type Smad2, when Smad2*P was overexpressed there was a low but detectable level...
of basal phosphorylation. In contrast to wild type Smad2, however, the phosphorylation state of Smad2*P was not significantly altered by either soluble TGF-β or coexpression of the active TjRI but is enhanced by coexpression of active MEKK-1. These results suggest that MEKK-1 activity can result in the phosphorylation of Smad2 (either by MEKK-1 itself or a yet to be defined kinase) and that this phosphorylation event can occur at a site or sites distinct from the C-terminal SSXS motif that is the site of type 1 receptor kinase-mediated phosphorylation. Given the ability of active MEKK-1 to stimulate the transcriptional activity of Gal4 Smad2C and Smad2C*P (Figs. 2A and 4A), the sites of MEKK-1-induced phosphorylation likely reside in the linker region or the C-terminal MH2 domain of Smad2. Although the SSXS motif appears to be dispensable for the MEKK-1-mediated stimulation of Smad2 demonstrated here, these data do not exclude some role for this site in the wild type Smad2 protein stimulated with active MEKK-1.

**MEKK-1 Stimulates Smad-Smad Interactions, Nuclear Localization of Smad Proteins, and Interaction of Smad2 with a Required Transcriptional Coactivator—Homo- and heterotypic interactions between Smad2 and Smad4 are thought to be critical for nuclear localization and subsequent stimulation of transcriptional events in response to TGF-β (1–3). To determine if MEKK-1 can regulate Smad-Smad interactions, we employed mammalian two-hybrid and coimmunoprecipitation approaches. In the mammalian two-hybrid system, protein-protein interactions are detected by fusing one test protein to the Gal4 DNA binding domain, fusing a second test protein to the strong transcriptional activation domain of the VP16 protein, and coexpressing these with the GAL4-dependent reporter. An interaction between these two proteins results in activation of a Gal4-dependent luciferase reporter by the VP16 activation domain. By using Gal4 and VP16 fusion constructs of Smad2 and Smad4, we investigated the ability of MEKK-1 to regulate Smad-Smad interactions in endothelial cells. As shown in Fig. 5A, this system demonstrates some basal level of interaction between Smad2 and itself, as well as with Smad4, in the absence of any stimulation (see “control” condition in pM2 with VP-Smad2 or VP-Smad4 compared with pVP alone). This result likely reflects both an increased association between the Smad proteins due to overexpression and the presence of small amounts of TGF-β in the culture system. However, the constitutively activated form of MEKK-1 markedly enhances both homotypic Smad2-Smad2 and heterotypic Smad2-Smad4 interactions. Interestingly, active MEKK-1 did not significantly stimulate Smad4-Smad4 interactions in this assay (data not shown). Expression of active MEK-1, which did not stimulate the transcriptional activity of Smad2 or Smad4, had no effect on Smad-Smad interactions in this endothelial two-hybrid system (Fig. 5A).

To demonstrate Smad-Smad interactions in cell extracts, we performed a series of coimmunoprecipitations. Epitope-tagged Smad2 and Smad4 were coexpressed with active TjRI, MEK-1, TAK-1, or MEKK-1 in endothelial cells, and the interactions between Smad2 and Smad4 were assessed by immunoprecipitating Smad2 and probing the immunocomplexes for Smad4. As can be seen in Fig. 5, there is no detectable interaction between these two proteins in this assay in the absence of stimulation (control), but in the presence of a constitutively activated form of the TGF-β type 1 receptor (TjRI), significant amounts of Smad4 coimmunoprecipitate with Smad2. This is consistent with previous data demonstrating TGF-β receptor-stimulated interaction between these two Smad proteins (22). As shown in Fig. 5, active MEKK-1 also stimulates this interaction between Smad2 and Smad4, whereas active MEK-1 or TAK did not stimulate any detectable Smad2-Smad4 associa-
tion under these conditions. These data are consistent with the mammalian two-hybrid data presented above and demonstrate that MEKK-1 can selectively and specifically stimulate homo-
typic Smad2-Smad2 and heterotypic Smad2-Smad4 interac-
tions in BAEC. To determine if MEKK-1 enhancement of Smad2 and Smad4 interactions leads to changes in the subcellular localization of these proteins, we utilized immunofluorescence microscopy. Epitope-tagged Smad2 or Smad4 was transfected into BAEC and visualized by immunofluorescence. As can be seen in Fig. 6, both Smad2 and -4 demonstrate diffuse staining in unstimu-
lated cells, a pattern consistent with a predominantly cytoplas-
mic localization of these proteins. When a constitutively acti-
vated form of MEKK-1 is cotransfected with either Smad2 or
Smad4, both proteins exhibit predominant nuclear staining.
Cotransfection of active MEK-1 did not significantly alter the
pattern of Smad staining in these cells. To ensure that these effects were not simply the result of overexpression of Smad proteins, we expressed Smad7, a distinct Smad that is unre-
sponsive to MEKK-1 transcriptional stimulation (Fig. 2). As
shown in Fig. 6, Smad7 exhibits diffuse, predominantly cyto-
plasmic staining, which is unaltered in the presence of active
MEKK-1.

We and others(14, 24, 25) have recently shown that Smad2
and Smad4 are among the growing number of regulated trans-
criptional activators that interact with the mammalian tran-
scriptional coactivator, CREB-binding protein (CBP). To de-
terminate if MEKK-1 could stimulate Smad2 and Smad4
interactions with CBP, we employed the mammalian two-hy-
brid system. As shown in Fig. 7, MEKK-1 selectively stimulates
an interaction between Smad2 and the C-terminal 549 amino
acids of CBP. This interaction was specific for Smad2 as
MEKK-1 did not significantly stimulate interaction between
Smad4 and any region of CBP in this two-hybrid system (data
not shown). To confirm these results biochemically, we per-
formed immunoprecipitations with an anti-CBP/P300 antisera
on cell extracts derived from endothelial cells expressing
tagged Smad constructs and active TβR1, MEKK-1, TAK, or
MEK-1. As shown in Fig. 7, in the presence of either active
TBR1 or active MEKK-1, both Smad2 and Smad4 can be dem-
onstrated to coimmunoprecipitate with CBP/P300.

**Laminar Shear Stress Can Stimulate Smad2-mediated Transcriptional Activity in Endothelial Cells**—To determine whether endogenous MEKK-1 expressed in endothelial cells is
involved in the regulation of Smad2 activity, we examined the
ability of steady laminar shear stress, a physiologically rele-

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**Fig. 4.** Transcriptional activation of Smad2 by MEKK-1 correlates with a change in the phosphorylation state of Smad2 and does not require the C-terminal SSXS motif. A, Gal4 fusion proteins of Smad2 with the C-terminal SSXS motif deleted (pM2*P) or the MH2 domain of Smad2 with the SSXS deleted (pM2C*P) were examined for their transcriptional response to active MEK-1 or MEKK-1. The latter is significantly stimulated by active MEKK-1 expression. B, wild type Smad2 or mutant Smad2 lacking the SSXS motif (Smad2 mutant) tagged with a Flag epitope were expressed by transient transfection, and the cells were subsequently metabolically labeled with [32P]orthophosphate. Stimuli consisted of soluble TGF-β (5 ng/ml) for 30 min or cotransfection of the indicated activated receptor or kinases. Smad2 phosphorylation was assessed by immunoprecipitation with anti-Flag antiserum and autoradiography. The No tx lane is a no transfection control; the control lane is no treatment. The bottom panels confirm comparable protein expression of Flag-Smad2. The bar graphs represent quantification of phosphorylation by densitometry of the autoradiograms and corresponding Western blots. These results are representative of two independent experiments.
FIG. 5. Active MEKK-1 can stimulate homotypic Smad2-Smad2 and heterotypic Smad2-Smad4 interactions in BAEC. A, two-hybrid analysis of the ability of Gal4-Smad2 to interact with either Smad2 or Smad4 fused to the VP16 activation domain, respectively (VP-Smad2, VP-Smad4), when coexpressed in endothelial cells with the indicated active kinases. MEKK-1 can stimulate Smad2-Smad2 interactions and Smad2-Smad4 interactions in this system. (Note: these experiments are typically performed with 1/10th to 1/50th the amount of Gal4 fusion expression plasmid to minimize any background transcriptional activity of the Gal4 fusion proteins themselves.) B, both active TGF-β receptor (actTβR1) and active MEKK-1, but not active MEK-1, can stimulate Smad2 and Smad4 to interact as assessed by communoprecipitation (IP) in BAEC. Smad4 tagged with a hemagglutinin epitope and Smad2 tagged with a Flag epitope were coexpressed with the indicated kinases, and precipitations were performed with antibodies against the Flag epitope. Coprecipitating Smad4 was detected by Western blot with the anti-hemagglutinin antibody (upper panel). The lower two panels confirm comparable protein expression.

FIG. 6. Coexpression of active MEKK-1 with Smad proteins in BAEC stimulates the nuclear localization of Smad2 and Smad4 but not Smad7. Immunofluorescence was performed on monolayers of BAEC cotransfected with combinations of Smad expression constructs (indicated on the left) and activated kinases (indicated at the top). The cells were fixed, permeabilized, stained with antibodies against the epitopes on the Smad proteins, and then visualized with secondary antibodies coupled to fluorescein isothiocyanate by fluorescence microscopy. Regions of the confluent monolayer containing representative stained cells are shown (adjacent cells are untransfected and thus appear unstained). Smad2 and Smad4, but not Smad7, demonstrated a predominant nuclear localization in active MEKK-1-expressing cells and suggest that Smad proteins may be capable of transducing diverse stimuli in these cells.

DISCUSSION

The discovery of Smad proteins has been a major advance in our understanding of TGF-β superfamily signaling. Recent data suggest that these proteins may interact with a variety of other signaling pathways in cells and thus may be involved in the transduction or modulation of other stimuli as well (6, 8, 9, 28). We have demonstrated that a constitutively active form of MEKK-1 can selectively activate Smad-dependent transcription in cultured endothelial cells in the absence of exogenous TGF-β stimulation. MEKK-1 activation of Smad2-mediated transcription correlated with an increase in phosphorylation of Smad2 even in the absence of the C-terminal SSXS motif of Smad2 that is the site of TGF-β type-1 receptor-induced phosphorylation, further supporting the fact that MEKK-1 induced activation of Smad2 can occur independently of TGF-β activation of Smad2 by MEKK-1 resulted in an enhanced interaction with Smad4, nuclear localization of both Smad2 and Smad4, and a stimulation of Smad-transcriptional coactivator (CBP) interactions. These data thus describe a novel mechanism for the initiation of Smad protein signaling in endothelial cells.

The functional interactions between the classic mitogen (MAPK/ERK)- and stress (SAPK/JNK)-activated kinase pathways and Smad proteins are likely to be complex. Previous work has demonstrated that both pathways can be implicated in TGF-β transcriptional responses (29–31). Atfi and colleagues (29, 30) reported that a dominant negative form of MEKK-1 could inhibit TGF-β-mediated induction of the p3TP promoter in HepG2 cells, and this same group subsequently reported that dominant negative MEKK-1 could inhibit Smad3/4-mediated transcription. Recently Zhang and colleagues (8) demonstrated a direct interaction between Smad3/4 and c-Fos, two transcription factors that are among the targets of the MAPK/SAPK pathways. These data suggest that there are important stimulatory interactions between Smad proteins and the SAPK/JNK pathway in the context of TGF-β signaling. Our results demonstrating that MEKK-1, a MAPKKK in this pathway, can specifically stimulate Smad2-mediated transcrip-
tion suggest an additional stimulatory interaction between these pathways. Interestingly, both hepatocyte growth factor and epidermal growth factor that signal through receptor tyrosine kinases have also been reported to activate Smad2-mediated signaling independently of TGF-β. This effect was demonstrated to be due to a phosphorylation event on Smad2 but did not appear to require the presence of Smad4 (9). Our findings are consistent with a model whereby Smad2 is the target of MEKK-1 or a downstream kinase, and this stimulates Smad2 to interact with Smad4, translocate to the nucleus, interact with the transcriptional coactivators CBP/P300, and activate transcription. In this regard the events downstream of Smad2 activation mediated by active MEKK-1 appear similar to those induced by TGF-β stimulation. Thus, MEKK-1-mediated stimulation of Smad2 may be an additional or alternate mechanism of activation of Smad-mediated transcriptional events in endothelial cells.

A potential limitation of these studies is the use of constitutively activated forms of signaling kinases that can be associated with nonspecific or promiscuous effects. However, the MEKK-1-mediated activation we observe is selective since active MEK-1, a distinct upstream kinase in the MAPK pathway,
or active TAK-1, a kinase implicated in TGF-β signaling, failed to demonstrate the ability to activate Smad2 when overexpressed at comparable levels as activated forms. TAK-1 was identified as a TGF-β-activated kinase and has been reported to activate the TGF-β responsive promoter plasminogen activator inhibitor-1 in mink lung epithelial cells (12). In endothelial cells in our hands this activation required very high levels of active TAK-1 expression, and under these conditions, we observed activation of a variety of other promoters such as the cytomegalovirus and SV-40 promoters. Interestingly, active TAK-1 was able to activate an AP-1-dependent promoter when expressed in endothelial cells (data not shown), consistent with previous data demonstrating the ability of this kinase to stimulate JNK activity (11). However, under these conditions we did not observe reproducible Smad protein activation. Similarly, active MEK-1 could activate ELK-1-dependent transcription from other groups suggest that multiple mechanisms may exist to mediate Smad protein signaling in cells and that this important family of intracellular signaling molecules may be involved in more than just TGF-β superfamily signaling. Given the many stimuli that have been demonstrated to activate the SAPK/JNK pathway and MEKK-1 (17, 26, 34), Smad proteins may be involved in mediating the cellular response to diverse signals ranging from humoral effectors such as growth factors, to biomechanical stimuli such as physical stresses. As such, this class of endothelial-expressed signaling proteins may participate importantly in the orchestration of complex biological processes such as atherogenesis, vascular remodeling, angiogenesis, and vascular development.

Acknowledgments—We thank William Atkinson and Kay Case for invaluable technical assistance and Dra. Tucker Collins and Dean Falb for helpful discussions.

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