Regulation of Biglycan Gene Expression by Transforming Growth Factor-β Requires MKK6-p38 Mitogen-activated Protein Kinase Signaling Downstream of Smad Signaling*

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Several signaling pathways have been implicated in mediating TGF-β1-induced extracellular matrix production and fibrosis. We have shown recently that induction of biglycan (BGN) expression by TGF-β1 depended on a functional Smad pathway (Chen, W.-B., Lenschow, W., Tiede, K., Fischer, J. W., Kalthoff, H., and Ungefroren, H. (2002) J. Biol. Chem. 277, 36118–36128). Here, we present evidence that the ability of TGF-β1 to induce BGN mRNA, in addition to Smads, requires p38 MAPK signaling, because 1) pharmacological inhibitors of p38 dose-dependently inhibited the TGF-β effect without significantly affecting the transcriptional activity of a constitutively active mutant of the TGF-β type I receptor or Smad2 phosphorylation at concentrations up to 10 μM, 2) the up-regulation of BGN mRNA was preceded by a delayed increase in the phosphorylation of p38 and its upstream activator MKK6 in TGF-β1-treated PANC-1 cells, 3) inhibition of the p38 pathway by stable retroviral transduction with a dominant negative mutant of p38 or MKK6 reduced TGF-β1-induced BGN mRNA expression, and 4) overexpression of wild-type p38 or MKK6, but not MKK3, augmented the TGF-β1 effect on BGN mRNA. We further demonstrate that the (delayed) p38 activation by TGF-β1 is downstream of Smads and requires a functional Smad pathway, because blocking TGF-β1-induced p38 activity with SB202190 had no effect on Smad2 phosphorylation, but blocking Smad signaling by forced expression of Smad7 abolished TGF-β1 induction of p38 activation and, as shown earlier, BGN mRNA expression; finally, re-expression of Smad4 in Smad4-null CFPAC-1 cells restored TGF-β1-induced p38 phosphorylation and, as demonstrated previously, BGN mRNA accumulation. These results clearly show that TGF-β induction of BGN expression in pancreatic cells requires activation of MKK6-p38 MAPK signaling downstream of Smad signaling and provide a mechanistic clue to the up-regulation of BGN seen in inflammatory response-related fibrosis and desmoplasia.

TGF-β1 has emerged as a multifunctional cytokine involved in autocrine and paracrine regulation of proliferation, differentiation, wound healing, apoptosis, and immunomodulation (1, 2). TGF-β1, one of three mammalian TGF-β isoforms, TGF-β1–3, is a potent inducer of extracellular matrix formation and has been implicated as the key mediator of fibrogenesis and desmoplasia in a variety of tissues (3). In epithelial cells, TGF-β, besides its powerful antiproliferative function, induces morphological and biochemical changes toward a mesenchymal phenotype designated epithelial to mesenchymal transdifferentiation (EMT) (4). A hallmark of both fibrogenesis and EMT is the TGF-β1-induced up-regulation of matrix-associated proteins, such as certain integrins and their extracellular matrix ligands (collagens, fibronectin, proteoglycans). The TGF-β responses are initiated by the interaction of the TGF-β ligand with cell surface receptors that form a heterotetrameric complex of two type I and two type II serine/threonine kinase receptors (5, 6). The activated ligand-receptor complex, in turn, activates one or more downstream signaling pathways, the most prominent one being the Smad pathway (7, 8). This pathway is initiated with the phosphorylation of Smad2 or Smad3, which then heterodimerize with the co-Smad, Smad4, followed by translocation of the Smad2/3-Smad4 complex to the nucleus; here, it binds directly or via other DNA-binding proteins to the promoters of TGF-β-responsive genes to stimulate or repress their transcription (7, 8). Activation of Smad signaling is blocked by Smad7, which inhibits the phosphorylation of Smad2/3 by the type I receptor thereby preventing their association with Smad4 (9, 10). Evidence over the past few years suggests that TGF-β may also stimulate other downstream pathways, involving RhoA (11), as well as the MAP kinases, extracellular signal-regulated kinases (ERKs) (12), c-Jun N-terminal kinases (JNKs) (13, 14), and p38 MAPKs (15–17). However, the question whether MAP kinases depend on a functional Smad pathway and, if so, how both pathways interact, has only been addressed in a few cases (13–15, 18–20). Nevertheless, there is compelling evidence to suggest a functional separation among the individual signaling pathways (for review see Ref. 21). Smads, but not p38 MAPKs, appear to be required for TGF-β1-induced growth inhibition of epithelial cells through their ability to transcriptionally up-regulate the prof...
motors of p1S5N5K4b, p21CIP1, and c-myc (22–24). In contrast, TGF-β-induced apoptosis in mouse mammary gland epithelial cells is Smad-independent but requires activation of p38 (18). Still other biological outcomes, e.g., EMT, seem to require activation of both pathways (18).

Among the proteoglycans that are up-regulated by TGF-β in vitro is biglycan (BGN), a prototype member of the small leucine-rich proteoglycan family (reviewed in Refs. 25–27). BGN can be considered a marker gene for TGF-β activity that is reflected in vivo by the close spatial and temporal association of both proteins under physiological and various pathophysiological conditions. BGN has been implicated in the regulation of matrix assembly, cellular adhesion (28), migration (29), and growth factor, e.g., TGF-β, activity (30). Recently, BGN has been shown to directly inhibit the growth of cancer cells in a TGF-β-independent manner (31), a biological effect that it shares with its close homologue decorin (32). In conjunction with our recent observations that pancreatic tumor cells themselves synthesize and secrete BGN in response to TGF-β in vitro and that a functional Smad pathway is crucial for this to occur (33), we have proposed a novel tumor suppressor function for Smad4: growth inhibition via autoinhibitory BGN.

In the pancreatic carcinoma cell line PANC-1 the dramatic (up to 50-fold) TGF-β-induced increase in BGN mRNA and a concomitant, albeit smaller, rise in core-protein synthesis and release occurred in a BGN promoter-independent fashion and by far exceeded the Smad-dependent transcriptional activation of a heterologous reporter in these cells (33). This observation implies the existence of an additional signaling pathway(s) that function(s) to amplify Smad-mediated signaling. PANC-1 cells undergo EMT in response to TGF-β (34), which is characterized by adoption of a fibroblastoid morphology, a down-regulation of epithelial markers, and an up-regulation of mesenchymal markers, e.g., BGN. As mentioned above, EMT requires activation of both Smad and p38, and it is thus conceivable that this may be reflected at the level of particular genes involved in EMT. Finally, formation of fibrotic tissue with enhanced BGN production often results from a previous inflammatory reaction known to be associated with increased p38 MAPK signaling (35). Together, these observations led us to hypothesize that efficient BGN induction by TGF-β, in addition to the well characterized Smad pathway, requires activation of p38 MAPK. Using a combination of specific pharmacologic inhibitors, as well as overexpression of wild-type proteins from the p38 MAPK cascade and their dominant negative mutants, we present first-hand evidence that the p38 pathway is essential for TGF-β-mediated induction of BGN and that it cooperates with the Smad pathway. We further demonstrate that activation of p38 is secondary to and dependent on activation of Smad proteins. This is the first report demonstrating the involvement of the p38 MAPK pathway in the TGF-β control of BGN and small leucine-rich proteoglycan gene expression.

**EXPERIMENTAL PROCEDURES**

**Materials**—The p38 MAPK pathway inhibitors SB203580, SB202190, and SKF86024 and the MEK1 inhibitors PD98059 and U0126 were purchased from Calbiochem and dissolved in water (SB203580) or dimethyl sulfoxide (all others). The selective p38 MAPK inhibitor SB239063 was obtained from GlaxoSmithKline. Human recombinant TGF-β1 was obtained from R & D Systems, and anisomycin was from Sigma.

**Cell Lines and Cell Culture**—The human pancreatic adenocarcinoma cell lines PANC-1 and CFPAC-1 were purchased from the American Type Culture Collection (Manassas, VA) or obtained from Dr. W. von Bernstorff (University of Kiel), respectively. Both cell lines were maintained in RPMI 1640 supplemented with 10% FCS, 2 mM l-glutamine, and 1 mM sodium pyruvate (all from Invitrogen). PANC-1 and CFPAC-1 cells stably transduced with recombinant retrovirus were cultured in the presence of 700 µg/ml (PANC-1) or 350 µg/ml (CFPAC-1) genein (biologically active concentration; Invitrogen) or 2.5 µg/ml puromycin (Sigma).

**Antibodies**—The following antibodies were used: anti-phospho-p38 MAP kinase (Thr180/Tyr182), anti-p38 MAP kinase, anti-phospho-SAPK/JNK (Thr183/Tyr185), anti-SAPK/JNK, anti-phospho-MKK3/MKK6 (Ser189/Ser192), anti-phospho ATM-2 (Thr183) (all from Cell Signaling Technology, Heidelberg, Germany), anti-MKK6 (V-20; Santa Cruz Biotechnology, Inc.), anti-MKK3 (N-20; Santa Cruz Biotechnology, Inc.), anti-phospho-MKK2 (Ser358/Ser361) (Upstate Biotechnology, Inc., Lake Placid, NY/Biomol, Hamburg, Germany), anti-Smad2 (Zymed Laboratories Inc., Berlin, Germany), anti-Smad4 (B-8; Santa Cruz Biotechnology, Inc.), anti-Smad7 (N-19; Santa Cruz Biotechnology, Inc.), anti-JI-actin (AC-15; Sigma). FLAG-tagged proteins were detected with an anti-FLAG monoclonal antibody M2 (Sigma).

**RNA Isolation and Semiquantitative RT-PCR**—Total RNA was isolated from cells with RNA Clean (AGS, Heidelberg, Germany) according to the manufacturer’s instructions. The general RT-PCR protocol and the oligonucleotide primers used for amplification of BGN and GAPDH mRNAs were described in detail earlier (33, 36). For semiquantification of BGN, PAI-1, and GAPDH mRNAs we carried out a competitive approach using gene-specific internal standards (33). Briefly, for each target mRNA multiple reactions were run in parallel containing identical amounts of cDNA (corresponding to 100 ng of total RNA) but different concentrations of internal standard DNA. For this purpose, the standard DNA was serially diluted (0.9, 0.8, . . . , 0.09, 0.08, . . . and forth). To keep reactions in the exponential phase, the number of cycles with an annealing temperature of 59 °C was adjusted to 16 cycles for BGN and 8 cycles for GAPDH. Following electrophoretic separation of PCR products on agarose gels and staining with ethidium bromide photographs were taken and densitometrically scanned using the NIH Image software (version 1.62). TGF-β induction of BGN and PAI-1 mRNA was assessed from those reactions that showed an equimolar concentration of target and internal standard. The corresponding amount of target mRNA in these reactions was considered to be accurately determined when this ratio and the target to standard ratios of at least two neighboring reactions plotted against the corresponding standard dilutions on a semilogarithmic scale formed a linear relationship. Relative values for BGN and PAI-1 mRNA were normalized to GAPDH mRNA levels. For each stimulation experiment at least two independent competitive RT-PCR assays were performed yielding the same results.

**Construction of Retroviral Expression Vectors and Generation of Stable and Transient Transductants of the PANC-1 Cell Line**—For retroviral transduction, cDNAs for murine flag epitope-tagged kinase inactivating versions of p38α (p38AF, harboring T180A and Y182F amino acid substitutions) and MKK6 (MKK6Δα,β) in pcDNA3 (both generously provided by Dr. S. Ludwig, Würzburg, Germany) were amplified with T7 and SP6 primers using Turbo-Pfu polymerase (Stratagene) and subcloned in sense orientation into the Pmel site of the retroviral vector TJBAMoLink-neo (33). The p38AF and MKK3 were generated by RT-PCR with primers p38-forward, 5′-AAAATGTCCTCAGGAGAGCCCGCAG-3′ (stop codon underlined) and p38-reverse, 5′-TCAGGACCTCATCCTTCGGGTCC-3′ (stop codon underlined) and MKK3-forward, 5′-ACCAGTGGCAAGCCGCCGCGC-3′, respectively, using Turbo-Pfu polymerase and PANC-1 cDNA as template. The resulting fragments were gel-purified and ligated in sense orientation into Pmel-restricted TJBAMoLink-neo vector (MKK3) or Sma1-restricted pBABEpuuro (p38). In both cases sequencing revealed authenticity with the published mRNA sequences. The generation of a retrovirus encoding Smad4 was described in detail earlier (33). A retrovirus for Smad7 was prepared by transfecting the Smad7 cDNA from pcDNA3 (a generous gift from Dr. C.-H. Heldin, Institute for Cancer Research, Uppsala, Sweden) followed by directional cloning into BamHI/Pmel-restricted TJBAMoLink-neo. Positive clones (evaluated by PCR, restriction analysis, and sequencing of the plasmid-cDNA junctions) were transfected into human embryonic kidney (HEK) 293 cell producer cells and retroviral supernatants were harvested as described previously (37). Retroviral particles released by human embryonic kidney 293T cells were used to infect PANC-1 and CFPAC-1 cells. Pools or individual clones (obtained by limited dilution) of produc-
(4 × 10^4) were seeded in 6-well plates on day 1 and transfected on day 2 with an expression vector for MKK3/H/MK6 (a gift of Dr. S. Ludwig) using LipofectAMINE Plus (Invitrogen) according to the manufacturer’s instructions. Following a period of 24 h in normal growth medium to allow expression of proteins from the transfected plasmids, cells were starved (0.5% FCS) for another 24 h and stimulated with TGF-β1 (5 ng/ml) in the same medium for 24 h. Subsequently, cells were processed for RNA isolation and RT-PCR. For detection of luciferase activity, PANC-1 cells were seeded in 96-well plates at 1 × 10^4 cells/well. On the next day cells were cotransfected with the TGF-β responsive reporter p3TP-lux and a plasmid encoding a constitutively active mutant of TGF-β receptor type I/ALK5 (ALK5T204D) both plasmids kindly provided by Dr. J. Massagué, Memorial Sloan-Kettering Cancer Center, New York) using LipofectAMINE Plus. After removal of the transfection mixture, cells were incubated in normal growth medium for 24 h. For analysis of the effect of the MAPK inhibitors on ALK5T204D activity, different amounts of SB203580 were then added to the medium for another incubation period of 24 h. Following lysis in lysis buffer luciferase activities were determined with the Bright Glo luciferase assay system (Promega) in the MicroBeta TriLux 1450 system (Wallac, Inc., Gaithersburg, MD) for 2 s. The mean ± S.D. for each sample and treatment were determined from 6–8 wells processed in parallel. Because control experiments showed that the overall results were not affected by unequal transfection efficiencies, normalization to β-galactosidase activity was omitted.

Immunoprecipitation and Immunoblot Analysis—For immunoprecipitation experiments the cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed in 25 mM HEPES buffer, pH 7.5, 100 mM NaCl, 10% glycerol, 5 mM EDTA, and 1% Triton X-100 supplemented with Complete™ protease inhibitor mixture (Roche Diagnostics). Cleared lysates were subjected to immunoprecipitation using anti-FLAG-antibody, followed by adsorption with protein G-Sepharose. Protein G beads were then washed in lysis buffer five times, boiled in 2× Laemmli buffer (125 mM Tris-Cl, pH 6.8, 100 mM dithiothreitol, 20% glycerol, 4% SDS) and subjected to immunoblot analysis. For direct immunoblot analysis, cells were either lysed in radioimmune precipitation assay buffer (0.1% SDS, 1% Nonidet P-40, and 0.5% sodium deoxycholate and Complete™ in TM protease inhibitor mixture) or, for detection of phosphorylated proteins, were lysed directly in 2× Laemmli buffer. 40 μg of total cellular protein (measured with BCA protein assay reagent; Pierce) from cleared lysates or equal volumes of Laemmli lysates were separated by 12.5% SDS-PAGE and blotted onto Immobilon-P polyvinylidene difluoride membranes (Millipore). Membranes were blocked with PBS containing 5% non-fat dry milk, washed several times with PBS containing 0.1% Tween 20, and then incubated with the primary antibody. For detection of phosphorylated proteins, TBST (Tris-buffered saline + 0.1% Tween 20) plus 5% bovine serum albumin was used for membrane blocking, and TBST was used for washing. After washing, blots were incubated with the appropriate peroxidase-conjugated secondary antibodies and visualized by enhanced chemiluminescence (ECL or ECL plus; Amersham Biosciences). Immunoblots for the detection of activated MAP kinases were first incubated with the respective phosphospecific antibody and then stripped and reprobed with an antibody against the corresponding total protein or β-actin to confirm equal loading.

RESULTS

Effect of p38 MAPK Blockade on TGF-β Induction of BGN Expression—Several observations suggested that in PANC-1 cells an additional pathway is activated by TGF-β that, together with the Smad pathway, functions to amplify the TGF-β signal for the BGN mRNA up-regulation (see Introduction). To analyze whether p38 MAP kinases or ERKs are involved in the TGF-β effect on BGN expression, we used pharmacological inhibitors that specifically block these pathways. The pyridyl-nylimidazole-based compounds SB203580 and SB202190 inhibit the activity of p38 for its substrate but do not block its phosphorylation. Pre-incubation of the cells with SB203580 or SB202190 strongly suppressed the TGF-β effect on BGN mRNA (Fig. 1A) and proteoglycan synthesis (data not shown) in a dose-dependent manner. Successful inhibition of p38 was verified for SB202190 by reduced phosphorylation of p38α, a nuclear target of the p38 pathway (Fig. 1A, inset). SB202190 inhibition of TGF-β-induced BGN mRNA was also seen in other cell types, e.g. osteoblastic MG-63 cells (data not shown). Pre-incubation of PANC-1 cells with PD98059 or U0126, two inhibitors of MEK1, the upstream kinase that activates ERK1/2, did not inhibit TGF-β induction of BGN mRNA. Rather, the MEK1 inhibitors slightly enhanced the TGF-β effect (Fig. 1B), which would be compatible with an inhibitory effect of the ERKs on TGF-β-induced BGN expression (see “Discussion”).

SB203580 and SB202190 were shown recently (38, 39) to inhibit ALK5 function at higher concentrations because of similar critical concentrations in the kinase domains (ATP binding pocket) of ALK5 and p38 MAPK. To further exclude that the inhibitory effect of these compounds on TGF-β-induced up-regulation of BGN mRNA was because of inhibition of ALK5 rather than p38, we sought to assess the functional effect of SB203580 on ALK5 more directly. To do this, we transiently cotransfected PANC-1 cells with ALK5T204D, a constitutively active ALK5 mutant, along with the TGF-β responsive reporter p3TP-lux and measured ALK5T204D activity in the presence or absence of SB203580. As shown in Fig. 1C, concentrations of up to 10 μM SB203580 did not significantly affect ALK5T204D transcriptional activity. Finally, we used the chemically related compound SB239063, which is considered to be selective for p38 (40). Like SB203580 and SB202190, SB239063 potently inhibited the TGF-β effect on BGN with an IC50 of ~2 μM (Fig. 1D). Notably, SB239063 only marginally affected TGF-β induction of PAI-1 mRNA (Fig. 1D) indicating that SB239063 is not a general inhibitor of TGF-β signaling. From these results we conclude that the inhibition of TGF-β-induced BGN mRNA expression by SB203580 and SB202190 was not caused by inhibition of ALK5 activity. Instead, the data presented suggest that TGF-β mediated part of its effect on BGN expression via the p38 MAPK pathway.

TGF-β Activates p38 and the Upstream Kinases MKK3/6 in a Delayed Fashion—Several MAPK pathways have been shown to be activated in response to TGF-β in PANC-1 cells (41). Because pharmacological inhibitors of the p38 MAPK cascade blunted the TGF-β effect, we investigated whether p38 was activated in response to TGF-β by analyzing the phosphorylation state of p38 by immunoblotting using a phosphospecific antibody. From Fig. 2 it is evident that TGF-β1 induced an increase in p38 activation that was first noticed 1 h after TGF-β1 addition, peaked at 2–4 h, and returned to baseline levels thereafter. Interestingly, another member of the stress-activated protein kinases, SAPK/JNK, was not activated following TGF-β treatment in PANC-1 cells, although it was readily activated by anisomycin, a known activator for both JNK and p38 (Fig. 2). Because p38 is activated by the two upstream MAP kinases, MKK3 and MKK6 (42), we predicted that these, too, were activated by TGF-β in a temporal fashion similar to that of p38. This was confirmed by immunoblot analyses using a phosphospecific antibody against these two kinases (Fig. 2). In accordance with activation of p38 but not SAPK/JNK, MKK4, the upstream kinase of SAPK/JNK, was not activated in response to TGF-β (data not shown).

TGF-β Induction of BGN mRNA Was Reduced upon Dominant Negative Inhibition of p38 MAPK and Enhanced by Overexpression of Wild-type p38—To more specifically demonstrate the involvement of p38 MAPK in TGF-β-regulation of BGN, we inhibited p38 function by stable retrovirus-mediated expression of a phosphorylation-resistant mutant of p38α, p38AP, that has been shown to act in a dominant negative fashion (43). Analysis of a pool of PANC-1 transductants, as well as several individual clones by immunoprecipitation with anti-FLAG antibody followed by anti-p38 immunoblotting, indicated expression of the mutant construct (Fig. 3A, inset). The amount of immunoprecipitated protein correlated well with the degree of

a N. Laping, personal communication.
FIG. 1. The TGF-β effect on BGN mRNA is blocked by inhibitors of p38 MAPK. PANC-1 cells were seeded on day 1 in normal growth medium and switched to medium containing 0.5% FCS on day 2 for 24 h, followed by treatment with TGF-β1 (5 ng/ml) for another 24 h in the absence or presence of various MAPK inhibitors or vehicle (0.5 h of pre-incubation prior to the addition of TGF-β). Subsequently, cells were analyzed for BGN or PAI-1 expression by quantitative RT-PCR (A, B, and D) or reporter gene activity in a luciferase assay (C). A, effect of the p38 MAPK inhibitors SB203580 and SB202190. At the end of the incubation period total RNA was isolated from the cells and subjected to reverse transcription. Equal amounts of cDNA were amplified with specific primers for BGN and GAPDH, along with different concentrations of internal standard as described under “Experimental Procedures.” Following agarose gel fractionation and ethidium bromide staining, relative concentra-
inhibition of the TGF-β effect on BGN mRNA (Fig. 3A). Moreover, overexpression of wild-type p38 in a clone pool of transduced cells augmented the TGF-β effect on BGN (Fig. 3B). These data confirm the results of pharmacologic inhibition and emphasize the pivotal role of p38 in TGF-β regulation of BGN.

TGF-β Regulation of BGN Expression Involves MKK6—p38 is activated by the MAPK kinases MKK3 or MKK6, which were activated in a similar temporal pattern as p38 in response to TGF-β (see Fig. 2). To test whether these MAPK kinases were part of the TGF-β signaling pathway targeting BGN, we used SKF86002, which has been reported to inhibit the activity of MKK6 for p38 (19). Notably, SKF86002 blocked the TGF-β effect on BGN mRNA as efficiently as the p38 inhibitors (Fig. 4A). To more selectively interfere with MKK6 activity, we stably overexpressed a kinase-negative dominant negative MKK6 mutant (MKK6ΔN). This approach efficiently blocked the TGF-β effect on BGN mRNA in a dose-dependent fashion (Fig. 4B). Like for p38, (transient) overexpression of the wild-type MKK6 protein enhanced the TGF-β effect on BGN (Fig. 4C, upper panel). Given the fact that transfection efficiency was only ~30% (as determined with an expression vector encoding green fluorescent protein) (data not shown), an even stronger effect of MKK6 on TGF-β signaling can be anticipated if 100% of the cells were transfected. In contrast, (stable) overexpression of wild-type MKK3 had no effect (Fig. 4C, lower panel). Together, these data strongly implicate MKK6, rather than MKK3, as the relevant MAPK kinase in the TGF-β control of BGN expression.

Activation of the Smad Pathway Is Required for TGF-β-Induced p38 Activation in Pancreatic Cells—We have shown previously (33) that a functional Smad pathway is crucial for the TGF-β effect on BGN expression in pancreatic cells. The question therefore arises how the Smad and p38 pathway interact to bring about the rise in BGN expression. The results obtained so far are consistent with either of the following two models. In model 1, TGF-β (through its type I receptor) concomitantly but independently activates Smad complex and p38 MAPK, which subsequently converge on a common (nuclear) target, e.g. ATF-2 (16). In model 2, the activated Smad complex induces expression of an (unknown) protein, which subsequently activates p38 MAPK. As shown earlier, phosphorylation of Smad2 commenced ~15 min after TGF-β addition (33) whereas appreciable phosphorylation of p38 was only realized 1 h after TGF-β addition suggesting that Smad activation precedes activation of p38 (model 2). In concordance with this assumption, inhibition of p38 by SB202190 was unable to block TGF-β-induced phosphorylation of Smad2 (Fig. 5A); even at concentrations as high as 50 μM no major decrease in Smad2 phosphorylation was seen when compared with cells that were treated with TGF-β alone, further dismissing the possibility that inhibition of ALK5, rather than p38, accounted for the TGF-β effect on BGN expression. A corollary of model 2 is that p38 activation, like BGN mRNA up-regulation, is dependent on functional Smad4 expression. To test this prediction, we initially analyzed the p38 response in the Smad4-deficient, TGF-β-non-responsive, pancreatic carcinoma cell line CFPAC-1, which expresses functional TGF-β receptors. As predicted by this model, TGF-β failed to activate p38 in CFPAC-1 cells (Fig. 5B), as well as in control cells expressing an empty retrovirus (Fig. 5C), though a robust activation of p38 by anisomycin (Fig. 5B) indicated that there was no defect in the p38 MAPK pathway per se. In contrast, in CFPAC-1 stably transduced with the same retrovirus encoding wild-type Smad4, TGF-β-induced activation of p38 was restored (Fig. 5C). As demonstrated earlier the capability to up-regulate BGN mRNA in response to TGF-β was also restored in these cells (33) (Fig. 6B). Finally, we analyzed p38 activation in PANC-1 cells in which Smad signaling has been blocked by ectopic expression of the antagonistic Smad7. Overexpression of Smad7 was shown previously by us to efficiently suppress TGF-β-induced BGN mRNA accumulation (33). As predicted from the above experiments, TGF-β-induced p38 phosphorylation, too, was inhibited by Smad7 (Fig. 7D). Collectively, these data prove that intact Smad signaling is required for p38 activation and that activation of the p38 pathway occurs downstream of Smad signaling, hence that model 2 is correct. Fig. 6 summarizes and integrates current and previous data on signaling events involved in TGF-β regulation of BGN expression.

**DISCUSSION**

In this study we have demonstrated that activation of the p38 MAPK pathway, in addition to Smad signaling (33), is crucial for TGF-β regulation of BGN expression in pancreatic carcinoma cells. This conclusion was derived from experiments in which the expression of known components of the p38 signaling pathway, namely p38α MAPK and its upstream activators MKK3 and MKK6, was either enhanced or blocked by specific drugs or dominant inhibitory mutants. In addition, we have presented evidence that Smad proteins are required for p38 activation and that their activation temporally precedes p38 activation. The finding that the Smad and the p38 pathways are serially connected is probably the most intriguing aspect of this study. It was confirmed by inhibition and gene replacement experiments in which one pathway was blocked or activated, respectively, followed by a monitoring of the activation state of the other; whereas inhibition of p38 with SB202190 had no effect on Smad2 activation, inhibition of Smad signaling by ectopic expression of Smad7 inhibited TGF-β-induced p38 activation (this study) and BGN mRNA up-regulation (33), and reconstitution of Smad signaling by re-expression of Smad4 in Smad4-deficient, TGF-β-, and p38-unresponsive CFPAC-1 cells restored the TGF-β response of p38 (this study) and BGN mRNA induction (33). This scenario was initially suspected from the observation that Smad2 is phosphorylated much more rapidly (33) than p38 (this study) in response to TGF-β. With re-
spect to the kinetics of p38 activation by TGF-β, two different signaling mechanisms seem to exist; in some cells, e.g. C2C12 mouse myoblasts, activation of p38 is rapid (within the first 30 min) and transient (basal levels restored by 1 h) (15, 16, 44). This rapid activation of p38 has been associated with TAK1, a member of the MAPKK family (45, 46), which is connected to ALK5 via bridging proteins XIAP and TAB1 and has been suggested to mediate TGF-β-induced p38 activation without participation of Smad proteins (16, 45, 47). In other cell types (e.g. keratinocytes, osteoblasts, gingival fibroblasts, and pancreatic acinar cells) maximal p38 activation occurs only 1–2 h after TGF-β stimulation and persists for several hours (48–53). Takekawa et al. (20), in a publication that appeared shortly before submission of our manuscript, analyzed TGF-β-induced p38 activation in various cell types including pancreatic carcinoma cells, and their findings on the time course of p38 activation are in good agreement with our data. These authors convincingly demonstrated that delayed activation of p38 by TGF-β was independent of TAK1 but instead involves activation of the MAPKKK MTK1/MEK kinase 4 through Smad-dependent intermediate GADD45 expression (20). TGF-β/Smad-stimulated GADD45 expression was found to be necessary for p38 activation in PAN1-1 cells. Although not directly shown in this cell type, inhibition of MTK1 by a dominant interfering mutant abolished GADD45-induced p38 activation triggered by constitutive active ALK5. Also, in cotransfection experiments carried out by these authors in COS-7 cells, GADD45 only stimulated MTK1 but not TAK1 kinase activity. This is in line with experiments from our group employing PAN1-1 cells that

**Fig. 2.** Activation of p38 MAPK and MKK3/6 by TGF-β1. PANC-1 cells were starved for 24 h in medium containing 0.5% FCS and subsequently treated in the same medium with 5 ng/ml TGF-β1 or 150 nM anisomycin (Co) for the indicated time periods. Cell lysates were analyzed by immunoblotting for the phosphorylation status of various endogenous MAP kinases using phosphospecific antibodies. **Upper panel**, detection of phosphorylated p38α (p-p38) and total p38 protein (t-p38) in cell lysates. **Middle panel**, detection of phosphorylated SAPK/JNK (p-JNK) and total SAPK/JNK protein (t-JNK). Note the absence of constitutive and TGF-β-induced JNK phosphorylation. **Lower panel**, detection of phosphorylated MKK3/6 (p-MKK3/6) and total MKK6 protein (t-MKK6). Note the delayed activation that is similar to that of p38.

**Fig. 3.** Effect of dominant negative inhibition and enforced exogenous expression of p38 MAPK on TGF-β induction of BGN mRNA. A, determination of TGF-β-induced BGN mRNA expression in PAN1-1 cells stably overexpressing a p38 mutant (p38α, known to act in a dominant negative fashion or the empty retroviral vector. Cells were stimulated with TGF-β1 (5 ng/ml) for 24 h and analyzed by semiquantitative RT-PCR for BGN expression as described in the legend to Fig. 1 and under “Experimental Procedures.” BGN mRNA concentrations were expressed relative to unstimulated vector controls set arbitrarily at 1. Inset, combined immunoprecipitation (IP) and immunoblot (IB) analysis of a pool of a large number of stable transductants (pool), as well as of two individual clones (12 and 14). Note that higher expression of the p38 mutant correlates with a stronger inhibitory effect. B, TGF-β effect on BGN transcript levels in a pool of PAN1-1 cells stably overexpressing wild-type p38 from the retroviral vector pBABEpuro. Inset, immunoblot analysis of total p38 protein expression in lysates from p38-transduced PAN1-1 cells. An antibody to β-actin was used to control for equal loading.
overexpress a dominant negative TAK1 mutant, TAK1_K63W, which failed to block TGF-β induction of BGN mRNA. Also, we showed earlier that the TGF-β effect on BGN expression was cycloheximide-sensitive and was not mediated via enhanced transcriptional activity from the BGN promoter (33). With regard to the kinetics of p38 activation and BGN mRNA up-regulation it is noteworthy that the initial rise in BGN mRNA occurs between 4 and 8 h after TGF-β addition (31), which is similar to that observed for thrombospondin-1 (20) and nicely matches the observation that activation of p38 was maximal at 2–4 h following TGF-β addition. These data are consistent with a model in which the TGF-β-induced activation of Smads results in the transcriptional induction of an (hitherto unknown) protein that subsequently activates the p38 pathway (Fig. 6). Recent studies showed that additional signals may be required for proper activation of p38 by TGF-β. Functional integrin β1 and αv signaling was necessary for TGF-β-dependent (17) and -independent (54) p38 MAPK activation, respectively. Interestingly, αv integrin-mediated p38 activation and urokinase plasminogen activator up-regulation required MKK3 but not MKK6. Consequently, the possibility has been considered that integrin-induced p38 activation may be mediated strictly by MKK3 (54). Because we showed that overexpression of wild-type MKK6, but not wild-type MKK3, was capable of enhancing TGF-β-induced BGN up-regulation (and supposedly p38 activation), the reverse conclusion would argue against a role of integrins in TGF-β control of BGN expression. In agreement with this assumption, blocking integrin β1 function in PANC-1 cells did not affect TGF-β-mediated BGN mRNA up-regulation (data not shown). Although MKK3 and MKK6 are 80% homologous to each other and, in many cases, mediate the same signals for p38 MAPK activation, they have been reported to exhibit differential involvement in certain cellular events. Be-

Fig. 4. Effect of pharmacologic and gene expression-mediated inhibition of MKK6 activity or ectopic expression of MKK6 or MKK3 on TGF-β induction of BGN expression. A, PANC-1 cells were treated with TGF-β1 (5 ng/ml) for 24 h in the absence or presence of the MKK6 inhibitor SKF86002 (0.5 h of pre-incubation prior to the addition of TGF-β) and analyzed for BGN expression by quantitative RT-PCR as described in Fig. 1 and “Experimental Procedures.” This experiment was repeated two times with almost identical results. B, RT-PCR analysis of BGN expression in three individual clones of PANC-1 cells stably overexpressing a dominant negative MKK6 mutant (MKK6Ala) or the empty retroviral vector. BGN mRNA concentrations were expressed relative to unstimulated vector controls set arbitrarily at 1. Inset, immunoprecipitation (IP)/immunoblot (IB) analysis of MKK6Ala expression. Duplicate samples are shown for each clone and the controls. Note that higher expression of the MKK6 mutant correlates with a stronger inhibitory effect. wt, wild-type. C, upper panel, PANC-1 cells were transiently transfected with a wild-type (wt), FLAG-tagged MKK6 DNA, or empty pcDNA3 vector (vector) followed by incubation in normal growth medium for 24 h. After another 24-h incubation in starved medium, cells were stimulated with TGF-β1 (5 ng/ml) for 24 h and subsequently processed for BGN RT-PCR. Lower panel, PANC-1 cells infected with a retrovirus encoding wild-type MKK3 or empty retrovirus (vector) were stimulated with TGF-β1 for 24 h followed by measurement of BGN mRNA using RT-PCR. The MKK3 protein level in the transduced cells was determined by immunoblot analysis.

3 H. Ungefroren, unpublished data.
Fig. 5. Ordering the p38 MAPK and the Smad pathways. A, effect of the p38 MAPK inhibitor SB202190 on activation of the Smad pathway as assessed by phosphorylation of Smad2. PANC-1 cells were treated with 5 ng/ml TGF-β1 in the absence or presence of the indicated concentrations of SB202190 for 8 h. Cells were lysed in Laemmli buffer and analyzed by immunoblotting with anti-phospho-Smad2 (p-Smad2; lower panel) and anti-Smad2 antibody (t-Smad2; upper panel) for total Smad2 protein (t-Smad2). B, TGF-β-induced p38 activation in the TGF-β-unresponsive Smad4-deficient pancreatic carcinoma cell line CFPAC-1. CFPAC-1 cells were treated for various times with TGF-β1 (5 ng/ml) or for 2 h with anisomycin (150 ng/ml) as control (Co), and cell extracts were prepared. The phosphorylation status of endogenous p38 was analyzed by immunoblotting as described in the legend to Fig. 2. C, TGF-β1-induced p38 activation in CFPAC-1 cells stably transduced with Smad4 or empty vector. D, effect of exogenous expression of Smad7 on the TGF-β-mediated phosphorylation of p38 MAPK. PANC-1 cells were transiently transduced with a Smad7-encoding retrovirus or empty retrovirus (vector). 24 h post-infection cells were starved and then treated or not with TGF-β1 (5 ng/ml) for 4 h. Subsequently, cells were lysed in Laemmli buffer and subjected to immunoblot analysis for phospho-p38 and total p38. Two independent experiments were performed (C and D) with similar results.

Fig. 6. Proposed model of Smad and p38 signaling in TGF-β induction of BGN expression. This scheme summarizes available data on the signaling events involved in conveying the TGF-β signal from the receptor complex to the nucleus, ultimately resulting in the accumulation of cytoplasmic BGN mRNA transcripts. Upon TGF-β binding TGF-β receptor I/ALK5 is activated and subsequent phosphorylates Smad2/3. Smad2 or Smad3 then forms an active heterodimeric complex with Smad4, which is translocated into the nucleus to induce expression of an as yet unknown protein (protein X). This protein activates p38 MAPK via activation of the MAPKKK upstream of MKK6 or of a protein further upstream of the MAPKKK level. Active p38 translocates to the nucleus and induces BGN mRNA accumulation through nuclear mRNA transcript processing, stability, and export. Recent results from Takekawa et al. (20) suggest that the gene/protein X may be GADD45β, which binds and activates the MAPKKK MKK1/MEK kinase 4 (see “Discussion”). Signal routing that in other cellular systems have been shown to be involved in TGF-β-mediated activation of p38 are marked by dashed arrows.
ERK activation induced by mitogenic stimuli in this cell type (41) it appears that the TGF-β effect on BGN results from both direct activation of p38 and, to a smaller extent, simultaneous inhibition of ERKs.

As mentioned above, TGF-β stimulation of PANC-1 and other TGF-β-responsive pancreatic cancer cell lines led to EMT, a biological response that requires activation of both the Smad and the p38 pathway. Up-regulation of BGN, too, depends on sequential activation of both pathways as shown here and may contribute to the phenotypic changes associated with EMT. EMT involves an increase in tumor cell migration, invasion, and scattering (34). Interestingly, up-regulation of BGN (39) and p38 activation (61) have independently been implicated in cellular migration and adhesion. The realization that the synthesis of BGN, like fibronectin (39), is induced by TGF-β via p38 may thus point to an important role of this proteoglycan in tumor spread and metastasis and may contribute to the tumor-promoting effect of TGF-β in later stages of carcinogenesis (62). In conclusion, we have shown for the first time that TGF-β regulation of the small interstitial proteoglycan BGN requires activation of the p38 MAPK signaling pathway, which, in turn, depends on functional Smad signaling.

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