We have recently shown that induction of biglycan (BGN) expression by transforming growth factor-β1 (TGF-β1) required sequential activation of both Smad and p38 mitogen-activated protein kinase signaling (Ungefroren, H., Lenschow, W., Chen, W.-B., and Kalthoff, H. (2003) J. Biol. Chem. 278, 11041-11049). Here, we have analyzed the receptors through which TGF-β1 controls expression of BGN and GADD45β, the latter of which is postulated to link early Smad signaling to delayed activation of p38. Ectopic expression of a dominant-negative mutant of the TGF-β type II receptor in PANC-1 cells abrogated TGF-β-induced BGN up-regulation. Similarly, inhibition of the TGF-β type I receptor/ALK5 with either SB431542 or by enforced stable expression of a kinase-dead mutant greatly attenuated the TGF-β effect on both BGN and GADD45β expression in PANC-1 and MG-63 cells. The enhancing effect of ALK5 on TGF-β-mediated GADD45β and BGN expression and on GADD45β promoter activity was also dependent on its ability to activate Smad signaling, because an ALK5 mutant defective in Smad activation (TβRImL45) but with an otherwise functional kinase domain failed to mediate these responses. The TGF-β/ALK5 effect on p38 activation and BGN expression and GADD45β expression (alone and in the absence of TGF-β stimulation) and suppressed upon anti-sense inhibition of GADD45β expression. These results show that TGF-β induces BGN expression through the Smad-activating function of ALK5 and GADD45β and suggest that the sensitivity of MyD118 to activation by TGF-β, which varies between tissues, ultimately determines the strength of the TGF-β effect on BGN.

TGF-β1 and its signaling effectors regulate basic cellular functions such as proliferation and apoptosis and act as key determinants of tumor cell behavior (1–3). Most, if not all, of the cellular activities of TGF-β are mediated by specific receptor complexes that are assembled upon ligand binding and comprise the TGF-β type II receptor (TβRII) and a type I receptor (4). Whereas only one type II receptor is known so far, there are at least three type I receptors, activin receptor-like kinase (ALK1/TSR-1 (5), ALK2/Tsk7L (6), and TGF-β type I receptor/ALK5 (7) that have been shown to bind TGF-β in the presence of TβRII. ALK5 has an ubiquitous distribution and represents the principle type I receptor that mediates most cellular responses to TGF-β. The activated ligand-receptor complex activates one or more downstream signaling pathways, the most prominent one being the Smad pathway (1–4). However, other signaling pathways can be activated by the ALK5 kinase, including mitogen-activated protein kinases (MAPKs), ERK1/2 (8), and p38 MAPK (9). In the case of p38 signaling the activation by ALK5 may be Smad-independent (10) or Smad-dependent (4, 11). Recently, transcriptional induction by TGF-β of GADD45β (encoded by MyD118), a protein involved in the response to genotoxic stress (12), has been shown to be Smad-dependent and to mediate the (delayed) activation of p38 by TGF-β (13, 14) via binding and activation of the MAPKK MTK1/MEKK4 (14, 15). However, although GADD45β has been identified as a positive modulator of TGF-β-induced apoptosis (Ref. 12 and references therein), its role in other major TGF-β responses, e.g. matrix gene expression is less clear.

Biglycan (BGN) belongs to the family of small leucine-rich proteoglycans and is functionally involved in matrix assembly, cellular migration, and the regulation of growth factor, e.g. TGF-β activity (reviewed in Refs. 16 and 17). BGN is a major constituent of the bone matrix where it is synthesized by osteoblasts, and studies in BGN-deficient mice indicated a crucial role for this proteoglycan in bone metabolism and mechanical properties (18, 19). BGN is markedly up-regulated in fibrotic lesions of lung (20), of hepatic (21) and renal (22) tissues, in corneal scars (23), and in the stroma of solid tumors, e.g. pancreatic carcinoma (24). These compartments, like the bone matrix, are highly enriched in TGF-β. This correlation is not accidental, because BGN is exceptionally sensitive to induction by TGF-β and as such can serve as a marker of fibrotic tissue deposition in the stroma (23). However, BGN-producing cells differ in their BGN response to TGF-β stimulation, e.g. osteoblastic MG-63 cells increase BGN expression only moderately by 2- to 3-fold, whereas pancreatic PANC-1 cells respond to TGF-β under the same conditions with a 10-fold stronger induction (24, 25). Previous studies from our group indicated that in both pancreatic and bone cells the TGF-β-induced rise in cytoplasmic BGN mRNA involves an as yet undefined nuclear post-transcriptional mechanism rather than an increase in transcriptional activity of the BGN gene promoter (25, 26). However, at present it is unknown if the greater inducibility of
BGH by TGF-β in (pancreatic) epithelial versus mesenchymal cells resides in qualitative and/or quantitative differences in receptor-proximal signaling, or at the transcriptional/post-transcriptional level.

TGF-β signaling to BGH has revealed an unexpected degree of complexity. The TGF-β effect on BGH is strictly dependent on activation of Smads (25) and p38 (27). The delayed kinetics of both p38 activation and BGH up-regulation and the sensitivity of the TGF-β effect on BGH to the protein synthesis inhibitor cycloheximide (25) suggested an indirect effect involving early Smad-mediated transcriptional activation of a gene X, which subsequently activates p38 and finally BGH. These findings further predict that signaling intermediates crucial for TGF-β activation of p38 are likely to be important for TGF-β regulation of BGH, too. As part of a larger project aimed at characterizing the “missing links” in the series of signaling events commencing with the activation of TGF-β receptors and culminating in BGH mRNA accumulation, we initially focused on the role of ALK5 and its functional domains, respectively, responsible for transducing the TGF-β signal to BGH. We present evidence that induction of both GADD45β and BGH expression as well as activation of p38 by TGF-β requires the Smad-activating function of ALK5. We further show that intermittent activation of GADD45β expression is mandatory for TGF-β/ALK5 regulation of p38 activation and BGH induction. The results of this study establish the ALK5-GADD45β-p38 pathway to be crucial for TGF-β regulation of BGH and provide for the first time data on the regulation of GADD45β expression by the ALK5 kinase.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—TGF-β1 was purchased from R&D Systems (Wiesbaden, Germany). Antibodies to TβRII (t-21), ALK5 (V-22), and GADD45β (N-20) were obtained from Santa Cruz Biotechnology, to phospho-p38, p38, and phospho-Smad2 from Cell Signaling Technology (Heidelberg, Germany), to β-actin and FLAG (M2) from Sigma, and to HA (clone 12CA5) from Roche Diagnostics (Mannheim, Germany). SB431542 was purchased from Tocris (Ellisville, MO). Pharmacological inhibitors were added to cells 30–60 min before the addition of TGF-β, which was used at a concentration of 5 ng/ml in all experiments. All other reagents used were of analytical grade purity.

Cell Lines and Cell Culture—Human pancreatic cancer PANC-1 and osteosarcoma MG-63 cells were maintained as described earlier (26, 27). Colon carcinoma SW48 cells were a kind gift of M. Lühr (University of Erlangen) and were cultured in Gibco’s modified Eagle’s medium supplemented with 10% fetal calf serum and 2 mM L-glutamine (Invitrogen). Cells stably transduced with various retroviral vectors were cultured in the presence of 700 (PANC-1) or 250 (MG-63) μM Geneticin (Invitrogen).

RNA Isolation and RT-PCR Analysis—Total RNA from PANC-1 and MG-63 cells was isolated withpeqGOLD RNAPure (Peqlab, Erlangen, Germany). The general RT-PCR protocol, the conditions for competitive quantitative PCR, the PCR primer sequences for BGH and plasminogen activator-inhibitor (PAI-1), as well as the procedure of data analysis provided by the supplier and subsequently analyzed by immunoblotting as described in detail earlier (25).

RESULTS

TβRII Function Is Required for TGF-β Induction of BGH Expression—To assess the role of TβRII for TGF-β-induced BGH expression, we measured TGF-β-stimulated BGH expression in SW48, a colon carcinoma cell line harboring a loss-of-function mutation in TβRII as a result of a mismatch repair deficiency with microsatellite instability (30). These cells failed to up-regulate BGH mRNA after a 24-h treatment with 5 ng/ml TGF-β1 (Fig. 1A). Prolonged treatment with or higher concentrations of this growth factor were also ineffective in inducing BGH expression (data not shown). Under the same conditions, TGF-β-responsive PANC-1 cells responded with a 20-fold induction of BGH mRNA over basal levels (Fig. 1A). To further probe the role of TβRII in TGF-β regulation of BGH, we retrovirally transduced PANC-1 cells in a stable fashion with a TβRII mutant (TβRII-D404G). This mutant has been shown to act in a dominant negative fashion by inhibiting the appearance of wild type receptor at the cell surface (31). A clone pool (heterogenous mixture of G418-resistant cells with respect to the transgene) was tested for transduction efficiency with the mutant as revealed by immunoblotting (Fig. 1B). Notably, TGF-β-induced expression of BGH and of PAI-1, another TGF-β-responsive gene dependent on Smad, but independent of p38 activation (25), was completely suppressed (Fig. 1, C and D). These data indicate that TβRII is required for BGH induction by TGF-β in gastrointestinal epithelial cells.

The ALK5 Kinase Activity Is Required for p38 Activation and BGH Induction—Next, we tested whether ALK5 is involved in TGF-β induction of BGH and treated PANC-1 cells with SB431542, a novel imidazole compound for selective si...
ALK5 group of TGF-β/BMP type I receptors (ALK5, ALK4, and ALK7) (32) (Fig. 2A). This inhibitor effectively suppressed the BGN response to TGF-β in both PANC-1 (Fig. 2A) and MG-63 cells (data not shown). To more specifically inhibit ALK5, we expressed in PANC-1 and MG-63 cells by stable retroviral transduction a kinase-deficient ALK5 mutant (kdALK5) known to act in a dominant negative fashion. The mutant protein was strongly expressed in the PANC-1 transductants (Fig. 2B, upper panel) and was functional, because TGF-β-induced phosphorylation of Smad2 was markedly attenuated in kdALK5-expressing cells but not in the corresponding vector-transduced control cells (Fig. 2B). Interestingly, ectopic expression of kdALK5 also blocked phosphorylation, and thus activation, of p38 MAPK (Fig. 2B). In agreement with the results from the pharmacological inhibition, BGN mRNA was markedly reduced in both kdALK5-expressing PANC-1 (Fig. 2C) and MG-63 cells (Fig. 2D). The data clearly indicate that the ALK5 kinase activity is crucial for TGF-β regulation of BGN in both pancreatic and bone cells.

p38 MAPK Activation and BGN Up-regulation by TGF-β Requires the Smad-activating Function of ALK5—Previous data indicated that an early activation of the Smad pathway was required for and preceded the activation of p38 and BGN expression by TGF-β (14, 25, 27), suggesting that, besides the kinase activity, the Smad-activating function of ALK5 is also important. To confirm this directly on the receptor level, we employed an ALK5-derived mutant (TβRIIImL45) that has retained a functional kinase domain but due to additional mutations in the L45 loop was unable to activate Smads (10). To avoid activation of the endogenous receptors we used the constitutively active (ca) counterparts ALK5-TD (33) and TβRIIImL45(TD) (10). As expected, co-transfection of PANC-1 cells with HA-tagged caALK5 or HA-tagged caTβRIIImL45 along with FLAG-tagged Smad2 or FLAG-tagged p38 followed by anti-FLAG immunoprecipitation and anti-Smad or anti-p38 immunoblotting, respectively, revealed that caALK5, but not caTβRIIImL45, phosphorylated Smad2 and p38 (Fig. 3A). Next, we verified the inability of caTβRIIImL45 to activate the Smad pathway in a transcriptional reporter assay using the Smad-specific reporter plasmid p6SBE-Luc. Transcriptional induction of p6SBE-Luc by this mutant was weak in comparison to that of caALK5 (Fig. 3B). Finally, BGN mRNA levels in (unstimulated) MG-63 cells stably expressing caALK5 approached those in TGF-β-treated control cells, whereas they remained below basal levels in their caTβRIIImL45-expressing counterparts (Fig. 3C), despite approximately equal expression of the mutant proteins (not shown). Very similar results with respect to BGN expression were obtained with PANC-1 cells ectopically expressing caALK5 or caTβRIIImL45 (data not shown; these cells were also used for analysis of TGF-β/ALK5 regulation of GADD45β expression, see Fig. 4C). These data strongly support the assumption that the Smad-activating function of ALK5 accounts for most, if not all, of p38 activity, which is consistent with the delayed type of p38 activation seen in both

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We also verified the inability of caALK5 or caTβRIIImL45 to activate the Smad pathway in a transcriptional reporter assay using the Smad-specific reporter plasmid p6SBE-Luc. Transcriptional induction of p6SBE-Luc by this mutant was weak in comparison to that of caALK5 (Fig. 3B). Finally, BGN mRNA levels in (unstimulated) MG-63 cells stably expressing caALK5 approached those in TGF-β-treated control cells, whereas they remained below basal levels in their caTβRIIImL45-expressing counterparts (Fig. 3C), despite approximately equal expression of the mutant proteins (not shown). Very similar results with respect to BGN expression were obtained with PANC-1 cells ectopically expressing caALK5 or caTβRIIImL45 (data not shown; these cells were also used for analysis of TGF-β/ALK5 regulation of GADD45β expression, see Fig. 4C). These data strongly support the assumption that the Smad-activating function of ALK5 accounts for most, if not all, of p38 activity, which is consistent with the delayed type of p38 activation seen in both pancreatic and bone cells.

p38 MAPK Activation and BGN Up-regulation by TGF-β Requires the Smad-activating Function of ALK5—Previous data indicated that an early activation of the Smad pathway was required for and preceded the activation of p38 and BGN expression by TGF-β (14, 25, 27), suggesting that, besides the kinase activity, the Smad-activating function of ALK5 is also important. To confirm this directly on the receptor level, we employed an ALK5-derived mutant (TβRIIImL45) that has retained a functional kinase domain but due to additional mutations in the L45 loop was unable to activate Smads (10). To avoid activation of the endogenous receptors we used the constitutively active (ca) counterparts ALK5-TD (33) and TβRIIImL45(TD) (10). As expected, co-transfection of PANC-1 cells with HA-tagged caALK5 or HA-tagged caTβRIIImL45 along with FLAG-tagged Smad2 or FLAG-tagged p38 followed by anti-FLAG immunoprecipitation and anti-Smad or anti-p38 immunoblotting, respectively, revealed that caALK5, but not caTβRIIImL45, phosphorylated Smad2 and p38 (Fig. 3A). Next, we verified the inability of caTβRIIImL45 to activate the Smad pathway in a transcriptional reporter assay using the Smad-specific reporter plasmid p6SBE-Luc. Transcriptional induction of p6SBE-Luc by this mutant was weak in comparison to that of caALK5 (Fig. 3B). Finally, BGN mRNA levels in (unstimulated) MG-63 cells stably expressing caALK5 approached those in TGF-β-treated control cells, whereas they remained below basal levels in their caTβRIIImL45-expressing counterparts (Fig. 3C), despite approximately equal expression of the mutant proteins (not shown). Very similar results with respect to BGN expression were obtained with PANC-1 cells ectopically expressing caALK5 or caTβRIIImL45 (data not shown; these cells were also used for analysis of TGF-β/ALK5 regulation of GADD45β expression, see Fig. 4C). These data strongly support the assumption that the Smad-activating function of ALK5 accounts for most, if not all, of p38 activity, which is consistent with the delayed type of p38 activation seen in both
cells stably expressing caALK5, but not in PANC-1 cells stably expressing caTβRImL45 (Fig. 4C). To test in a more direct fashion whether ALK5 can transcriptionally activate MyD118, we monitored caALK5-mediated activation of G45β in pancreatic and bone cells. **GADD45β Activates p38 MAPK and Enhances BGN Expression**—Having demonstrated that GADD45β expression is induced by TGF-β via ALK5, we next investigated whether ectopic overexpression of GADD45β could activate p38 and up-regulate BGN in the absence of TGF-β stimulation. To test this prediction, PANC-1 cells were stably transduced with a GADD45β-encoding retrovirus or empty retroviral vector used as control. In GADD45β-expressing cells (clones #7 and #8, detected by anti-GADD45β antibody, Fig. 5A) p38 was activated in the absence of TGF-β treatment (Fig. 5B). The enhanced levels of phosphorylated p38 reflected increased p38 kinase activity as determined with an in vitro kinase assay using ATF-2 as substrate (data not shown). To explore whether the ability of GADD45β to activate p38 is sufficient to enhance BGN expression, we measured BGN mRNA levels in the
GADD45β transductants in the absence of TGF-β stimulation. Strikingly, BGN expression was dramatically increased in both clones (Fig. 5C), while expression of PAI-1 remained unaffected (Fig. 5D), indicating the specificity of the effect for BGN. Another consequence of ectopic expression of GADD45β and subsequent p38 activation in some cell types may be induction of apoptosis (13). However, we did not observe a higher rate of apoptotic cells in the PANC-1 cultures overexpressing GADD45β. These results show that ectopic overexpression of GADD45β can specifically mimic the TGF-β effect on BGN.

**Antisense-mediated Inhibition of GADD45β Blocks TGF-β Induction of Both p38 Activation and BGN Expression**—To address this question of whether endogenous GADD45β induced by TGF-β is responsible for p38 activation and BGN up-regulation, PANC-1 cells were stably infected with antisense GADD45β cDNA thereby blocking the expression of the endogenous MyD118. Several individual clones were assayed by quantitative RT-PCR before and after TGF-β stimulation. Antisense GADD45β effectively suppressed endogenous GADD45β expression albeit to a varying degree. For further analysis we chose two clones, one with moderate and one with high suppression (Fig. 6A). B, caTβRɪmL45 is unable to activate a Smad-responsive reporter. PANC-1 cells were transiently transfected with empty vector, caALK5, or caTβRɪmL45 along with pSBE-Luc and pRL-TK as described under “Experimental Procedures” and treated, or not, with TGF-β. Reporter gene activity was measured 24 h after transfection. The data shown are from one representative experiment out of three experiments performed in total each with very similar results. Data represent the mean ± S.D. of six wells processed in parallel and are expressed relative to the value in untreated vector-transfected cells set arbitrarily at 1. C, caTβRɪmL45 is unable to mimic the TGF-β effect on BGN in MG-63 cells. Wild type MG-63 cells and polyclonal cultures of MG-63 cells stably expressing empty retroviral vector, caALK5, or caTβRɪmL45 were treated with TGF-β for 24 h and subjected to quantitative RT-PCR for BGN. D, TGF-β weakly activates p38 in MG-63 cells. Confluent cultures of MG-63 cells were serum-starved for 16 h and stimulated with TGF-β1 for the indicated times or for 1 h with anisomycin as control (Co). Cellular lysates were assayed by immunoblotting for phospho-p38 and total p38.

**FIG. 3.** The Smad activating function of ALK5 is required for TGF-β-induced activation of p38 and p38-dependent gene expression. A, the ability of ALK5 to phosphorylate Smad2 and p38 depends on its Smad-activating function. PANC-1 cells were transiently transfected with either 10 μg of pFLAG-Smad2 or pFLAG-p38 together with empty vector, or HA-tagged versions of caALK5, or caTβRɪmL45. After 24 h empty vector-transfected cells were stimulated, or not, with TGF-β for 2 h followed by lysis and immunoprecipitation (IP) of FLAG-tagged and HA-tagged proteins. Immunoprecipitates were subjected to SDS-PAGE and immunoblotting (IB) with antibodies recognizing phosphorylated forms of Smad2 (p-Smad2) and p38 (p-p38), Smad2 (t-Smad2) and p38 (t-p38), or ALK5. B, caTβRɪmL45 is unable to activate a Smad-responsive reporter. PANC-1 cells were transiently transfected with empty vector, caALK5, or caTβRɪmL45 along with pSBE-Luc and pRL-TK as described under “Experimental Procedures” and treated, or not, with TGF-β. Reporter gene activity was measured 24 h after transfection. The data shown are from one representative experiment out of three experiments performed in total each with very similar results. Data represent the mean ± S.D. of six wells processed in parallel and are expressed relative to the value in untreated vector-transfected cells set arbitrarily at 1. C, caTβRɪmL45 is unable to mimic the TGF-β effect on BGN in MG-63 cells. Wild type MG-63 cells and polyclonal cultures of MG-63 cells stably expressing empty retroviral vector, caALK5, or caTβRɪmL45 were treated with TGF-β for 24 h and subjected to quantitative RT-PCR for BGN. D, TGF-β weakly activates p38 in MG-63 cells. Confluent cultures of MG-63 cells were serum-starved for 16 h and stimulated with TGF-β1 for the indicated times or for 1 h with anisomycin as control (Co). Cellular lysates were assayed by immunoblotting for phospho-p38 and total p38.
the antisense GADD45β expression (Fig. 6D). These data clearly indicate that TGF-β/ALK5-dependent expression of endogenous GADD45β is necessary and specific for TGF-β-mediated induction of BGN.

DISCUSSION

In this study we have analyzed the role of ALK5 in TGF-β regulation of GADD45β and BGN in PANC-1 and MG-63 cells. Using a combination of kinase-deficient and kinase-active mutants as well as specific pharmacological inhibitors, we present evidence that both the kinase activity and the Smad-activating function of ALK5 are crucial for up-regulation of both GADD45β and BGN expression. We go on to show that GADD45β acts as a signal transducer in the signaling pathway leading from activated ALK5 to BGN via intermittent activation of p38 MAPK. This conclusion was drawn from the observation that ectopic expression of GADD45β in PANC-1 cells mimicked the TGF-β effect on p38 and BGN, whereas antisense-mediated suppression of TGF-β-induced GADD45β expression dose-dependently inhibited p38 activation and BGN up-regulation by this growth factor. Fig. 7 integrates GADD45β into the signaling pathway involved in TGF-β regulation of BGN expression.

We have previously reported that TGF-β regulation of BGN required both functional Smad4 expression (25) and activation of p38 (27) and have shown that p38 activation occurred downstream of Smad activation (27). We sought to confirm the Smad dependence of p38 and BGN directly at the receptor level by investigating, if the ability of activated ALK5 to induce p38 and BGN was due to direct Smad activation by the ALK5 kinase. For this purpose we expressed an ALK5-derived mutant with an intact kinase domain but deficient in its ability to activate R-Smads by phosphorylation (10). This mutant was unable to activate p38 and p38-dependent gene expression, e.g. BGN, in transiently transfected and stably transduced PANC-1 and MG-63 cells, respectively. The data obtained with this mutant together with the Smad4 reconstitution experiments reported earlier (27) clearly indicate that the Smad-activating function of ALK5 is required for p38 activation and BGN induction and that p38 activation is Smad-dependent in these cells rather than being Smad-independent as described in NMuMG cells.
GADD45β expression activates p38 and induces BGN expression. A, detection of ectopic GADD45β in individual clones (S#7 and S#8) of PANC-1 cells stably infected with a GADD45β encoding retrovirus by immunoblot analysis. Basic endogenous levels of GADD45β in unstimulated empty vector-transduced controls (left lane) and wild type cells (data not shown) were hardly detectable, but were estimated by quantitative RT-PCR to be 10-times lower than in vector-transduced cells treated for 1.5 h with TGF-β (compare Fig. 6B). B, ectopic expression of GADD45β activates p38 in the absence of TGF-β treatment in PANC-1 cells. As control, vector-transduced cells were stimulated with TGF-β for 1.5 h. The phosphorylated p-38 (p-p38) and total p38 (t-p38) are shown in the upper and lower panels, respectively. C and D, ectopic expression of GADD45β induces BGN, but not PAI-1 mRNA. Unstimulated PANC-1 cells stably transduced with either an empty retrovirus (vector) or a GADD45β encoding retrovirus (clones #7 and #8) were subjected to quantitative RT-PCR for BGN and PAI-1.

Earlier studies using cycloheximide indicated that de novo protein synthesis is required for TGF-β regulation of BGN in PANC-1 cells (25). Given the crucial role of intermittent p38 activation for BGN induction and the recent discovery that TGF-β/ALK5-mediated activation of p38 is accomplished through Smad-regulated transcriptional activation of MyD118 (13, 14), we hypothesized that suppressing TGF-β-stimulated endogenous GADD45β expression would result in inhibition of both p38 activation and BGN induction. To this end, antisense-mediated suppression of GADD45β expression in PANC-1 cells prevented p38 activation and blocked the TGF-β effect on BGN in a dose-dependent fashion. Having shown that ALK5 mediates the TGF-β effect on p38 and BGN, we analyzed whether ALK5 was also able to activate MyD118. Notably, the GADD45β gene remained silent in TGF-β-treated PANC-1 cells upon SB431542-mediated inhibition of the ALK5 kinase activity and in PANC-1 cells stably expressing caTβRlMl45, but was activated in caALK5-expressing cells. The differential responsiveness of the GADD45β gene was confirmed by promoter-reporter gene assays in MG-63 cells, which showed that kinase-active ALK5, but not kinase-active TβRI/Ml45 directly activated the upstream GADD45β promoter. Under similar conditions, PANC-1 cells were unresponsive to this construct (data not shown), the reason of which is not clear but may reflect tissue-specific differences in promoter usage (see below).

In a parallel approach, we observed that stable overexpression of GADD45β in PANC-1 cells was able to rescue activation of p38 and BGN induction. GADD45β has also been implicated in TGF-β-induced apoptosis (10, 13), and Yoo et al. (15) demonstrated that ectopic expression of GADD45β is sufficient to activate p38 and to trigger apoptosis.

In addition to elucidating the molecular pathway acting upstream of p38 and BGN, we have revealed histogenetic differences in the signaling pathways involved in TGF-β regulation of BGN. In PANC-1 cells strong activation of MyD118 transcription by TGF-β correlated with greater induction of p38 activation and a dramatic rise of BGN expression within 24 h, whereas in MG-63 cells a comparatively small increase in GADD45β mRNA corresponded with a lower level of p38 activation and only a moderate up-regulation of BGN, further lending support to the ALK5-GADD45β-BGN connection. It thus appears that the powerful induction of BGN mRNA by TGF-β in PANC-1 cells is the consequence of a stronger activation of MyD118, an issue that may be resolved by defining the characteristics of MyD118 transcriptional regulation. Major and Jones (29) have found that the GADD45β 5′-promoter sequence (which is contained in the G45β-1 construct) responds to TGF-β with an ~2-fold increase in transcriptional activity in HaCaT and Mv1Lu cells. In addition, these authors identified a second TGF-β-responsive module encompassing the highly conserved third intron of the GADD45β gene, which conferred 3-fold greater transcriptional induction by TGF-β than the 5′-promoter sequence (29). In MG-63 cells, caALK5-induced activity of the 5′-promoter sequence and TGF-β-stimulated GADD45β
FIG. 6. Antisense-mediated inhibition of GADD45β blocks TGF-β-mediated p38 activation and BGN expression. A, expression of GADD45β antisense RNA suppresses TGF-β-induced GADD45β mRNA accumulation. Individual clones of PANC-1 cells stably transduced with an empty retroviral vector (vector) or an GADD45β antisense encoding retrovirus were screened for GADD45β mRNA expression. Following a 1-h incubation of the cells with or without TGF-β, total RNA was prepared and subjected to both semiquantitative (left panel) and quantitative (right panel) RT-PCR using primers specific for the sense GADD45β transcript. B, expression of GADD45β antisense RNA suppresses TGF-β-induced activation of p38. Phosphorylation of endogenous p38 in total cell extracts prepared from the same cells in A, untreated or treated with TGF-β for 1.5 h, was analyzed by immunoblotting. The phosphorylated p-38 (p-p38) and total p38 (t-p38) are shown in the upper and lower panels, respectively. C and D, expression of GADD45β antisense RNA suppresses TGF-β induction of BGN, but not PAI-1 expression. PANC-1 cells stably transduced with either an empty retrovirus (vector) or a GADD45β encoding retrovirus (clones #7 and #8) were treated with or without TGF-β for 24 h and subjected to quantitative RT-PCR for BGN and PAI-1.

FIG. 7. Current model of the signal flow involved 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 BGN mRNA. Upon activation of TβRII and ALK5, the Smad-activating function of ALK5 residing in the L45 loop (L45) phosphorylates an R-Smad, which subsequently forms a complex with Smad4, and this complex translocates to the nucleus to induce expression of GADD45β. GADD45β then activates p38 MAPK via activation of a MAPKKK, possibly MTK1/MEKK4. Active p38 is shuttled to the nucleus and induces BGN mRNA accumulation through nuclear mRNA transcript processing, stability, and/or export.
mRNA levels were both increased ~2-fold, suggesting that bone cells utilize this 5′-promoter. In contrast, PANc-1 cells are more likely to utilize the intronic enhancer, since endogenous GADD45β transcript levels were induced 10-fold by TGF-β. Experiments are in progress to clarify if the greater induction of GADD45β in PANc-1 cells is indeed caused by activation of this intronic promoter. Interestingly, transcription from this enhancer was strongly dependent on Smad4, which is in close agreement with the observation that re-expression of Smad4 in Smad4-deficient pancreatic cancer cell lines restored not only TGF-β-stimulated GADD45β expression (14) but also TGF-β-mediated p38 activation (27) and BGN expression (25).

Our observation that the enforced expression of GADD45β alone can stimulate the BGN response opens the real possibility that BGN is up-regulated independently from TGF-β in physiological situations in which GADD45β is induced, e.g. during terminal differentiation (12), environmental stress-induced apoptosis (12), acute hypertonicity (34), or during an inflammatory response to invading microorganisms (35, 36). Of clinical relevance for the treatment of pancreatic carcinoma is the observation that GADD45 family proteins are rapidly induced by genotoxic agents (12, 37). Their use in the course of anticancer therapy may thus promote BGN synthesis through TGF-β-p38 signaling, which may play an important role in tumor inhibition. Taken together, the results presented in this study establish a central role for ALK5-induced GADD45β in TGF-β regulation of BGN.

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REFERENCES
1. Massagué, J., Blain, S. W., and Lo, R. S. (2000) Cell 103, 295–309
2. Moustakas, A., Pardali, K., Gaal, A., and Heldin, C. H. (2002) Immunol. Lett. 82, 85–91
3. Derynck, R., Akhurst, R. J., and Balmain, A. (2001) Nat. Genet. 29, 117–129
4. Derynck, R., and Zhang, Y. E. (2003) Nature 425, 577–584
5. ten Dijke, P., Ichijo, H., Franzen, P., Schulz, P., Saras, J., Toyoshima, H., Heldin, C. H., and Miyazono, K. (1993) Oncogene 8, 2879–2887
6. Ebner, R., Chen, R. H., Lawler, S., Ziemcheck, T., Lee, A., Lopez, A. R., and Derynck, R. (1993) Science 260, 1344–1348
7. Franžén, P., ten Dijke, P., Ichijo, H., Yamashita, H., Schulz, P., Heldin, C. H., and Miyazono, K. (1993) Cell 75, 681–692
8. Ellender, V., Hendler, S. F., Bock, W., Saffair, T., Menke, A., Ruhlrand, C., Adler, G., and Gress, T. M. (2001) Cancer Res. 61, 4222–4228
9. Bakin, A. V., Rinehart, C., Tomlinson, A. K., and Arteaga, C. L. (2002) J. Cell Sci. 115, 3139–3206
10. Yu, L., Hebert, M. C., and Zhang, Y. E. (2002) EMBO J. 21, 3749–3759
11. Ichijo, H., Toyokaito, S., Kowantz, M., Moustakas, A., Ichijo, H., and ten Dijke, P. (2002) J. Biol. Chem. 277, 5751–5758
12. Lieberman, D. A., and Hoffman, B. (2002) Leukemia 16, 527–541
13. You, J., Giass, M., Jirmanova, L., Balliet, A. G., Hoffinan, B., Narance, A. J., Jr., Lieberman, D. A., Bottinger, E. P., and Roberts, A. B. (2003) J. Biol. Chem. 278, 43001–43007
14. Takekawa, M., Tateyashia, K., Ichijo, H., Adachi, M., Imai, K., and Sato, H. (2002) EMBO J. 21, 6473–6482
15. Takekawa, M., and Sato, H. (1998) Cell 95, 521–530
16. Iozzo, R. V. (1999) J. Biol. Chem. 274, 18843–18846
17. Kinlessa, M. G., Bressler, S. L., and Wight, T. N. (2004) Crit. Rev. Eukaryot. Gene Expr. 14, 203–254
18. Young, M. F., Bi, Y., Ameye, L., and Chen, X. D. (2002) Glycoconj. J. 19, 257–262
19. Xu, T., Bianco, P., Fisher, L. W., Longenecker, G., Smith, E., Golstein, S., Bonadio, J., Boskey, A., Hageaard, A. M., Somner, B., Satomura, K., Dominguez, P., Zhao, C., Kulkarni, A. B., Robey, P. G., and Young, M. F. (1998) Nat. Genet. 20, 78–82
20. Westergren-Thorsson, G., Hermans, J., Sarnarstrand, B. L., Oldberg, A., Hein-egard, D., and Malamst, A. (1983) J. Clin. Invest. 62, 632–637
21. Gressner, A. M., Krull, N., and Bachem, M. G. (1994) Pathol. Res. Pract. 190, 864–882
22. Schaefer, L., Raslik, I., Grone, H. J., Schonherr, E., Macakova, K., Ugorcakova, J., Budry, S., Schaefer, R. M., and Kresse, H. (2002) J. Biol. Chem. 277, 34181–34187
23. Schaefer, L., Raslik, I., Grone, H. J., Schonherr, E., Macakova, K., Ugorcakova, J., Budry, S., Schaefer, R. M., and Kresse, H. (2002) J. Biol. Chem. 277, 15571–15575
24. Ungefroren, H., and Krull, N. B. (1996) J. Biol. Chem. 271, 15787–15795
25. Ungefroren, H., Lenschow, W., Tiede, K., Fischer, J. W., Kalthoff, H., and Ungef-ronen, H. (2002) J. Biol. Chem. 277, 36118–36128
26. Ungerehen, H., and Krull, N. B. (1996) J. Biol. Chem. 271, 15787–15795
27. Ungerehen, H., Lenschow, W., Chen, W. B., Faath, H., and Kalthoff, H. (2003) J. Biol. Chem. 278, 11041–11049
28. Dai, D. J., Turnacaglu, K. G., Schutte, M. S., and Kern, S. E. (1998) Cancer Res. 58, 4592–4597
29. Major, M. B., and Jones, D. A. (2003) J. Biol. Chem. 278, 5278–5287
30. Vincent, F., Nagashima, M., Takeonishi, S., Khan, M. A., Gemma, A., Hagi-wara, K., and Bennett, W. P. (1997) Oncogene 15, 117–122
31. Knaus, P. I., Lindemann, D., DeCoteau, J. F., Perlman, R., Vankele, H., Hille, M., Kadin, M. E., and Lodish, H. F. (1996) Mol. Cell. Biol. 16, 3480–3489
32. Inman, G. J., Nicolas, F. J., Callahan, J. F., Harling, J. D., Gaster, L. M., Reith, A. D., Laping, N. J., and Hill, C. S. (2002) Mol. Pharmacol. 62, 65–74
33. Wieser, R., Wrana, J. L., and Massagué, J. (1995) EMBO J. 14, 2199–2208
34. Chakravarty, D., Cai, Q., Ferraris, J. D., Michea, L., Burg, M. B., and Kultz, D. (2002) Am. J. Physiol. 283, F1020–F1029
35. Yang, J., Zhu, H., Murphy, T. L., Ouyang, W., and Murphy, K. M. (2001) Nat. Immunol. 2, 157–164
36. Lui, B., Ferrandino, A. F., and Flavell, R. A. (2004) Nat. Immunol. 5, 38–44
37. Chat, Z., Clark, S., Birkeland, M., Sung, C. M., Lago, A., Liu, R., Kirkpatrick, R., Johanson, K., Winkler, J. D., and Hu, E. (2002) Cancer Lett. 188, 127–140
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