MEKK1 Is Required for Inducible Urokinase-type Plasminogen Activator Expression*

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Urokinase-type plasminogen activator (uPA) regulates the remodeling of extracellular matrix and controls reparative processes such as wound healing and liver regeneration. Here we show inducible uPA expression is controlled by MEKK1, a MAPK kinase kinase that regulates the ERK1/2 and JNK pathways. MEKK1 is activated in response to growth factors and cytoskeletal changes. We have found MEKK1 to be necessary for uPA up-regulation in response to treatment with phorbol 12-myristate 13-acetate or basic fibroblast growth factor. We demonstrate that growth factor-treated MEKK1-deficient fibroblasts display greatly reduced uPA expression and activity compared with control fibroblasts. Further, we show that growth factor-induced uPA expression requires MEKK1-dependent MKK1 and JNK activity and that transfection of MEKK1 into knockout cells restores inducible uPA expression and activity. Therefore, we conclude that MEKK1 expression is required for PMA- or FGF-2-induced signals to control uPA expression and function.

Tissue remodeling in response to stress is a critical homeostatic function in multiple organ systems. One physiological process involving tissue remodeling is that of wound healing. Wound healing is a complex process involving multiple cell types and regulated proteolytic degradation of extracellular matrix components (1). The loss of activity of some key proteases results in an impaired ability to repair wounds (2–4). Among the important enzymes expressed and secreted during the wound healing response is the serine protease uPA (2, 5, 6). Known uPA substrates are diverse, and include the zymogen plasminogen (7). uPA activity promotes a proteolytic cascade by converting plasminogen to its active form, plasmin, which can then cleave and activate matrix metalloproteinases (8), thereby enhancing tissue remodeling processes by degrading extracellular matrix components. Beyond wound healing, uPA is required for liver regeneration (9), whereas deregulation of uPA activity plays a major role in human diseases including cancer metastasis (7, 10). As uPA can have a profound impact on homeostasis, both activity and expression are subject to tight regulation. uPA activity is controlled post-translationally through cleavage to produce an activated form (7) as well as through localization by binding to its cognate receptor (uPAR) (7). uPA is also regulated at the transcription level. AP-1 transcription factor duplexes play a major role in control of inducible uPA expression through binding of enhancer regions in the promoter of the uPA gene (11). AP-1 complex components and assembly are, in turn, subject to regulation by MAPK activity. Transcription factor and AP-1 component c-Jun may be phosphorylated and consequently activated by the MAPK c-Jun NH$_2$-terminal kinase (JNK) (12). JNK may also phosphorylate the CREB (cAMP-responsive element-binding protein) family member ATF-2. ATF-2/c-Jun heterodimers have been shown to be a predominant form of AP-1 that promotes uPA expression (11), and therefore JNK activity is an integral part of uPA regulation. Another MAPK, extracellular signal-regulated kinase (ERK1/2), is also key to AP-1 regulation, as its activation leads to the induction of c-Fos, which may join with c-Jun to form a second AP-1 heterodimer complex for the regulation of uPA expression (12).

MEKK1 is a 196-kDa serine-threonine kinase that functions as an MAPKKK in the JNK pathway and can modulate the ERK1/2 pathway (13). Although regulation of these MAPK pathways has been attributed to other MAPKKKs, genetic studies have begun to define a specific role for MEKK1 in tissue homeostasis. Targeted disruption of the MEKK1 gene in mice results in a failure to activate JNK in response to aortic banding, and MEKK1--/-- mice display reduced tolerance to hypertensive cardiac insult (14). Indeed, MEKK1-deficient mice show an increased propensity to cardiac rupture and congestive heart failure consistent with defective tissue remodeling capability. We have previously shown MEKK1 to be activated in response to an array of stimuli, including epidermal growth factor receptor ligation (15) and cytoskeletal alteration by nocodazole and taxol (13, 16). Further, we have found serum and lysophosphatidic acid-induced JNK activation results in a failure to activate JNK in response to aortic banding, and MEKK1--/-- mice display reduced tolerance to hypertensive cardiac insult (14). Indeed, MEKK1-deficient mice show an increased propensity to cardiac rupture and congestive heart failure consistent with defective tissue remodeling capability. We have previously shown MEKK1 to be activated in response to an array of stimuli, including epidermal growth factor receptor ligation (15) and cytoskeletal alteration by nocodazole and taxol (13, 16). Further, we have found serum and lysophosphatidic acid-induced JNK activation results in a failure to activate JNK in response to aortic banding, and MEKK1--/-- mice display reduced tolerance to hypertensive cardiac insult (14). Indeed, MEKK1-deficient mice show an increased propensity to cardiac rupture and congestive heart failure consistent with defective tissue remodeling capability.

In this work, we demonstrate that MEKK1 regulates uPA expression in response to growth factor receptor ligation and by phorbol ester stimulation. Our data are consistent with MEKK1 being a junction that integrates different signals to common pathways required for uPA expression.
MEKK1 Defines uPA

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—The anti-phospho JNK and anti-phospho-ERK monoclonal antibodies and the rabbit polyclonal antibody against ERK were purchased from Cell Signaling Technology (Danvers, MA). The rabbit polyclonal antibody against uPA was a generous gift of the Finsen Laboratory (Copenhagen, Denmark). HRP-anti-mouse serum was purchased from Amersham Biosciences. Protein A-HRP conjugate was purchased from Zymed Laboratories (San Francisco, CA). The constructs for full-length uPA and the uPA promoter were purchased from American Type Culture Collection (Manassas, VA).

Cell Lines—Both primary and immortalized mouse embryo fibroblasts (MEFs) were obtained as described previously (17). Immortalized, serum-starved MEFs were obtained as described previously (17). The constructs for full-length uPA and the uPA promoter were purchased from American Type Culture Collection (Manassas, VA). The parental strain and MEKK1−/− embryos (MEFs) for uPA expression. We tested fibroblasts derived from both parental strain and MEKK1−/− embryos (MEFs) for uPA expression.

Northern blot Analysis—Stimulated cells were lysed, and RNA was extracted with RNeasy (Qiagen, Valencia, CA) according to manufacturer's recommendations. RNA analysis by Northern hybridization was performed as described previously (33). The membrane was hybridized with a 460-bp uPA cDNA fragment (EcoRI digest).

Protein expression and secretion from wild type fibroblasts was assessed by color change at 405 nm. Following this incubation, the gel was stained for 2 h in Coomassie Blue stain (40% methanol, 10% acetic acid and 2.5 g/liter R250 Coomassie Blue dye (Sigma)) and destained with multiple changes of destain solution (30% methanol, 10% acetic acid) until light bands appeared on a blue background.

RESULTS

MEKK1 Regulates PMA-induced uPA Expression—Wound healing requires coordinated proteolysis of various wound matrix components (1). We observed that MEKK1-deficient mice developed wounds, through over-grooming or fighting, that were persistent and healed poorly (not shown). Importantly, these persistent wounds were not observed in mice deficient for MEKK2, a related MAPKKK. This apparent healing defect is very similar to one reported for mice deficient in the protease uPA. Carmeliet and colleagues (18) observed facial wound healing defects in uPA-deficient mice. uPA activity regulates the activation of other proteases necessary for the healing process and has been linked to activation of immunoregulatory cyto-}

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tine TGF-β (19). MEKK1 signaling through ERK1/2 and JNK pathways controls AP-1 activity (12), and uPA expression is transcriptionally regulated through the binding of AP-1-PEA3 complexes to sequences in the uPA promoter (11), which suggested to us that MEKK1 might regulate uPA expression. Thus, we investigated the role of MEKK1 in the regulation of uPA expression. We tested fibroblasts derived from both parental strain and MEKK1−/− embryos (MEFs) for uPA expression. PMA induces a dynamic reorganization of the actin cytoskeleton and is a well characterized, potent stimulator of uPA expression (20, 21). As we have demonstrated previously that MEKK1 regulates both ERK1/2 and JNK signaling (13), we predicted that MEKK1-deficient cells would show impaired PMA-induced uPA induction. We observed a dramatic reduction in PMA-induced uPA mRNA in MEKK1-deficient fibroblasts as measured by Northern blot analysis (Fig. 1A). Consistent with decreased uPA induction observed in Northern blot analysis was mirrored in reporter assays using luciferase-based uPA promoter constructs (Fig. 1, A–C), which indicated that MEKK1 is required for PMA to stimulate uPA promoter activity. Activation of the uPA promoter requires the kinase activity of MEKK1, as luciferase activity of MEKK1−/− fibroblasts transfected with a kinase-inactive MEKK1 mutant mimicked that of control cells (Fig. 1C).

Loss of uPA induction in response to PMA is a function of MEKK1 deficiency because the stable expression of MEKK1 in knockout cells restored regulation of the uPA promoter (Fig. 1, B and D). Thus, our data indicate that MEKK1 is essential for PMA-induced uPA expression.

MEKK1-deficient Fibroblasts Lose uPA Activity—Because our data indicated that MEKK1 plays a role in regulation of uPA expression, we wanted to determine whether the apparent loss of uPA message observed in MEKK1−/− cells would result in a corresponding decrease in uPA activity. Consistent with decreased uPA message in MEKK1−/− cells, a colorimetric assay for measuring uPA activity confirmed that MEKK1 deficiency is also associated with a reduction in secreted urokinase activity (Fig. 2A). In addition, zymographic analysis demonstrated the requirement of MEKK1 for regulation of uPA activity. We co-polymerized plasminogen and casein in SDS-PAGE and then used this gel to separate proteins of conditioned medium from wild-type and MEKK1−/− fibroblast cultures. In a subsequent incubation in reaction buffer, in-gel uPA activated plasminogen to form active plasmin, which then cleaved casein. Upon staining of the gel with Coomassie Blue, areas of casein proteolysis could then be observed as clear bands. Our results demonstrate that PMA stimulates uPA protein expression and secretion from wild type fibroblasts.
MEKK1 Deficiency Inhibits uPA Expression

(UO1126 or the JNK inhibitor SP600125. Analysis of uPA activity revealed it to be highly sensitive to MEK inhibition, as U0126 treatment blocked FGF-2-induced uPA expression (Fig. 5). Interestingly, treatment with p38 inhibitor SB203586 enhanced FGF-2-induced uPA activity (Fig. 5). We conclude that regulation of both the ERK1/2 and JNK signaling pathways, a property of MEKK1 signaling, is necessary for FGF-2-induced uPA expression. Altogether, our data are consistent with JNK and ERK1/2 activities, modulated by MEKK1, functioning in concert to regulate uPA expression.

**DISCUSSION**

Protease-dependent tissue remodeling plays an important role in normal homeostasis and wound healing and is a factor in significant human pathologies including myocardial infarction (24) and cancer metastasis (7). Further, uPA has been linked to arterial neointima formation and vascular wound

**MEKK1 is required for uPA expression.** PMA-induced uPA expression was assessed by Northern blot (A) and luciferase reporter assays (B–D). A, wild-type and MEKK1+/− fibroblasts were treated with 100 nM PMA for 18 h. RNA (5 μg) was probed for uPA and β-actin. B, wild-type and MEKK1+/− immortalized fibroblasts were transfected with a uPA-promoter/luciferase (uPA/Luc) construct and treated with 100 nM PMA for 18 h. Fold increase in uPA induction was assessed by luciferase assay. C, MEKK1+/− fibroblasts were transiently transfected with empty vector, wild-type MEKK1, or kinase-inactive MEKK1 K1253M together with uPA/Luc and treated with PMA. D, MEKK1+/− fibroblasts clones that stably express wild-type or kinase-inactive MEKK1 were treated with PMA. Both C and D were assayed for uPA induction as described in B. The results shown are the mean ± S.E. of at least three independent experiments.

(Fig. 2B). In contrast, uPA activity is markedly inhibited in MEKK1+/− fibroblasts in response to PMA. Immunoblotting confirmed that induction of secreted uPA protein expression in both primary and immortalized fibroblasts is MEKK1-dependent (Fig. 2B). We further verified the requirement of MEKK1 in uPA expression by immunofluorescence analysis. Stimulation of wild-type fibroblasts with PMA resulted in an increased accumulation of uPA in the Golgi complex (Fig. 2C). Although MEKK1+/− fibroblasts exhibit a basal level of uPA expression, the presence of Golgi-associated uPA was not observed in PMA-stimulated MEKK1+/− fibroblasts (Fig. 2C). To confirm the specificity of MEKK1 in regulating fibroblast uPA expression, we repeated the zymography assay with MEKK2+/− fibroblasts. Our zymography assays clearly showed that PMA-induced uPA activity absolutely requires MEKK1 activity, as expression of a kinase-inactive MEKK1 mutant did not rescue uPA activity (Fig. 3C). Our results indicate that MEKK1 is absolutely required for FGF-2-induced uPA up-regulation in fibroblasts.

**MEKK1 Regulates MAPK Signaling Downstream of FGF-2 Receptor Ligation—Control of uPA expression is largely dependent on the binding of the consensus AP-1-PFA3 sequence within the uPA promoter (21, 23). Both ERK1/2 and JNK signaling impact AP-1 activity (12), and we have previously shown that MEKK1 activity plays a role in both pathways (13). To determine whether MEKK1 expression is necessary for FGF-2-induced ERK1/2/JNK signaling, we stimulated either wild-type or MEKK1-deficient fibroblasts and assessed MAPK activity by immunoblot with antibodies specific for activated ERK or JNK (Fig. 4A). We clearly show that ligand-induced ERK and JNK phosphorylation is dramatically reduced in the MEKK1-deficient samples (Fig. 4A). Importantly, other FGF-2-induced pathways, such as phosphatidylinositol 3-kinase activation and JNK phosphorylation, remain intact in MEKK1-deficient cells (Fig. 4B). This finding demonstrates that disruption of MEKK1 expression specifically impacts MAPK signaling.

We wanted to determine whether ERK1/2, JNK, or both of these signaling pathways were responsible for FGF-2-induced uPA activity. We therefore repeated the zymography assay using wild-type fibroblasts treated with the MEK inhibitor U01126 or the JNK inhibitor SP600125. Analysis of uPA activity revealed it to be highly sensitive to MEK inhibition, as U01126 treatment blocked FGF-2-induced uPA expression (Fig. 5). Interestingly, treatment with p38 inhibitor SB203586 enhanced FGF-2-induced uPA activity (Fig. 5). We conclude that regulation of both the ERK1/2 and JNK signaling pathways, a property of MEKK1 signaling, is necessary for FGF-2-induced uPA expression. Altogether, our data are consistent with JNK and ERK1/2 activities, modulated by MEKK1, functioning in concert to regulate uPA expression.
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Fig. 2. uPA protease activity is reduced in MEKK1−/− but not MEKK2−/− fibroblasts. Secreted uPA protease activity specifically is regulated by MEKK1. A, concentrated tissue culture medium conditioned with wild-type or MEKK1−/− fibroblasts (untreated or treated with 100 nm PMA for 24 h) was incubated with urokinase-specific chromogen S2444 for 24 h. PMA-induced uPA activity was determined by measuring the change in absorbance at 405 nm. Results shown are the mean ± S.E. of at least three independent experiments. B and D, proteins of media from wild-type (WT), MEKK1−/−, or MEKK2−/− fibroblast cultures were separated by SDS-PAGE co-polymerized with plasminogen and casein (see "Experimental Procedures"). and uPA activity was assessed by casein proteolysis. NS, no stimulus. B, secreted uPA protein levels were assessed by immunoblot with anti-uPA antibodies. The displayed zymograms are representative of at least three independent experiments. C, wild-type and MEKK1−/− fibroblasts were serum-starved overnight and then treated with PMA as described previously. Fibroblast uPA was bound by anti-uPA antibodies and anti-rabbit Cy3.

Fig. 3. MEKK1 activity required for uPA induction by FGF-2. A, FGF-2-induced uPA activity was assessed by Northern blot. Wild-type, MEKK1−/−, and MEKK1−/− fibroblasts were treated with FGF-2 (10 ng/ml, 10 h). A, RNA (5 μg) was probed for uPA and β-actin. B, zymogram analysis of uPA activity from tissue culture media of treated or untreated fibroblasts. C, zymogram of tissue culture media from fibroblasts transiently transfected with empty vector, wild-type MEKK1, or kinase-inactive MEKK1 K1253M.

As uPA activates plasmin, the loss of uPA expression observed in MEKK1−/− fibroblasts would be predicted to result in a reduced capacity to remodel matrix components. This defect could be manifested by an impaired ability to heal wounds, and in fact, this very defect has been observed in uPA-deficient mice (18). Our observation that facial wounds persisted in our MEKK1-deficient mouse colony suggests that MEKK1 is involved in the wound healing process. Mice lacking MEKK1−/− have an inhibited wound healing response that mimics uPA deficiency and that is not observed in MEKK2 knockout mice. What properties of MEKK1 make it an important MAPKKK for tissue remodeling? First, MEKK1 is activated in response to changes in cytoskeletal structure (13, 16), cell shape, and adherence (13, 16) and in response to epidermal growth factor and lysophosphatidic acid (13, 15). In contrast, other MAPKKKs, like MEKK2, appear to be involved primarily in responses to specific growth factor and cytokine receptors but not in changes to the cytoskeleton, adherence, or cell shape (28). Second, MEKK1 interacts with Rac and Cdc42, important GTPases in the control of the cytoskeleton, cell shape, and migration. Finally, MEKK1 regulates the JNK and ERK1/2 pathways, MAPKs that have been shown clearly to be involved in the regulation of uPA expression. We have demonstrated that MEKK1-deficient fibroblasts do not have the ability to up-regulate uPA in response to FGF-2 and PMA. Further, we provide evidence that FGF-2-induced uPA expression specifically requires MEKK1-dependent JNK and ERK1/2 activities. It is intriguing that, whereas other MAPKKKs are known to regulate ERK1/2 or JNK in response to specific stimuli, MEKK1 remains indispensable for uPA up-regulation in response to these growth factors. Growth factor-dependent uPA expression is blocked in MEKK1-deficient fibroblasts, demon-
MEKK1 Deficiency Inhibits uPA Expression

Fig. 4. MEKK1 regulates MAPK signaling downstream of stimulation with FGF-2. Wild-type (WT) KK1−/− fibroblasts were serum-starved overnight, stimulated with FGF-2 (10 ng/ml) for the indicated times, and then lysed. A, equal amounts of lysate were separated by 10% SDS-PAGE, and MAPK signaling assessed by immunoblot with antibodies to phosphorylated forms of ERK and JNK. An anti-ERK2 immunoblot was performed to confirm equal loading. B, FGF-2-induced activation of the AKT signaling pathway was assessed by anti-phospho-AKT immunoblot with equal loading confirmed by total AKT immunoblot.

Fig. 5. uPA up-regulation requires both ERK1/2 and JNK activity. Wild-type MEFs were treated with 20 μM U0126, SP600125, SB203580, or Me2SO vehicle for 60 min. Cells were then stimulated for 24 h with 1 ng/ml FGF-2 or left untreated. The activity of secreted uPA in the media was assessed by casein/plasminogen zymography. The displayed zymograms are representative of at least two independent experiments. Inh., inhibitor.

Fig. 6. Model depicting MEKK1 pathway controlling uPA expression. MEKK1−/− fibroblasts are defective in MAPK activation-dependent uPA expression (see “Discussion” for details).

transcription has been shown to be driven by SP1 binding to the proximal promoter (30). SP1-dependent transcription is regulated differently than that reliant upon AP-1 (12, 31) and may in part be regulated through ERK1/2 signaling (32). Indeed, our results showing basal uPA activity to be sensitive to MEK but not JNK inhibition is consistent with SP1-dependent constitutive transcription being controlled by the ERK1/2 pathway, independently of JNK activity (Fig. 4). There are additional MAPKKKs capable of activating the JNK and ERK1/2 pathways that are regulated by upstream stimuli different from those that activate MEKK1. The MEKK1 and MEKK2 knockouts have defined their selective roles in regulating different physiological functions. The pathological consequences observed with MEKK1 or MEKK2 deletion are different, even though the downstream MAPK signaling pathways remain intact. For this reason, MAPKKK knockouts, like those of MEKK1 and MEKK2, can be more subtle than total loss of a signaling pathway, such as found for the JNKs, but can be much more telling in regards to physiological regulation in adult animals. The MEKK1 knockout defines MEKK1 as a critical MAPKKK controlling the expression of uPA from multiple stimuli. MEKK1 expression is required for uPA induction downstream of both receptor tyrosine kinase (FGF receptor) and protein kinase C activation (PMA), and thus these pathways converge at or upstream of MEKK1 (Fig. 6). Our data place MEKK1 at the nexus of the signaling pathways that control uPA expression and, as such, reveal the utility of modulating MEKK1 as a means of regulating uPA dependent cell functions. The control of proteolytic degradation of extracellular matrix required for tumor metastasis is just one example of such a function. The importance of MEKK1 in these processes validates the use of MAPKKKs as targets for inhibition by small molecule inhibitors in specific diseases including cancer metastasis.

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