Cross-talk between the p42/p44 MAP Kinase and Smad Pathways in Transforming Growth Factor β1-induced Furin Gene Transactivation*

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Furin, a predominant convertase of the cellular constitutive secretory pathway, is known to be involved in the maturation of a number of growth/differentiation factors, but the mechanisms governing its expression remain elusive. We have previously demonstrated that transforming growth factor (TGF) β1, through the activation of Smad transducers, regulates its own converting enzyme, furin, creating a unique activation/regulation loop of potential importance in a variety of cell fate and functions. Here we studied the involvement of the p42/p44 MAPK pathway in such regulation. Using HepG2 cells transfected with fur P1 LUC (luciferase) promoter construct, we observed that forced expression of a dominant negative mutant form of the small G protein p21ras (RasN17) inhibited TGFβ1-induced fur gene transcription, suggesting the involvement of the p42/p44 MAPK cascade. In addition, TGFβ1 induced sustained activation/phosphorylation of endogenous p42/p44 MAPK. Furthermore, the role of MAPK cascade in fur gene transcription was highlighted by the use of the MEK1/2 inhibitors, PD98059 or U0126, or co-expression of a p44 antisense construct that repressed the induction of fur promoter transactivation. Conversely, over-expression of a constitutively active form of MEK1 increased unstimulated, TGFβ1-stimulated, and Smad2-stimulated promoter P1 transactivation, and the universal Smad inhibitor, Smad7, inhibited this effect. Activation of Smad2 by MEK1 or TGFβ1 resulted in an enhanced nuclear localization of Smad2, which was inhibited upon blocking MEK1 activity. Our findings clearly show that the activation of the p42/p44 MAPK pathway is involved in fur gene expression and led us to propose a co-operative model whereby TGFβ1-induced receptor activation stimulates not only a Smad pathway but also a parallel p42/p44 MAPK pathway that targets Smad2 for an increased nuclear translocation and enhanced fur gene transactivation. Such an uncovered mechanism may be a key determinant for the regulation of furin in embryogenesis and growth-related physiopathological conditions.

Furin is a mammalian subtilisin/Kex2p-like Ca2+-dependent endoprotease involved in the processing of various types of higher molecular mass precursor substrates, containing the minimal basic amino acid RXR motif. This prototype of the pro-protein convertase family (for reviews, see Refs. 1 and 2) is primarily located in the trans-Golgi apparatus (3), although some proportion of the furin molecules can recycle from the cell membrane to endosomes (4). The biological importance of this convertase stems from the large number and variety of bioactive proteins and peptides that can be generated through its activity. These include key molecules involved in normal and physiopathological conditions including growth/differentiation processes. Furin cleaves a C-terminal to an unique processing site (RXK/RK/R) found in many growth/differentiation-related peptides and proteins including TGFβ1, activin A, BMP family members, Nodal, lefty-1, and lefty-2 as well as growth factor pro-receptors such as insulin-like growth factor receptor and the hepatocyte growth factor receptor (e-Met) (2, 5, 6). Silencing of the expression of mouse furin results in embryonic lethality because of hemodynamic insufficiency associated with several development defects including disruption of development of the heart and vascular system and failure to undergo axial rotation (7). These findings highlight the role of furin in growth and development and in the physiological maturation of substrates involved in these processes including members of the TGFβ family.

The results from previous studies have demonstrated that pro-TGFβ1 is efficiently processed by furin releasing the genuine mature growth factor (8) and that among the pro-protein convertase members, furin more closely satisfies the requirements needed for an authentic TGFβ1 converting enzyme (9). In fibroblastic and synovial cells, the furin cleavage product, TGFβ1, up-regulated gene expression of its own converting enzyme, resulting in an increase in endogenous TGFβ1 processing activity and release of the bioactive peptide (10). TGFβ1 did not increase furin mRNA stability and treatment of synovial cells with actinomycin D, before TGFβ1 addition prevented the increase in fur gene expression. This observation suggested that furin concentrations could be regulated at the transcriptional level, resulting in the increase in local concentrations of bioactive growth factors. However, the molecular mechanisms by which TGFβ1 exerts its effects have not been fully elucidated.

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† The abbreviations used are: TGF, transforming growth factor; BMP, bone morphogenetic protein; ERK, extracellular signal-regulated kinase; MEK-1, mitogen-activated protein kinase kinase kinase-1; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; PBS, phosphate-buffered saline.
In the last few years significant progress has been made in the signaling mechanisms utilized by TGFβ. A major discovery comes from the recent uncovering and cloning of specific Smad signaling proteins consisting of pathway-restricted Smads (Smad2 and Smad3), the common mediator Smad4, and the inhibitory Smads (Smad6 and Smad7) (for reviews see Refs. 11–13) that directly inhibit the TGFβ type I receptor serine-threonine kinase and the transcriptional machinery. TGFβ signals through sequential activation of two cell surface serine-threonine kinase receptors, which phosphorylate Smad2 and Smad3 within their conserved C-terminal SSXS motif (14, 15). These activated Smads, together with Smad4, translocate to the nucleus and, in association with other transcription factors, activate the transcription of target genes (16, 17). Among the participating transcription factors are the winged helix factor FAST (now known as FoxH1) (18) in the case of Smad2 (19) or AP-1 in the case of Smad3 (reviewed in Refs. 20–22). Naturally occurring inhibitors, Smad6 and Smad7, block and hence control TGFβ superfamily signaling by competitively interacting with the activated type I receptors (23, 24).

As increasing information is collected regarding the detailed molecular mechanism of Smad protein signaling, a number of functional interactions between these proteins and other signaling pathways have been reported. For example, recent work has demonstrated that the linker region of BMP pathway-restricted Smad1 was phosphorylated by ERK2 (25), a member of the classic ERK-activated protein kinase pathway, leading to the inhibition of nuclear translocation of the Smad1-Smad4. In contrast, other studies have demonstrated positive functional interaction between the two stress-activated protein kinase pathways and Smads. For instance, it has been demonstrated that the mitogen-activated protein kinase kinase-1 (MEKK1), an upstream activator of the stress-activated protein kinase/c-Jun N-terminal kinase pathway, enhances Smad protein transcriptional co-activator interactions in endothelial cells (26). Also, other groups have demonstrated that TGFβ1 activates Smad and TAK1 pathways, resulting in the formation of an active transcription complex composed of Smad3-Smad4 and the p38 nuclear target ATF-2 (27, 28).

In a recent study, we provided evidence for the central role of Smads in the transcriptional activation of the TGFβ1-inducible fur P1 promoter activity (29). Using HepG2 cells transfected with LUC (luciferase) promoter constructs, we observed that among the three furin promoters, the P1 promoter was the strongest and the most sensitive to TGFβ1 and that Smads were essential for mediating such responsiveness. We also observed that the proximal P1 promoter region (positions −8734 to −7925) that contains one SBE (Smad binding element) and one ARE (activin-responsive element) binding site carries most of the Smad responsiveness. These results highlight the central role of Smads in the expression of furin, an important gear of the complex TGFβ1 maturation/activation machinery.

In light of the emerging evidence for the interactions between the Smad and the MAPK pathways and the role of furin in growth and differentiation events, it was of interest to explore the possible integration between these two pathways for the regulation of this convertase. Using HepG2 cells transfected with fur P1 LUC (luciferase) promoter construct, we observed that forced expression of a dominant negative mutant of the small G protein p21ras (RasN17) inhibited TGFβ1-induced fur gene transcription. Furthermore, the role of the p42/p44 MAPK cascade in fur gene transcription was emphasized by the use of the MEK1/2 inhibitors, PD98059 or U0126, or co-expression of a p44 antisense construct that blunted the induction of fur gene transcription by TGFβ1. Conversely, forced expression of a constitutively active form of MEK1 (MEKA) increased unstimulated, TGFβ1-stimulated, and Smad-stimulated promoter P1 transactivation, and the universal Smad inhibitor, Smad7, inhibited this effect. Our findings clearly show that activation of the p42/p44 MAPK pathway is involved in fur gene expression and suggest functional interactions between the Smad and the p42/p44 MAPK cascade pathway in this regulation.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The human liver cell line (HepG2) was obtained from American Type Culture Collection and maintained in a complete medium composed of minimal essential medium (Life Technologies, Inc.) with 10% fetal bovine serum (Intergen Company, Rochester, NY) and 40 μg/ml gentamycine (Shering Canada Inc., Pointe-Claire, Canada). The HepG2 cells were trypsinized and reseeded twice weekly.

**Materials**—Recombinant human TGFβ1 was a generous gift from Dr. Anthony P. Puchnio. Dr. Torik A. Y. Ayoubi generously provided human promoter luciferase constructs pGL2-P1. Plasmids pCMV5B (empty vector), pCMV5B-FlagSmad1, and pCMV5B/mSmad4 were described previously (14, 30). Plasmid pCMV5-Smad7 (Smad7) was kindly provided by Dr. Dean Falb (Millennium Pharmaceuticals Inc., Cambridge, MA). Constructions of Rous sarcoma virus Neo (control vector) and Rous sarcoma virus Ras Asn17 (dominant negative of p21ras, RasN17) were described previously (31). MEKα subclone into expression vector pECE and p44-mutant antisense in pDNA (kindly provided by Dr. Jacques Pouyssegur) were described previously (32, 33). Finally, plasmids pCSMT (empty vector) and pCSMT-FAST (FAST-1), now known as FoxH1 (18), were a generous gift from Dr. Malcolm Whitman. Antiserum E1B, which specifically recognizes p42/p44 MAPK on Western blots, was a kind gift from Drs. Fergus McKenzie and Jacques Pouyssegur (34). Rabbit polyclonal antibodies directed against phosphorylated and active forms of p42/p44 MAPK were from New England Biolabs (Mississauga, Canada). The MEK1/2 inhibitors PD98059 and U0126 were purchased from Calbiochem-Novabiochem Corp. (San Diego, CA). Goat polyclonal anti-Smad2 antibodies were purchased from Santa Cruz Biotechnology. Inc. (Santa Cruz, CA) and revealed with fluorescein-conjugated rabbit affinity purified Fab’/2 fragment to goat IgG (ICN Biochemicals, Costa Mesa, CA).

**Luciferase Assays**—HepG2 cells were transiently transfected by CaPO4 precipitation technique using a mammalian cell transfection kit (Speciality Media Inc., Lavallette, NJ) as described previously (35). Briefly, 24 h prior to transfection, HepG2 cells were plated using a plating density of 27,000 cells/well in 24-well plates (Falcon Labware, Mississauga, Canada) in complete medium. The cells were fed fresh complete medium for 3–4 h before transfection. HepG2 cells were transfected with 0.5 μg plasmid/well for each plasmid, and the cells in control wells were transfected with appropriate control vectors to compensate for potential squelching. Gently vortexed DNA/CaPO4 precipitate suspension was added slowly, dropwise, while gently swirling the medium in the plate. The plates were returned to the incubator until the following morning when the cells were rinsed with PBS and serum-starved (0.2% fetal bovine serum) for 6–8 h prior to overnight stimulation with 0 or 5 ng/ml TGFβ1. In experiments using PD98059, the MEK1-specific inhibitor was added simultaneously with TGFβ1 to a final concentration of 10 μM, and control cells received a final concentration of 10 μM of 0.5%. The cells were then lysed, and luciferase activity was assayed as described previously (35). β-Galactosidase activity was monitored as an internal control of transfection efficiency using a β-galactosidase enzyme assay system (Promega, Madison, WI). The results were expressed as ratios of luciferase activity over β-galactosidase activity.

**Northern Analysis—Total RNA was extracted from cultured cells according to the TRI-Reagent protocol described previously (Molecular Research Center Inc., Cincinnati, OH). Northern analysis of furin gene expression and measure of RNA loading and integrity used in this publication has previously been extensively detailed elsewhere (10).**

**Signal intensity was quantitated by densitometry with a Amersham Biomolecular Imaging System (Piscataway, NJ). The densitometric values are expressed as the ratio of fur/GAPDH densitometric quantification with control values set at 1.**

**Protein Expression and Immunoblotting—As described previously, the cells were lysed in SDS sample buffer (62.5 m Tris-HCl, pH 6.8, 2.3% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.005% bromphenol blue, 1 mM phenylmethylsulfonyl fluoride); proteins (40 μg) from whole cell lysates were separated by SDS-polyacrylamide gel electrophoresis in 10% gels (36). The proteins were detected immunologically following electrotransfer onto nitrocellulose membranes. The blots were then...
incubated with different antibodies in blocking solution overnight at 4°C and then incubated with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit (1:1000) IgG in blocking solution for 1 h. The blots were visualized by the Amersham Pharmacia Biotech ECL system. The protein concentrations were measured using a modified Lowry procedure with bovine serum albumin as standard (37).

Indirect Immunofluorescence Microscopy—HepG2 cells were plated at a density of 125,000 cells/well in 6-well plates (Falcon Labware) on sterile coverslips in complete medium and then transfected in suspension with 2 μg of Smad2 DNA construct/well with 4 μl/well of FuGENE6 (Roche Molecular Biochemicals) according to manufacturer’s protocol. Twenty-four hours post-transfection, HepG2 cells were serum-starved (0.2% fetal bovine serum) overnight prior to 30 min of preincubation with 10 μM of PD98059 or 0.05% MeSO vehicle followed by a 1-h incubation with 10 ng/ml TGFβ1. Transfected and stimulated HepG2 cells were then prepared for immunofluorescence staining as described previously (38). Briefly, the cells were washed with cooled PBS and then fixed for 15 min in precooled (−20°C) methanol/acetic acid (30/70). After a 15-min rehydration in PBS, the cells were permeabilized in PBS with 0.25% Triton for 5 min, and nonspecific binding was blocked by incubation for 20 min in PBS with 2% bovine serum albumin. Fixed cells were then incubated for 1 h with anti-Smad2 primary antibody (1:50). The cells were again washed five times with PBS followed by incubation in PBS with bovine serum albumin for 60 min with fluorescein-conjugated second antibody (anti-goat, 1:100). Finally, the cells were washed five times and stained 5 min with 0.025% Evan’s Blue (Sigma) and then fixed in PBS with bovine serum albumin. The protein concentrations were measured using a modified Lowry procedure with bovine serum albumin as standard (37).

RESULTS

Involvement of the Ras-MAPK Cascade in TGFβ1-induced fur Gene Activation—Previous reports have demonstrated in several cell systems that TGFβ1 increased furin mRNA expression starting 3 h after stimulation, and the peak effect was observed at 18−24 h. No effects were observed at the earlier 1-h time point (10, 29). Treatment of cells with actinomycin D before or after stimulation indicated that furin gene expression was regulated at the transcriptional level. As an attempt to determine the nature of the signaling response involved, we have examined the involvement of the Smad transducers. We have shown that Smad2 and Smad4, possibly in complex with the winged helix transcription factor FAST (FoxH1), participate in the constitutive and TGFβ1-inducible transactivation of the fur P1 promoter in HepG2 cells (29). Because TGFβ1 is widely documented to be implicated in the regulatory mechanisms of cellular growth and differentiation (39−41), it was of interest to determine whether the classical proliferation/differentiation module Ras/Raf/MEK/p42/p44 MAPK is also involved in fur P1 promoter transactivation. For this, we co-transfected HepG2 cells with a pGL2-P1 luciferase reporter gene and either a control or a dominant interfering p21WAF1 vector (RasN17), and cells were stimulated overnight in the presence (5 ng/ml) or the absence of TGFβ1. We observed that RasN17 inhibited both constitutive (77% inhibition) and TGFβ1-induced (91% inhibition) P1 promoter activation (Fig. 1). These results indicate that Ras-dependent signaling pathways are involved in the regulation of fur P1 promoter.

In addition to the p42/p44 MAPK cascade, Ras also controls other signaling pathways such as those linked to phosphatidylinositol 3-kinase or Rac/Rho proteins (42). Hence, to specifically analyze the contribution of the p42/p44 MAPK cascade in TGFβ1-induced P1 promoter activation, we first examined the phosphorylation of p42/p44 isoforms in cells stimulated by TGFβ1. The cell lysates were prepared from serum-starved HepG2 cells stimulated for the indicated time periods with 5 ng/ml TGFβ1. Western blot analysis with an antibody recognizing the biophosphorylated and active MAPK isoforms revealed that TGFβ1 induces a relatively small but reproducible increase in MAPK activities within 30 min (1.5 ± 0.5-fold increase; p ≤ 0.05; n = 3) with a maximal effect observed 2 h post-stimulation (7.3 ± 2.7-fold increase; p ≤ 0.05; n = 3) (Fig. 2). Therefore, the changes in p42/p44 phosphorylation by TGFβ1 precede the previously reported up-regulation in furin mRNA levels (10).

Next, we employed a pharmacological inhibitor of MEK1/2, PD98059 that prevents the activation of MEK1/2, thereby inhibiting p42/p44 phosphorylation. In the presence of PD98059, the ability of TGFβ1 to induce P1 promoter transcription is dose-dependently inhibited (Fig. 3A). As a control, the MeSO vehicle, at the concentration used in these experiments (0.05%), did not alter either basal or induced P1 transactivation in HepG2 cells. To confirm that these effects were through the inhibition of MEK, the experiments were repeated using a structurally unrelated MEK inhibitor, U0126, which specifically inhibits the function of activated MEK1/2 (43). The effects of this inhibitor were similar to those observed using PD98059 (Fig. 3B), strongly suggesting that the effects of PD98059 are due to its ability to inhibit MEK, thereby inhibiting TGFβ1-induced phosphorylation of downstream p42/p44 kinases. To confirm this, p42/p44 phosphorylation was measured by Western blot assays. As demonstrated in Fig. 3C, low concentrations (2.5 μM and 10 μM) of PD98059 or U0126 efficiently suppressed TGFβ1-induced p42/p44 phosphorylation.

Northern blot analysis was also performed to examine the effects of MEK inhibitors on TGFβ1-induction of furin mRNA expression. As shown in Fig. 3D, the addition of 10 μM of PD98059 or U0126 inhibited the ability of TGFβ1 to induce furin mRNA expression by 48 and 77%, respectively. So far, our results indicate that TGFβ1 induction of furin gene expression is mediated through the MEK/p42/p44 MAPK pathway as one of the events downstream of Ras.

Inhibition of the MEK/MAPK Cascade Blocks Smad-induced fur Promoter Activation—Because both Smads (29) and p42/p44 MAPKs are involved in fur regulation, we next investigated the possible interplay between these signaling pathways. HepG2 cells were transfected with pCSMT plasmid encoding...
MEK1/2 inhibitor PD98059 at a concentration of 10 μM inhibited Smad2 transactivation, which is clearly inhibited by TGFβ1 stimulation in HepG2 cells. As expected, in the presence of the MEK inhibitor PD98059, no significant increase in luciferase activity was observed in response to TGFβ1 stimulation in HepG2 cells. This is a representative experiment of three.

To provide further insights into the possible interplay existing between the classic p42/p44 kinase cascade and Smad2-dependent pathway, HepG2 cells were co-transfected with or without MEK1, Smad2, or Smad4, and pGL2-P1 promoter luciferase activity was measured. As shown in Fig. 5A, MEK1 increased constitutive and TGFβ1-induced fur promoter P1 transactivation by 0.9- and 9.8-fold, respectively. The highest transactivation was observed when Smad2 (18.0 ± 0.9-fold) and Smad4 (43.4 ± 9.6-fold) were combined with MEK1 in the presence of TGFβ1. To determine the contribution of receptor-activated Smads in MEK1-induced activation of fur promoter, MEK1 was co-expressed with Smad7, a Smad antagonist that interferes with the phosphorylation/activation of Smad2 and Smad3 (24) or Smad2(3SA), a dominant negative form of Smad2 (14). The basal levels of luciferase activity observed as well as the increase in the presence of MEK1 alone probably reflects endogenous TGFβ1 production by HepG2 cells, as previously demonstrated by us (29) and others (45). As shown in Fig. 5B, TGFβ1, MEK1, and TGFβ1 plus MEK1-induced activation of fur P1 promoter were potently inhibited by co-expression of Smad7, indicating that TGFβ1 receptor-activated Smad proteins are indeed playing a role in MEK1-mediated transcriptional activation of P1. Moreover, Smad2(3SA) blocked MEK1-induced transactivation in the presence of TGFβ1 (Fig. 5C), suggesting cooperation between MEK1 and activated endogenous Smad2. Taken together, these results indicate that maximal transcriptional activation of pGL2-P1 promoter by TGFβ1 stimulation requires the action of p42/p44 MAPKs as well as the TGFβ1-specific receptor-regulated Smad2 and common Smad4.

**Effect of MEK1 on Smad-regulated fur Gene Transcription**—To provide further insights into the possible interplay existing between the classic p42/p44 kinase cascade and Smad2-dependent pathway, HepG2 cells were co-transfected with or without MEK1, Smad2, or Smad4, and pGL2-P1 promoter luciferase activity was measured. As shown in Fig. 5A, MEK1 increased constitutive and TGFβ1-induced fur promoter P1 transactivation by 0.9- and 9.8-fold, respectively. The highest transactivation was observed when Smad2 (18.0 ± 0.9-fold) and Smad4 (43.4 ± 9.6-fold) were combined with MEK1 in the presence of TGFβ1. To determine the contribution of receptor-activated Smads in MEK1-induced activation of fur promoter, MEK1 was co-expressed with Smad7, a Smad antagonist that interferes with the phosphorylation/activation of Smad2 and Smad3 (24) or Smad2(3SA), a dominant negative form of Smad2 (14). The basal levels of luciferase activity observed as well as the increase in the presence of MEK1 alone probably reflects endogenous TGFβ1 production by HepG2 cells, as previously demonstrated by us (29) and others (45). As shown in Fig. 5B, TGFβ1, MEK1, and TGFβ1 plus MEK1-induced activation of fur P1 promoter were potently inhibited by co-expression of Smad7, indicating that TGFβ1 receptor-activated Smad proteins are indeed playing a role in MEK1-mediated transcriptional activation of P1. Moreover, Smad2(3SA) blocked MEK1-induced transactivation in the presence of TGFβ1 (Fig. 5C), suggesting cooperation between MEK1 and activated endogenous Smad2. Taken together, these results indicate that maximal transcriptional activation of pGL2-P1 promoter by TGFβ1 stimulation requires the action of p42/p44 MAPKs as well as the TGFβ1-specific receptor-regulated Smad2 and common Smad4.

**Involvement of MEK1 in TGFβ-induced Smad2 Nuclear Translocation**—It has been previously demonstrated that Smad2 translocates to the nuclear compartment in response to the activated TGFβ type 1 receptor, TGR1 (14) or by TGFβ1 stimulation (46). To determine whether disruption of the MAPK pathway can alter the subcellular localization of Smad2, we used indirect immunofluorescence microscopy with Smad2-specific antibodies. HepG2 cells were transfected with Smad2 and were treated with 10 ng/ml TGFβ1 for 1 h. As expected, Smad2 in unstimulated cells demonstrated a diffuse, mainly cytoplasmic staining (47), a pattern consistent with overexpression and nonactivated Smad2. When HepG2 cells were stimulated with TGFβ1, up to 49.6 ± 3.1% of them exhibited a predominantly nuclear Smad2-specific staining compared with 10.1 ± 1.4% for control unstimulated cells. Interestingly, the use of PD98059 significantly reduced (78%) the amount of TGFβ1-induced Smad2 nuclear staining with 19.0 ± 0.9% of cells that exhibited a predominant nuclear staining (Fig. 6). To further support the role of MEK1 in Smad2 subcellular localization, Smad2 was co-transfected with constitutively active MEK1 mutant. Interestingly, overexpressing MEK1 in unstimulated HepG2 cells results in 3.7-fold increase in Smad2 nuclear localization that is further increased to 5.3-fold upon TGFβ1 stimulation (Fig. 6B). These observations clearly demonstrated functional interaction between Smad2 and MEK/p42/p44 MAPK cascade.

**DISCUSSION**

The findings presented herein clearly show that TGFβ1-induced receptor activation stimulates not only a Smad pathway but also a parallel p42/p44 MAPK pathway that targets Smad2 for an increased nuclear translocation and enhanced fur transactivation.
gene transactivation. Even though other reports such as the one from Hayashida et al. (48) have raised the possibility of interactions between the Smad and MAPK pathways for TGFβ-stimulated collagen gene expression, few studies have indeed reported positive cross-talk between the growth/differentiation MAPK pathway and the Smad pathway for the regulation of TGFβ-related functions. For instance, work from de Caestecker et al. (46) indicated that hepatocyte growth factor and epidermal growth factor mediate Smad-dependent reporter gene activation and induce phosphorylation of Smad 2 by kinases downstream of MEK1. More recently, Watanabe et al. (49) have shown that in differentiated chondrocytes, rapid and sustained activation of both p42/p44 and p38 MAPK is required for high levels of aggrecan gene expression, whereas Smad 2 was also involved in the initial activation of this gene that occurs in undifferentiated cells. In contrast, Kretzschmar et al. (25) have demonstrated that the MAPK/ERK1/2 pathway can negatively regulate the BMP-Smad-1-dependent transcriptional response.

Also, results from Brown et al. (26) indicated that the stress-activated SAPK/JNK pathway, but not the p42/p44 MAPK pathway, can activate Smad-2-mediated transcriptional activation in bovine endothelial cells. Although Yue and Mulder (50) have suggested that the Ras/MAPK pathways are essential for TGFβ1 induction of TGFβ1 in lung and intestinal epithelial cells, they proposed that Smads only contribute to this biological response in an indirect manner. The exact reasons for these discrepancies remain unknown, but it is clear that multiple interactions between MAPK and Smad pathways can occur depending on the cell type and possibly the extent of MAPK activation.

To determine whether the classical p42/p44 MAPK cascade is involved in TGFβ1-induced activation of the fur gene, our first initiative was to determine whether the ability of TGFβ1 to stimulate fur gene expression was a p21ras-dependent or p21ras-independent mechanism. Interestingly, constitutive and TGFβ1-induced transcription of the reporter gene was effi-

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**Fig. 3.** Regulation of TGFβ1-induced fur gene transcription by the p42/p44 MAPK module. HepG2 cells were incubated overnight in the absence (white bars) or the presence (black bars) of TGFβ1 (5 ng/ml). Decreasing concentrations of MEK-specific inhibitors PD98059 or U0126 were added simultaneously and vehicle control cells received a final concentration of 0.05% Me2SO (D). In A and B, the cells were transiently transfected with furin promoter P1 luciferase construct 24 h before TGFβ addition. A, PD98059 treatment; B, U0126 treatment. The data are expressed as the means ± S.E. (n = 6). In parallel experiments, C p42/p44 phosphorylation was analyzed as described above. D, mRNA was analyzed by Northern blot. The results are expressed as ratios of fur:GAPDH with the control value set at 1. BN M, medium.
ciently inhibited by dominant negative RasN17 expression in HepG2 cells. Partial blockage of constitutive P1 promoter activity may reflect the requirement of p42/p44 MAPK cascade or 

FIG. 4. Regulation of Smad-induced fur P1 promoter transactivation by the p42/p44 MAPK module. HepG2 cells were transiently co-transfected with fur P1 promoter construct pGL2-P1 and either the winged helix FAST (FoxH1) transcription factor construct pCSMT-FAST-1 (A), combinations of Smad1 or Smad2 with common Smad, Smad4 (B) or Smad2 or FAST-1 in the presence or the absence of p44-AS construct or control pcDNA vector (C). The cells were incubated overnight in the absence (white bars) or the presence (black bars) of TGFβ1 (5 ng/ml). MEK1 specific inhibitor PD98059 (PD) was added simultaneously to a final concentration of 10 μM in B, and the control cells were incubated in medium only (M) or Me2SO vehicle (D). Luciferase activity is expressed as fold-increase relative to the unstimulated control. The data are expressed as the means ± S.E. (n = 3–5).

FIG. 5. Effect of MEKA on Smad-regulated fur gene transcription. HepG2 cells were transiently co-transfected with fur P1 promoter construct and either overexpressed Smad2, Smad4, MEKA plasmid constructs or individual Smads in combination with MEKA (A), combined overexpressed MEKA with inhibitor Smad7 construct (B), or either overexpressed Smad2 or Smad4 or individual Smads in combination with MEKA in the presence or the absence of Smad2–3SA (C). The cells were incubated overnight in the absence (white bars) or presence (black bars) of TGFβ1 (5 ng/ml). Luciferase activity representing fur P1 promoter activation is expressed as the fold increase relative to the unstimulated control. The data are expressed as the means ± S.E. (n = 3–5).
**FIG. 6.** TGFβ1-induced Smad2 nuclear translocation is modulated by the MEK1/ERK MAPK module. Immunofluorescence was performed on monolayers of HepG2 cells transfected with Smad2 construct and treated as indicated. The cells were fixed, permeabilized, stained with goat anti-Smad2 polyclonal antibody, and then visualized with secondary antibodies coupled to fluorescein isothiocyanate by fluorescence microscopy. A, regions of the confluent monolayer containing representative stained cells are shown (adjacent untransfected cells are revealed only by Evan’s Blue stain). B, graphic representation of three independent experiments expressed as the percentage of positive nuclei following ligand treatment. The data are expressed as the means ± S.E. (n = 3–6). Student t test: *, p = 0.016; **, p < 0.001.

other Ras effectors such as those linked to phosphatidylinositol 3-kinases for basal fur transactivation. Also, it is possible that the inhibition of basal P1 promoter activation by Ras DN is due to interference with basal activation of TGFβ signal transduction pathways. In support of this, recent experiments using TGFβ-neutralizing antibodies have demonstrated that autocrine production of TGFβ accounts for 45% of basal furin P1 promoter activation in the HepG2 cell system (29). The requirement of Ras for TGFβ-induced fur transactivation extends previous study that identified other GTPases of the Rho family as intermediates of TGFβ1-initiated signaling leading to transcriptional activation of a TGFβ reporter construct (p3TP-Lux) in the same cell line (51).

In addition to the fur gene, Ras has been shown to be involved in the expression of other genes/proteins regulated by TGFβ. For example, RasN17 was shown to inhibit the autoinduction of TGFβ1 in lung and intestinal epithelial cells (50), the TGFβ-induced cell cycle-related p27Kip1 and p21 Cip1 in intestinal epithelial cells (52), and TGFβ-induced urokinase expression in transformed keratinocytes (53). In this context, the participation of Ras in the cellular cascade leading to the regulation of the fur gene by TGFβ would be consistent with the view of Ras as an integrator of a wide variety of TGFβ-related growth/differentiation events.

Although Ras is involved in the activation of multiple pathways, we next demonstrated that the p42/p44 MAPK pathway is also involved in TGFβ-induced fur activation. In initial experiments, we have observed that TGFβ1 stimulation of HepG2 cells results in a relatively delayed but sustained p42/p44 MAPK phosphorylation. Next, we observed that a biochemical blockade of p42/p44 MAPK activation or mRNA reduction through antisense technology blocked TGFβ1-induced transcriptional activation of furin P1 promoter and activation of p42/p44 MAPKs. In contrast, MEKα increased unstimulated and TGFβ1-stimulated P1 transactivation. Taken together, these observations provide strong evidence that the Ras-MEK-p42/p44 MAPK signaling plays an important role in the regulation of fur gene expression. In a similar way, the absolute requirement of p42/p44 MAPKs cascade in TGFβ1-induced functions such as the attenuation of haptoglobin gene expression in intestinal epithelial cells has been reported (54).

In our study, the delayed (starting 30 min) and sustained stimulation of p42/p44 MAPK phosphorylation by TGFβ is consistent with a possible indirect mechanism of activation. In fact, most studies using different cell types including phorbol 12-myristate 13-acetate-differentiated THP-1 cells, epithelial cells, or chondrocytes have reported more rapid activation of p44 MAPK occurring within 5–10 min of TGFβ1 addition (49, 55, 56). In human mesangial cells, however, a more delayed (30 min) kinetic of activation has been observed (48). Although the significance of this difference in timing of activation has not been elucidated, Hayashida et al. (48) have ruled out the involvement of new protein synthesis and/or release of platelet-derived growth factor for the delayed activation observed in mesangial cells. In our system, platelet-derived growth factor would unlikely mediate the observed early p42/p44 activation because the kinetic of platelet-derived growth factor production in cells typically occurs later (i.e. at 2–4 h time points) after
TGFβ stimulation (57, 58). However, it would appear logical to propose that autocrine regulation of growth factors by TGFβ accounts for at least some of the signal amplification observed 2–4 h after TGFβ stimulation.

The mechanisms regulating fur expression are not fully understood. In previous studies, we found that TGFβ-induced fur gene regulation occurs at the level of gene transcription (10) and that Smad2 possibly with winged helix transcription factor FAST (FoxH1) participate in this transactivation (29). Here we demonstrate that forced expression of Smad2-Smad4 or MEKA leads to the activation of the fur P1 promoter, mimicking the effect of TGFβ1. In addition, MEKA-induced transactivation was abrogated in cells co-expressing the Smad inhibitor Smad7, and similarly, Smad2-Smad4-induced transactivation was blocked using chemical MEK inhibitors. This argues that both Smad and p42/p44 MAPK pathways are essential for mediating TGFβ1-induced transactivation of furin. In this regard, several cross-talk interactions are possible between the Smad and MAPK pathways, depending on the cellular environment and the targeted biological function (21, 25, 26, 46, 49, 59). As more information is being gathered regarding direct involvement of the stress-activated protein kinase/c-Jun N-terminal kinase pathway in Smad activation, little information is available regarding direct involvement of the p42/p44 MAPK pathway. In our study, we observed that inhibition of MEK by PD98059 blocked most of the enhanced Smad-2 nuclear localization induced by TGFβ. In contrast, activation of p42/p44 MAPKs by activated MEK1 resulted in an enhanced nuclear localization of Smad2. One explanation for this cross-talk is a direct interaction between MEK1 or p42/p44 MAPKs and Smad2. It has been recently demonstrated that growth factors, namely hepatocyte growth factor and epidermal growth factor, can also mediate both Smad-dependent and Smad-independent transcriptional regulation of Smad2 (46). This correlates with an increase of Smad2 phosphorylation that is markedly reduced in the absence of the C-terminal SSXS motif of Smad2, which is the site of TGFβ type I receptor-induced phosphorylation. It has been shown that Smad7 can bind to type I TGFβ1 receptor and inhibits its capacity to phosphorylate Smad2 (22, 23). In our study, the ability of Smad7 to inhibit MEK1-mediated transcripational activation suggests that phosphorylation at the SSXS motif is needed for MEKα-induced activation of Smad2. This does not rule out the possibility that other potential phosphorylation motifs for the proline-directed kinases MEK/p42/p44 MAPK found within the Smad2 linker region may also participate in Smad activation as demonstrated for Smad1 (25, 59).

It was surprising to observe an enhanced Smad2 nuclear localization by MEKA in the absence of exogenous TGFβ stimulation. One possible explanation for this is the induction of autocrine TGFβ production by activated MEK. In support of this, a recent study by Yue and Mulder (50) indicated a requirement of Ras/MAPK pathway for the induction of TGFβ1 by TGFβ. In this context, p42/p44 MAPK activation by MEKA may result in the induction of TGFβ that in turn activates the Smad pathway for an enhanced Smad2 nuclear localization and increased furin expression. Also, because nuclear translocation of the Smad3 proteins was shown to occur through direct binding to the nuclear transporter importin β (60), it would be tempting to speculate that the increase in Smad nuclear translocation comes from direct or indirect modification by activated MEK/p42/p44 kinases of proteins involved in Smad nuclear transport. In this regard, phosphorylation of the importin 58/97 heterodimer by activated CK2 kinases was shown to increase its affinity for the ligand, leading to enhanced nuclear transport of the complex (61).

Evidence is now accumulating favoring a crucial role for furin in various health and disease states including proliferative and inflammatory diseases (10, 62). It was demonstrated that the transcription of the fur gene can be regulated by TGFβ in several cells generating an enzyme/substrate amplification loop that leads to an increase in local concentrations of TGFβ1 (10). Also, Hoshino et al. (63) have demonstrated changes in the expression of furin and TGFβ in regulating and differentiating hepatocytes as well as developmental changes in furin expression in rat pancreatic islets (64). These observations suggested that furin concentrations could be regulated in growth/differentiation events leading to increased bioactive growth-related factors. In addition to members of the vast TGFβ family, these precursor proteins include, among others, several key growth factor precursors such as platelet-derived growth factor A and B chains, growth factor proreceptors such as the insulin receptor and the hepatocyte growth factor receptor (c-Met), several integrin α-subunits, and cadherin family members that share a common RXK/RK furin recognition motif at the junction between the prorregion and the mature polypeptide (65). The findings outlined in the present study support the involvement of MEK/p42/p44 MAPK signaling in TGFβ1-induced and Smad-regulated furin expression. The cross-talk between these two signaling pathways may serve as a growth/differentiation integration signal involved in the bioavailability of a multitude of furin-activated precursors, especially in developmental and physiopathological conditions where temporal or sustained increase in furin substrates were found coupled with changes in cell proliferation/differentiation events. Our current model is depicted in Fig. 7.

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Cross-talk between the p42/p44 MAP Kinase and Smad Pathways in Transforming Growth Factor β1-induced Furin Gene Transactivation
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