Regulation of Transforming Growth Factor- β 1-Dependent Integrin β 6 Expression by p38 Mitogen-Activated Protein Kinase in Bile Duct Epithelial Cells

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ABSTRACT

Bile duct epithelial cells (BDECs) contribute to liver fibrosis by expressing α V β 6 integrin, a critical activator of latent transforming growth factor β (TGF- β). β 6 integrin (Itg β 6) mRNA induction and α V β 6 integrin expression in BDECs are partially TGF- β -dependent. However, the signaling pathways required for TGF- β -dependent Itg β 6 mRNA induction in BDECs are not known. We tested the hypothesis that the p38 mitogen-activated protein kinase (MAPK) signaling pathway contributes to TGF- β 1 induction of Itg β 6 mRNA by activating SMAD and activator protein 1 (AP-1) transcription factors. Pretreatment of transformed human BDECs (MMNK-1 cells) with two different p38 MAPK inhibitors, but not a control compound, inhibited TGF- β 1 induction of Itg β 6 mRNA. Inhibition of p38 also reduced

TGF- β 1 activation of a SMAD-dependent reporter construct. Expression of a dominant-negative SMAD3 (SMAD3 Δ C) significantly reduced TGF- β 1-induced Itg β 6 mRNA expression. Expression of JunB mRNA, but not other AP-1 proteins, increased in TGF- β 1-treated MMNK-1 cells, and induction of JunB expression was p38-dependent. Consistent with a requirement for de novo induction of JunB protein, cycloheximide pretreatment inhibited TGF- β 1 induction of Itg β 6 mRNA. Expression of a dominant-negative AP-1 mutant (TAM67) also inhibited TGF- β 1 induction of Itg β 6 mRNA. Overall, the results suggest that p38 contributes to TGF- β 1-induced Itg β 6 mRNA expression in MMNK-1 cells by regulating activation of both SMAD and AP-1 transcription factors.

Introduction

Bile duct epithelial cells (BDECs) are injured chronically in cholestatic liver diseases such as primary sclerosing cholangitis and primary biliary cirrhosis. In addition to being targets of disease processes, it is increasingly clear that BDECs actively participate in the pathogenesis of cholestatic liver disease by producing proinflammatory and profibrogenic mediators such as transforming growth factor β 1 (TGF- β 1) and the α V β 6 integrin (Sedlacek et al., 2001; Hahm et al., 2007;

Sullivan et al., 2010). These mediators stimulate other cell types including portal fibroblasts to produce collagen, leading to liver fibrosis (Bataller and Brenner, 2005).

The α V β 6 integrin is selectively expressed by epithelial cells in multiple tissues and plays a role in physiological processes such as fetal development and wound healing (Breuss et al., 1995), as well as pathological processes including tumor cell invasion and fibrosis (Marsh et al., 2008; Patsenker et al., 2008). Most notably, the α V β 6 integrin binds to and facilitates the activation of latent TGF- β 1 (Munger et al., 1999), a cytokine and important profibrogenic mediator (Bataller and Brenner, 2005). Several studies using mice deficient in the β 6 integrin (Itg β 6) subunit have demonstrated a crucial role for this integrin in the activation of TGF- β 1 during fibrosis induced by chronic tissue injury. For example, in rodent models of lung and liver fibrosis, Itg β 6 deficiency reduced the deposition of extracellular matrix in these tissues (Jenkins et al., 2006; Hahm et al., 2007).

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ABBREVIATIONS: BDEC, bile duct epithelial cell; TGF- β , transforming growth factor β ; Itg β 6, β 6 integrin; MAPK, mitogen-activated protein kinase; AP-1, activator protein 1; qPCR, quantitative polymerase chain reaction; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; BSA, bovine serum albumin; TBST, 50 mM Tris, 150 mM NaCl, 0.1% Tween 20, pH 7.4; siRNA, small interfering RNA; ATF-2, activating transcription factor 2; CMV, cytomegalovirus; SB203580, 4-[4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-1H-imidazol-5-yl]pyridine; SB202190, 4-[4-(4-fluorophenyl)-5-(4-pyridinyl)-1H-imidazol-2-yl]phenol; SB202474, 4-ethyl-2(p-methoxyphenyl)-5-(4'-pyridyl)-1H-imidazole.

The *Itgβ6* gene, which encodes the limiting subunit of the α Vβ6 integrin, is expressed at low levels in normal liver. However, in rodent models of cholestasis, levels of both hepatic *Itgβ6* mRNA and α Vβ6 protein are increased (Hahm et al., 2007; Patsenker et al., 2008; Popov et al., 2008; Sullivan et al., 2010), and α Vβ6 protein expression colocalizes with BDECs (Hahm et al., 2007; Patsenker et al., 2008; Sullivan et al., 2010). Various genetic and pharmacologic interventions targeting the α Vβ6 integrin have been shown to reduce the activation of TGF-β1 and fibrosis in mice and rats during cholestasis (Jenkins et al., 2006; Patsenker et al., 2008; Sullivan et al., 2010). Taken together, these studies suggest that the induction of *Itgβ6* expression is a critical step in the fibrogenic response associated with chronic cholestasis. However, the mechanism of *Itgβ6* mRNA induction in BDECs is not known.

We have shown previously that neutralizing TGF-β reduces *Itgβ6* mRNA expression during cholestasis (Sullivan et al., 2010), suggesting the presence of a feed-forward amplification loop of TGF-β activation. Of importance, the mechanism whereby TGF-β regulates *Itgβ6* in BDECs is not completely understood. Mature TGF-β1 binds its type II receptor, which is expressed by BDECs (Lu et al., 2003). This binding event initiates downstream canonical signaling involving activation of TGF-β type I receptor, C-terminal phosphorylation of the regulatory (R-)SMAD transcription factors (SMAD2 and SMAD3), the heterodimerization of these proteins with SMAD4, and translocation to the nucleus where this complex regulates transcription (Derynck and Zhang, 2003). The importance of SMAD-dependent transcription in fibrosis has been demonstrated previously (Schnabl et al., 2001; Zhao et al., 2002). Of importance, TGF-β-induced *Itgβ6* mRNA in keratinocytes is SMAD4-dependent (Levy and Hill, 2005). TGF-β1 also activates noncanonical signaling pathways such as the p38 mitogen-activated protein kinase (MAPK), which can regulate gene expression through activation of downstream transcription factors such as activator protein 1 (AP-1). Noncanonical signaling pathways have been shown to be involved in profibrogenic gene induction (Shaulian and Karin, 2001; Derynck and Zhang, 2003; Javelaud and Mauviel, 2005). However, the involvement of the p38 MAPK pathway in the regulation of *Itgβ6* gene induction has not been determined.

This study aimed to investigate the signaling mechanisms of TGF-β1-dependent expression of the profibrogenic β6 integrin in BDECs. We used transformed human BDECs (MMNK-1 cells) to test the hypothesis that TGF-β1 activation of the SMAD and p38 MAPK signaling pathways coordinate the induction of the β6 integrin mRNA.

Materials and Methods

Antibodies and Reagents. TGF-β1 (PeproTech, Rocky Hill, NJ), 4-[4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-1H-imidazol-5-yl]pyridine (SB203580) (LC Laboratories, Woburn, MA), 4-[4-(4-fluorophenyl)-5-(4-pyridinyl)-1H-imidazol-2-yl]phenol (SB202190) and 4-ethyl-2(p-methoxyphenyl)-5-(4'-pyridyl)-1H-imidazole (SB202474) (EMD Chemicals, Gibbstown, NJ), cycloheximide (Sigma-Aldrich, St. Louis, MO), and dimethyl sulfoxide (DMSO; Research Organics, Cleveland, OH) were used to treat cultured cells. SMAD (SBE4; SABiosciences, Frederick, MD) and AP-1 (pAP-1-Luc; Stratagene, La Jolla, CA) responsive luciferase reporter constructs mixed 40:1 with renilla transfection control vector (pRL-CMV; Pro-

mega, Madison, WI), Fugene 6 transfection reagent (Roche Diagnostics, Indianapolis, IN), and a Dual-glo luciferase assay kit (Promega) were used for luciferase activity experiments. Dominant-negative SMAD2 (SMAD2ΔC) and SMAD3 (SMAD3ΔC) mutant as well as a control (pRK7) plasmid were obtained from addgene (Cambridge, MA), donated by Dr. Rik Derynck (Zhang et al., 1996). A dominant-negative AP-1 (TAM67) mutant and control (pCMV) plasmids were a gift from Dr. Michael J. Soares (University of Kansas Medical Center, Kansas City, KS) (Brown et al., 1993). A TriPecta RNAi kit (Integrated DNA Technologies, Inc., Coralville, IA) containing ATF-2 siRNA, control siRNA, and triFECTin transfection reagent were used for siRNA knockdown of ATF-2. TRI reagent (Molecular Research Center, Cincinnati, OH), High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA), and a MyCycler thermal cycler (Bio-Rad Laboratories, Hercules, CA) were used for the isolation of RNA and synthesis of cDNA. TaqMan gene expression assays, TaqMan master mix, and a StepOnePlus sequence detection analyzer (Applied Biosystems) were used for quantitative PCR (qPCR). Primary antibodies including rabbit anti-human p38, phospho-p38 (Thr180/Tyr182), (12F8) rabbit monoclonal antibody, SMAD2, phospho-SMAD2 (Ser465/Ser467), SMAD3, phospho-SMAD3 (Ser423/425), ATF-2, phospho-ATF2 (Thr71), JunB, and histone H3 (Cell Signaling Technology, Danvers, MA) were used for Western blotting and immunoprecipitation. Horseradish peroxidase-conjugated anti-rabbit secondary (Pierce/Thermo Fisher Scientific, Waltham, MA) antibody was used for Western blotting, and chromatin immunoprecipitation-grade protein G-conjugated magnetic beads were used for immunoprecipitation (Cell Signaling Technology).

Cell Culture and Treatments. MMNK-1 cells were kindly provided by Dr. Melissa Runge-Morris (Wayne State University, Detroit, MI), on behalf of Dr. Naoya Kobayashi (Okayama University, Okayama, Japan). MMNK-1 cells were maintained at 37°C at 5% CO₂ in Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich), 10 U/ml penicillin, and 10 μg/ml streptomycin (Sigma-Aldrich) in 75-cm² flasks (ISC BioExpress, Kaysville, UT) and were routinely passaged with 0.5% trypsin (Sigma-Aldrich) after cells reached 95% confluence. Before treatment, MMNK-1 cells were grown to 70% confluence and serum-starved for 1 h before stimulation with 5 ng/ml TGF-β1 or its vehicle [0.1% BSA (Thermo Fisher Scientific) in endotoxin-free PBS (Sigma-Aldrich)]. For inhibitor studies, MMNK-1 cells were serum-starved for 30 min, and then pretreated with 10 μM SB203580, SB202190, or SB202474 (LC Laboratories), 10 μg/ml cycloheximide (Sigma-Aldrich), or DMSO vehicle (final concentration, 0.1%) for an additional 30 min before treatment with 5 ng/ml TGF-β1 or vehicle. For dominant-negative mutant studies, cells were grown to 50% confluence, transfected with 1 μg of plasmid using Fugene6 transfection reagent, allowed to incubate for 24 h to reach 70% confluence, and treated as described above. For ATF-2 siRNA studies, cells were grown to 30% confluence, transfected with 10 nM control siRNA or ATF-2 siRNA (Integrated DNA Technologies, Inc.) using triFECTin transfection reagent (Integrated DNA Technologies, Inc.) according to the manufacturer's protocol, and allowed to incubate for 48 h to reach 70% confluence before treatment.

RNA Isolation, cDNA Synthesis, and Real-Time PCR. RNA was isolated from adherent cells using TRI reagent according to the manufacturer's protocol (Molecular Research Center). One microgram of total RNA was used for the synthesis of cDNA using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems) on a MyCycler thermal cycler (Bio-Rad Laboratories). Levels of *Itgβ6*, glyceraldehyde-3-phosphate dehydrogenase, ATF-2, and JunB mRNAs were determined using TaqMan gene expression assays and TaqMan gene expression master mix on a StepOnePlus sequence detection system (Applied Biosystems). The relative expression levels of *Itgβ6*, ATF-2, and JunB mRNAs were determined using the comparative Ct method using glyceraldehyde-3-phosphate dehydrogenase mRNA as the endogenous control.

Cytosolic and Nuclear Sample Preparation, Immunoprecipitation, Western Blotting, and Densitometry. Cells were harvested by scraping into ice-cold PBS and pelleted by centrifugation at 3500g at 4°C for 5 min. The cell pellet was then resuspended in lysis buffer (10 mM HEPES, 10 mM KCl, 300 mM sucrose, 1.5 mM MgCl₂, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 0.1% NP-40) containing protease and phosphatase inhibitors (Roche Diagnostics) and incubated for 10 min on ice. Nuclei were pelleted from lysate by centrifugation at 3500g for 10 min at 4°C, and supernatant was saved as the cytosolic fraction. Nuclei were then resuspended in nuclear lysis buffer (20 mM HEPES, 100 mM KCl, 100 mM NaCl, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride in 20% glycerol) for 30 min on ice. Nuclear debris was cleared by centrifugation at 20,000g for 2 min, and supernatant was saved as the nuclear fraction. Protein concentration was determined using a commercially available kit (D_C protein assay; Bio-Rad Laboratories) according to the manufacturer's protocol. For immunoprecipitation, 100 μ g of total nuclear protein was incubated with SMAD3 antibody 1:100 (Cell Signaling Technology) overnight with mild rotation at 4°C. Five micrograms of protein was diluted into 2 $\times$ NuPage LDS buffer containing 2.5% 2-mercaptoethanol (2 $\times$ LDS; Invitrogen, Carlsbad, CA) and saved as the input fraction. Magnetic protein G-conjugated beads were then added to the remaining sample and incubated at 4°C for 2 h. The magnetic beads were separated and immobilized using a magnetic tube rack, and the samples were then washed five times in 1 ml of nuclear lysis buffer. The magnetic beads were separated and resuspended into 2 $\times$ LDS, and samples were eluted at 95°C for 5 min before Western blotting. All Western blotting samples were denatured and reduced in 2 $\times$ LDS for 5 min at 95°C, separated by SDS-polyacrylamide gel electrophoresis using Criterion XT precast 4 to 12% Bis-Tris gels and XT MOPS running buffer (Bio-Rad Laboratories), and transferred onto polyvinylidene difluoride membranes (Millipore Corporation, Billerica, MA). Membranes were blocked for 60 min with 3% BSA in TBST (50 mM Tris, 150 mM NaCl, 0.1% Tween 20, pH 7.4). Membranes were then incubated overnight with primary antibodies diluted 1:1000 for p38, phospho-p38 (Thr183/Tyr185), phospho-SMAD2 (Ser465/Ser467), SMAD2, phospho-SMAD3 (Ser423/425), SMAD3, ATF-2, phospho-ATF2 (Thr71), JunB, or histone H3 (Cell Signaling Technology) in 1% BSA in TBST at 4°C. Membranes were washed six times in TBST for 5 min and then incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody diluted 1:1000 in 1% BSA in TBST for 1 h at room temperature. Membranes were washed six additional times, then incubated with Super Signal West Pico Chemiluminescence Substrate Solution (Thermo Fisher Scientific) and exposed to blue autoradiography film (ISC BioExpress). Developed films were scanned using an Epson Expression 1680 scanner (Epson America, Torrance, CA), and images were analyzed using Gel-Pro Analyzer 32 (Media Cybernetics, Inc., Bethesda, MD). For densitometry, a ratio of phosphorylated p38 or ATF-2 to the respective bands indicating total p38 or ATF-2 protein was generated.

Luciferase Assays. MMNK-1 cells grown to 50% confluence were transfected using Fugene 6 transfection reagent with 1 μ g of SMAD-responsive (SBE4) or AP-1-responsive (pAP-1-Luc) luciferase reporter constructs, each mixed 40:1 with control renilla construct. After 16 h of transfection, cells were treated with 5 ng/ml TGF- β 1 as described above. Luciferase and renilla activities were determined using a Dual-Glo luciferase kit (Promega) according to the manufacturer's protocol and read by an Infinite M200 plate reader (Tecan, Durham, NC). For each sample, luciferase relative light units were adjusted based on renilla activity as an estimation of transfection efficiency.

Statistics. Comparison of the effect of interventions with dominant-negative proteins on TGF- β -induced Itgb6 mRNA and luciferase reporter expression was made using a paired *t* test. For these experiments, at least three independent experiments were performed. Each individual experiment used a separate passage (i.e., split) of MMNK-1 cells. For each experimental replicate, MMNK-1

cells derived from the same flask (i.e., a single split) were distributed identically into wells of six-well culture plates, and "control" and "experimental" treatments were performed in parallel from a single cell passage. By design, comparing control and experimental groups using the paired *t* test is appropriate (Motulsky, 1995). Comparison of three or more groups was performed using an analysis of variance and Student-Neuman-Keuls post hoc test. The criterion for statistical significance was *P* < 0.05.

Results

TGF- β 1 Induction of Itgb6 mRNA in MMNK-1 Cells Is SMAD-Dependent. TGF- β 1 increased Itgb6 mRNA in human transformed BDECs (MMNK-1) (Fig. 1A). The cellular response to TGF- β is elicited in part through the canonical signaling pathway via the R-SMAD transcription factors, SMAD2 and SMAD3 (Derynck and Zhang, 2003). Stimulation of MMNK-1 cells with TGF- β 1 also increased nuclear levels of C-terminal phosphorylated SMAD2 and SMAD3 (Fig. 1B). TGF- β 1 treatment of MMNK-1 cells increased activation of a SMAD-dependent reporter construct (Fig. 1C). To test the hypothesis that TGF- β 1-dependent Itgb6 mRNA induction is SMAD-dependent, we used dominant-negative SMAD2 (SMAD2 Δ C) and SMAD3 (SMAD3 Δ C) mutants (Zhang et al., 1996). Transfection of MMNK-1 cells with SMAD3 Δ C significantly reduced (~25%) TGF- β 1-dependent induction of Itgb6 mRNA (Fig. 1D). However, the inhibition (~15%) of TGF- β 1-induced Itgb6 mRNA by expression of SMAD2 Δ C did not achieve statistical significance (Fig. 1E; *P* = 0.108). The data suggest SMAD3 is involved in TGF- β -induced Itgb6 mRNA expression in MMNK-1 cells.

Activation of p38 MAPK Contributes to TGF- β 1-Dependent Induction of Itgb6 mRNA in MMNK-1 Cells. TGF- β can also signal through SMAD-independent, noncanonical signaling pathways, leading to activation of the p38 MAPK (Derynck and Zhang, 2003). Treatment of MMNK-1 cells with TGF- β 1 increased levels of phosphorylated p38 MAPK (Fig. 2, A and B), an indicator of p38 MAPK activation. To determine the role of the p38 MAPK signaling pathway in TGF- β 1-dependent induction of Itgb6 mRNA, we used two selective pharmacologic inhibitors of p38 (SB203580 and SB202190). Pretreatment of MMNK-1 cells with SB203580 significantly reduced Itgb6 mRNA expression in MMNK-1 cells treated with TGF- β 1 by 40% (Fig. 2C). Likewise, pretreatment with a more potent p38 MAPK inhibitor, SB202190, significantly reduced Itgb6 mRNA expression in MMNK-1 cells treated with TGF- β 1 by 70% (Fig. 2D). In contrast, pretreatment with a structurally related negative control compound for these two inhibitors (SB202474) had no effect (Fig. 2E). The results indicate that p38 is involved in TGF- β 1-dependent induction of Itgb6 mRNA in MMNK-1 cells.

Activation of AP-1 Contributes to TGF- β 1 Induction of Itgb6 mRNA in MMNK-1 Cells. Activation of p38 MAPK directs transcriptional regulation in part through activation of AP-1 transcription factor family members, including FosB, ATF-2, JunB, and c-Jun (Kumar et al., 1997; Shaulian and Karin, 2001; Kumar et al., 2007). Indeed, in MMNK-1 cells stimulated with TGF- β 1, AP-1-dependent luciferase activity was increased (Fig. 3A). Of importance, MMNK-1 cells expressing a dominant-negative AP-1 mutant (TAM67), which inhibits all AP-1 family members (Brown et al., 1994), attenuated (~20%) the TGF- β 1-dependent induction of Itgb6

Fig. 1. Role of SMAD 3 in TGF- β 1-induced Itg β 6 mRNA expression in MMNK-1 cells. A, MMNK-1 cells were stimulated with 5 ng/ml TGF- β 1 or vehicle (0.1% BSA in PBS) for 4 h. Itg β 6 mRNA levels were determined by qPCR. B, MMNK-1 cells were treated with 5 ng/ml TGF- β 1 or vehicle for various times. Levels of C-terminally phosphorylated SMAD2 and SMAD3, total SMAD2, and total SMAD3 levels were determined in nuclear extracts by Western blotting. A representative Western blot is shown. C, MMNK-1 cells transiently transfected with a SMAD-responsive luciferase reporter construct (SBE4) were stimulated with 5 ng/ml TGF- β 1 or vehicle for 4 h and lysed, and luciferase activity was determined. Data are expressed as fold change $\pm$ S.E.M. compared with vehicle-treated MMNK-1 cells. D and E, MMNK-1 cells transiently transfected with SMAD3 Δ C (D) or SMAD2 Δ C (E) mutants were treated with 5 ng/ml TGF- β 1 or vehicle for 4 h, and Itg β 6 mRNA levels were determined by qPCR. Data are expressed as mean $\pm$ S.E.M. relative to vehicle-treated cells transfected with an empty control plasmid (pRK7) from at least three independent experiments. *, significantly different from 0.1% BSA vehicle-treated group, $P < 0.05$. #, significantly different from cells transfected with control plasmid, $P < 0.05$.

Fig. 2. Role of p38 MAPK in TGF- β 1-induced Itg β 6 mRNA expression in MMNK-1 cells. MMNK-1 cells were treated with 5 ng/ml TGF- β 1 or vehicle for 10, 30, or 60 min. A, representative Western blot showing phosphorylated p38 and total p38 levels. B, densitometry was performed on Western blots from three independent experiments. Data are expressed as a ratio of phosphorylated p38 to total p38 relative to vehicle-treated cells at 10 min. C to E, MMNK-1 cells were pretreated with 10 μ M SB203580 (C), 10 μ M SB202190 (D), 10 μ M SB202474 (E), or DMSO vehicle (0.1% final concentration) (C–E) for 30 min and then treated with 5 ng/ml TGF- β 1 or vehicle for 4 h. Itg β 6 mRNA levels were determined by qPCR. *, significantly different from 0.1% BSA vehicle-treated cells, $P < 0.05$. #, significantly different from cells pretreated with DMSO, $P < 0.05$. Data are expressed as mean $\pm$ S.E.M. from at least three independent experiments.

mRNA (Fig. 3B). The data indicate that AP-1 contributes to Itg β 6 mRNA induction in MMNK-1 cells.

ATF-2 Does Not Contribute to TGF- β 1 Induction of Itg β 6 mRNA in MMNK-1 Cells. ATF-2 is an AP-1 family member subject to phosphorylation and activation by p38 MAPK. TGF- β -dependent activation of p38 MAPK and subsequent phosphorylation of ATF-2 is reduced after treatment with SB203580 (Edlund et al., 2003). It is noteworthy that ATF-2 has been shown to bind SMAD3 and increase SMAD-dependent transcriptional activity (Kumar et al., 1997; Yamamura et al., 2000; Ionescu et al., 2003). TGF- β 1 treatment increased the levels of phosphorylated ATF-2 in MMNK-1 cells (Fig. 4, A and B), but did not increase ATF-2

mRNA levels (Fig. 4E). Pretreatment with SB203580 reduced the levels of phosphorylated ATF-2 in two independent experiments (data not shown). Coimmunoprecipitation experiments revealed an increased association of SMAD3 and ATF-2 in the nucleus of MMNK-1 cells treated with TGF- β 1 (Fig. 4C). It is noteworthy that SB203580 pretreatment reduced TGF- β 1-dependent SMAD luciferase activity by approximately 40% (Fig. 4D). To determine whether ATF-2 contributes to Itg β 6 mRNA induction, an siRNA approach was used. ATF-2 mRNA (Fig. 4E) and protein (Fig. 4F) levels were reduced in MMNK-1 cells transfected with ATF-2 siRNA compared with a length-matched control siRNA that has no complementary human sequence. Of importance,

Fig. 3. Role of AP-1 in TGF- β 1-dependent Itg β 6 mRNA induction in MMNK-1 cells. **A**, MMNK-1 cells transfected with an AP-1 luciferase reporter construct (pAP-1-Luc) were treated with 5 ng/ml TGF- β 1 or vehicle for 4 h and lysed, and luciferase activity was determined. **B**, MMNK-1 cells were transfected with a plasmid containing a dominant-negative AP-1 (TAM67) mutant for 24 h and then treated with 5 ng/ml TGF- β 1 or vehicle for 4 h. Itg β 6 mRNA levels were determined by qPCR. *, significantly different from 0.1% BSA vehicle-treated cells, $P < 0.05$. #, significantly different from cells transfected with a control plasmid (pCMV4), $P < 0.05$. Data are expressed as mean $\pm$ S.E.M. relative to vehicle-treated cells from three independent experiments.

Fig. 4. Role of ATF-2 in TGF- β 1-dependent Itg β 6 mRNA induction in MMNK-1 cells. **A**, MMNK-1 cells were treated with 5 ng/ml TGF- β 1 or vehicle (0.1% BSA in PBS) for 10 or 20 min. A representative Western blot indicating levels of phosphorylated ATF-2 and total ATF-2 is shown. **B**, densitometry was performed on Western blots from three independent experiments. Data are expressed as a ratio of phosphorylated ATF-2 to total ATF-2 relative to vehicle-treated cells. *, significantly different from 0.1% BSA vehicle-treated cells, $P < 0.05$. **C**, MMNK-1 cells were treated with 5 ng/ml TGF- β 1 or vehicle for 30 min and nuclear proteins were collected. SMAD3 immunoprecipitation was performed and levels of coimmunoprecipitated ATF-2 were determined by Western blot. A representative Western blot is shown. **D**, MMNK-1 cells transfected with a SMAD-dependent reporter construct were pretreated with 10 μ M SB203580 or vehicle (0.1% DMSO vehicle) for 30 min before treatment with 5 ng/ml TGF- β 1 for 4 h and lysed, and luciferase activity was determined. Data are expressed as fold change $\pm$ S.E.M. compared with DMSO-treated cells. *, significantly different from DMSO-treated cells, $P < 0.05$. **E** to **G**, MMNK-1 cells were transfected with an ATF-2 siRNA or control siRNA for 48 h before treatment with 5 ng/ml TGF- β 1 or vehicle for 4 h. ATF-2 mRNA (**E**), ATF-2 protein (**F**), and Itg β 6 mRNA (**G**) levels were determined. Representative Western blots are shown. Data are expressed as mean $\pm$ S.E.M. relative to vehicle-treated cells from three independent experiments. **E**, *, significantly different from the same group treated with control siRNA, $P < 0.05$. **G**, *, significantly different from the same group treated with 0.1% BSA vehicle, $P < 0.05$.

ATF-2 knockdown did not affect TGF- β 1-dependent induction of Itg β 6 mRNA in MMNK-1 cells (Fig. 4G). Although ATF-2 interacts with SMAD3 after TGF- β 1 treatment, the data suggest that ATF-2 is not required for TGF- β 1-induced Itg β 6 mRNA expression in MMNK-1 cells.

TGF- β 1 Increases JunB Expression in MMNK-1 Cells in a p38-Dependent Manner. In a transcription factor binding enzyme-linked immunosorbent assay screen (TransAM; Active Motif Inc., Carlsbad, CA) to identify other AP-1 family members that could contribute to TGF- β -induced Itg β 6 mRNA expression, we found that TGF- β 1 treatment increased the nuclear levels of JunB, but not FosB, c-Fos, Fra-1, c-Jun-P(S73),

or JunD (data not shown). Validating this result, we found that JunB mRNA and protein increased in TGF- β 1-treated MMNK-1 cells at 30 min (Fig. 5A) and 120 min (Fig. 5B), respectively. Of importance, pretreatment of MMNK-1 cells with SB203580 prevented the induction of JunB mRNA (Fig. 5C) and the accumulation of nuclear JunB protein (Fig. 5D). These results suggest that p38-dependent JunB induction could be a mechanism whereby TGF- β 1 contributes to Itg β 6 mRNA expression. It is noteworthy that pretreatment with the protein synthesis inhibitor cycloheximide reduced the TGF- β 1-dependent induction of Itg β 6 mRNA in MMNK-1 cells by approximately 30% (Fig. 5E). The data suggest that

Fig. 5. TGF- β 1-dependent Itg β 6 mRNA induction requires protein synthesis in MMNK-1 cells. MMNK-1 cells were treated with 5 ng/ml TGF- β 1 or vehicle for various times. A and B, JunB mRNA (A) and nuclear protein (B) levels were determined. C and D, MMNK-1 cells were pretreated with 10 μ M SB203580 or vehicle (0.1% DMSO) for 30 min before treatment with 5 ng/ml TGF- β 1 for 30 min (C) or 2 h (D), and mRNA (C) and nuclear protein (D) levels were determined. E, MMNK-1 cells were pretreated with 10 μ M cycloheximide (CHX) or vehicle (0.1% DMSO) for 30 min before treatment with 5 ng/ml TGF- β 1 or vehicle for 4 h, and Itg β 6 mRNA levels were determined by qPCR. Data are expressed as mean $\pm$ S.E.M. from three independent experiments. *, significantly different from 0.1% BSA vehicle-treated cells, $P < 0.05$. #, significantly different from cells pretreated with DMSO, $P < 0.05$.

p38 MAPK contributes to TGF- β 1-induced JunB mRNA and increased nuclear protein levels. Furthermore, full TGF- β 1-dependent Itg β 6 mRNA induction requires de novo protein synthesis in MMNK-1 cells.

Discussion

The α V β 6 integrin has been shown to facilitate activation of latent TGF- β 1 (Munger et al., 1999), which contributes to fibrosis in multiple models (Hahm et al., 2007; Popov et al., 2008; Sullivan et al., 2010). Cholestatic liver injury is associated with increased expression of Itg β 6 mRNA in liver and expression of α V β 6 by BDECs. Induction of mRNA encoding the β 6 subunit is probably a critical limiting factor for the surface expression of the α V β 6 integrin (Breuss et al., 1995). To this end, identifying the mediators and pathways required for the expression of this gene in BDECs could reveal novel targets for the treatment of liver fibrosis.

In agreement with our previous study (Sullivan et al., 2010), we found that treatment of MMNK-1 cells with TGF- β 1 increased Itg β 6 mRNA expression. It is initially counterintuitive to consider active TGF- β 1 as a key player in the induction of a gene essential for activation of its own latent form. However, it should be noted that there are multiple mechanisms whereby TGF- β 1 can become activated. For instance, other factors such as thrombospondin-1 may play a role in the initial activation of latent TGF- β 1 (Crawford et al., 1998). Moreover, there may be low basal levels of surface α V β 6 integrin expression in normal liver. Finally, it is highly likely that more than one mediator contributes to Itg β 6 mRNA expression during cholestasis. Of importance, the induction of the limiting β 6 subunit mRNA by active TGF- β 1 may form the basis of an amplification loop whereby TGF- β 1 drives the further activation of latent TGF- β 1 and ultimately liver fibrosis. Supporting this hypothesis are data from a previous study suggesting that inhibition of TGF- β signaling in vivo reduces Itg β 6 mRNA and α V β 6 expression in BDECs (Sullivan et al., 2010). In addition, pharmacologic inhibition of α V β 6 integrin reduces fibrosis in several rodent

models of cholestasis (Wang et al., 2007a; Patsenker et al., 2008; Sullivan et al., 2010). These studies further suggest that α V β 6 integrin is a key player in the development of fibrosis and enhance the argument for developing therapies targeting the α V β 6 integrin for the treatment of cholestasis-induced liver fibrosis.

TGF- β 1-induced intracellular signaling elicits SMAD-dependent gene induction in multiple cell types (Derynck and Zhang, 2003), including BDECs (Luo et al., 2005). Of importance, the pathogenesis of liver fibrosis in rodent models has been shown to involve SMAD-dependent induction of various profibrogenic genes including *PAI-1* and *CTGF* (Holmes et al., 2001; Schnabl et al., 2001; Levy and Hill, 2005; Wang et al., 2007b). A study has demonstrated that SMAD4 deficiency reduced TGF- β 1-dependent induction of Itg β 6 expression in keratinocytes (Levy and Hill, 2005). Likewise, we found that inhibition of SMAD3 reduced the TGF- β 1-dependent mRNA induction of Itg β 6. It is noteworthy that various genes have shown differential dependence on SMAD2 and SMAD3 for their induction. For example, TGF- β -dependent induction of matrix metalloproteinase-2 has been shown to be SMAD2-dependent, whereas connective tissue growth factor is SMAD3-dependent, and α -smooth muscle actin relies on both SMAD2 and SMAD3 for induction (Holmes et al., 2001; Phanish et al., 2006). Although the use of a dominant-negative SMAD2 showed the same inhibitory trend on TGF- β 1-dependent Itg β 6 mRNA induction, this did not meet the level of significance. Further studies are needed to clarify the individual roles of SMAD2 and SMAD3 in the induction of Itg β 6 by TGF- β 1 in BDECs.

It is increasingly clear that, in addition to activation of the SMAD transcription factors, TGF- β 1 activates other signaling pathways, including p38 MAPK (Derynck and Zhang, 2003; Ohshima and Shimotohno, 2003). Indeed, TGF- β 1 stimulation of MMNK-1 cells activated the p38 MAPK signaling pathway, and pretreatment of MMNK-1 cells with inhibitors of the p38 MAPK significantly reduced both TGF- β 1-dependent Itg β 6 mRNA expression and SMAD-luciferase reporter activation. The finding that two independent phar-

macologic inhibitors of p38, but not a structurally related negative control compound, reduced TGF- β 1-dependent Itg β 6 mRNA expression constitutes strong evidence of p38 involvement in Itg β 6 induction in MMNK-1 cells. However, we cannot exclude the possibility that “off target” effects of these inhibitors (Bain et al., 2007; Shanware et al., 2009) contribute to their activity in MMNK-1 cells.

Of importance, TGF- β 1 activation of the p38 MAPK has been shown to modify gene expression changes via cooperative enhancement of SMAD activity through multiple mechanisms. For example, TGF- β 1-dependent p38 MAPK activation stimulates sumoylation of SMAD4, promoting SMAD4 stabilization and sustaining transcriptional activity (Ohshima and Shimotohno, 2003). Alternatively, p38 can regulate AP-1 transcription factor components such as ATF-2, which have been shown to modify SMAD transcriptional activity by directly binding SMAD3 and SMAD4 (Kumar et al., 1997; Yamamura et al., 2000; Ionescu et al., 2003). Although we observed an association of ATF-2 with SMAD3 in TGF- β 1-treated MMNK-1 cells, knockdown of ATF-2 did not affect TGF- β 1-dependent Itg β 6 mRNA induction.

Other AP-1 family members such as Fos and Jun can alter transcriptional activation of TGF- β target genes (Liboi et al., 1988; Laiho et al., 1991). Of importance, inhibition of AP-1 by the expression of a dominant-negative AP-1 mutant (TAM67), which inhibits all AP-1 family members, reduced TGF- β 1-dependent Itg β 6 mRNA expression. The relatively modest inhibition of Itg β 6 mRNA expression by the dominant-negative AP-1 suggests that AP-1 activation by p38 is one of several pathways that contribute to Itg β 6 mRNA induction. Indeed, p38 activation of the SMAD3 transcription factor may be a parallel pathway regulating Itg β 6 mRNA induction, because the dominant-negative SMAD3 protein also reduced Itg β 6 mRNA induction.

In a screen to identify AP-1 family members that could be TGF- β 1-sensitive, only nuclear levels of JunB were increased in TGF- β 1-treated MMNK-1 cells. It is noteworthy that JunB has been shown to be induced in response to TGF- β stimulation (Coussens et al., 1994; Shaulian and Karin, 2001). JunB can also bind to SMAD3, and its expression is essential for TGF- β -dependent induction of select genes (Verrecchia et al., 2001; Selvamurugan et al., 2004). Indeed, TGF- β 1 stimulation of MMNK-1 cells increased JunB mRNA and nuclear protein levels in a p38-dependent manner. It is important to note that the induction of JunB mRNA and nuclear protein accumulation occurred before the induction of Itg β 6 mRNA, suggesting a requirement for JunB induction. Likewise, inhibition of protein synthesis reduced Itg β 6 mRNA expression, further suggesting that a key step in Itg β 6 mRNA induction is the de novo synthesis of this key regulatory protein. However, further studies are required to elucidate the mechanism whereby individual AP-1 family members participate in TGF- β 1-induced Itg β 6 mRNA expression.

In summary, TGF- β 1 increased the expression of Itg β 6 mRNAs in transformed human BDECs. Itg β 6 induction required activation of the p38 MAPK signaling pathway and also depended on AP-1 and SMAD3 transcription factors. Overall, the data suggest that both the SMAD and p38 MAPK pathways contribute to Itg β 6 mRNA expression in BDECs.

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Authorship Contributions

Participated in research design: Sullivan, Kassel, Manley, Baker, and Luyendyk.

Conducted experiments: Sullivan, Kassel, Manley, and Baker.

Performed data analysis: Sullivan, Kassel, Manley, Baker, and Luyendyk.

Wrote or contributed to the writing of the manuscript: Sullivan and Luyendyk.

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