Smad-dependent GADD45 β expression mediates delayed activation of p38 MAP kinase by TGF- β

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Transforming growth factor- β (TGF- β), when bound to its specific receptor, activates the transcription factor Smad by phosphorylation. TGF- β also activates the p38 MAPK pathway, but there seem to be disparate mechanisms for the early p38 activation and delayed p38 activation. In this report, we demonstrate that Smad-dependent expression of GADD45 β is responsible for the delayed activation of p38 by TGF- β . The GADD45 β protein binds and activates MTK1 (= MEKK4), which is a member of the MAPKKK family kinases and an upstream activator of the p38 MAPK cascade. Both TGF- β -induced GADD45 β expression and the delayed p38 activation require functional Smad proteins. Antisense inhibition of GADD45 β expression suppresses the TGF- β -induced delayed p38 activation, whereas overexpression of GADD45 β activates the p38 MAPK via MTK1. Expression of the angiogenesis inhibitor thrombospondin-1 (TSP-1) is induced by TGF- β via Smad-dependent p38 activation. Thus TGF- β -induced p38 activation, mediated by GADD45 β expression, may play an important role in the biological effects of TGF- β .

Keywords: GADD45 β /p38/Smad/TGF- β /thrombospondin-1

Introduction

Transforming growth factor- β (TGF- β) belongs to a family of multifunctional cytokines that regulate essential cellular functions, such as proliferation, differentiation, apoptosis, extracellular matrix production and angiogenesis (Derynck and Feng, 1997; Massagué, 1998). An altered cellular response to TGF- β has been postulated as a mechanism whereby cells undergo neoplastic transformation, because many cancers of epithelial and lymphoid origins are resistant to the negative growth-regulatory effects of TGF- β (Markowitz *et al.*, 1995; Hahn *et al.*, 1996; Thiagalingam *et al.*, 1996; Derynck *et al.*, 2001). TGF- β exerts its biological effects by binding to a cell

surface receptor complex composed of type I (T β RI) and type II (T β RII) receptor serine/threonine kinases (Wrana *et al.*, 1994). Upon ligand binding, T β RII phosphorylates and activates T β RI, which subsequently phosphorylates the cytoplasmic Smad2 and Smad3 proteins. Phosphorylated Smad proteins form stable complexes with a common partner, Smad4 (also known as DPC4). The activated Smad2/4 and Smad3/4 complexes translocate into the nucleus, where the Smad dimers induce transcriptional activation (or inhibition) of specific target genes in cooperation with other transcription factors (Macias-Silva *et al.*, 1996; Zhang *et al.*, 1996; Nakao *et al.*, 1997; Massagué and Wotton, 2000; ten Dijke *et al.*, 2000). Underscoring the importance of Smad proteins in TGF- β signaling, disruption of Smad pathway components abated transcriptional responses to TGF- β . Furthermore, Smad4 mutations are observed frequently (~50%) in human pancreatic carcinomas (Hahn *et al.*, 1996; Schutte *et al.*, 1996; Villanueva *et al.*, 1998).

Beside the Smad proteins, TGF- β also activates the p38 MAPK pathway, which may play an important role in TGF- β -induced gene expression (Hanafusa *et al.*, 1999; Sano *et al.*, 1999). In this regard, previous studies indicate that TAK1, a member of the MAPKKK family, is involved in TGF- β -induced p38 activation (Yamaguchi *et al.*, 1995; Shibuya *et al.*, 1996). T β RI interacts indirectly with TAK1 via bridging proteins XIAP and TAB1 (Yamaguchi *et al.*, 1999; Reffey *et al.*, 2001). It has been suggested further that TGF- β activates p38 MAPK via TAK1 without any involvement of the Smad transcription factors (Sano *et al.*, 1999; Yamaguchi *et al.*, 1999; Reffey *et al.*, 2001). Activation of TAK1 alone, however, seems insufficient to account for the *in vivo* activation of p38 in response to TGF- β . TGF- β induces TAK1 activity rapidly but only transiently; TAK1 activity peaks at ~10 min following TGF- β stimulation, and declines to basal level by 30 min (Yamaguchi *et al.*, 1995; Zhang *et al.*, 2000). Rapid activation of p38 by TGF- β , consistent with the kinetics of TAK1 activity, has been reported in some cell lines (Hannigan *et al.*, 1998; Hanafusa *et al.*, 1999; Sano *et al.*, 1999). In many other cell types (e.g. pancreatic acinar cells, keratinocytes, osteoblasts, gingival fibroblasts and rat hepatocytes), however, maximal p38 activation occurs only 1–2 h following TGF- β stimulation, and the activity persists for several hours (Ravanti *et al.*, 1999; Johansson *et al.*, 2000; Karsdal *et al.*, 2001; Herrera *et al.*, 2001; Simeone *et al.*, 2001; Schiffer *et al.*, 2001; Schrantz *et al.*, 2001). These findings suggest that there is an additional, TAK1-independent, signaling mechanism that mediates TGF- β -induced p38 activation.

We previously have demonstrated that the stress- and cytokine-inducible GADD45-family proteins (GADD45 α , β and γ) function as specific activators of MTK1 (also known as MEKK4), a MAPKKK upstream in the p38

pathway (Takekawa and Saito, 1998; Mita *et al.*, 2002). In this report, we provide evidence that GADD45 β is an important mediator of TGF- β -induced delayed activation of p38. TGF- β induces GADD45 β expression and p38 activation in pancreatic carcinoma cells, both in a Smad-dependent manner. Antisense inhibition of GADD45 β expression suppresses p38 activation induced by TGF- β , whereas ectopic GADD45 β expression activates p38 MAPK via MTK1 MAPKKK, demonstrating that Smad-induced GADD45 β is an essential upstream activator of p38 induced by TGF- β . We will demonstrate further that TGF- β -induced expression of thrombospondin-1 (TSP-1), a potent inhibitor of tumor cell growth and angiogenesis, is mediated by the Smad-GADD45 β -p38 pathway. TSP-1 secreted by TGF- β -stimulated pancreatic cancer cells has an anti-angiogenic activity *in vitro*. Based on these observations, we propose that TGF- β -induced p38 activation is mediated by Smad-dependent GADD45 β expression, and that this process may play an important role in tumor suppression by TGF- β .

Results

Smad4 is required for TGF- β -induced p38 activation in pancreatic cell lines

In order to clarify the molecular mechanism of p38 activation by TGF- β , we first examined the time course of p38 MAPK activation in TGF- β -responsive (Panc-1) and TGF- β -unresponsive (MiaPaCa-2, AsPc-1 and BxPc-3) pancreatic cancer cell lines (Freeman *et al.*, 1995; Schutte *et al.*, 1996; Grau *et al.*, 1997; Villanueva *et al.*, 1998; Giehl *et al.*, 2000). MiaPaCa-2 does not express T β RII, whereas AsPc-1 and BxPc-3 are null for Smad4 (Freeman *et al.*, 1995; Schutte *et al.*, 1996; Villanueva *et al.*, 1998; Barbera *et al.*, 2000). These cell lines were treated with either methyl methanesulfonate (MMS) or TGF- β , and the activity of p38 MAPK was monitored by immunoblotting using an antibody specific to the phosphorylated (activated) p38. Robust activation of p38 by MMS was observed in all the cell lines, indicating that there is no defect in the p38 MAPK *per se* (Figure 1A).

With TGF- β , however, p38 activation was observed only in the TGF- β -responsive Panc-1 cells: p38 was active at 1 h following TGF- β stimulation, and reached its maximum level by 2 h (Figure 1A). We call this a delayed response, because the p38 response is much faster in certain other cells (see Figure 2D). The total amount of p38 did not change during the period of observation. The delayed response was not limited to the pancreatic carcinoma cells, because a similar time course was observed in primary cultures of human dermal fibroblasts and in the immortalized human keratinocytes, HaCaT (Figure 1B).

In contrast, p38 activation was not observed in the TGF- β -non-responsive cells. It might be expected that T β RII-deficient MiaPaCa-2 cells did not activate p38 upon TGF- β treatment. More importantly, however, TGF- β also failed to activate p38 in the Smad4-null cell lines, AsPc-1 and BxPc-3, which express functional TGF- β receptors, suggesting that TGF- β -induced p38 activation requires the Smad proteins in these pancreatic cell lines.

To confirm the involvement of Smad proteins in the TGF- β -induced p38 activation, we exploited the previous

Fig. 1. Activation of p38 MAPK by TGF- β . (A) TGF- β -induced p38 activation in pancreatic carcinoma cells. TGF- β -responsive (Panc-1) and -unresponsive (MiaPaCa-2, AsPc-1 and BxPc-3) pancreatic carcinoma cells were treated with TGF- β (10 ng/ml) or MMS (100 μ g/ml), and cell extracts were prepared at the indicated time points. The phosphorylation status of endogenous p38 was analyzed by immunoblotting using an antibody specific to phosphorylated p38 (P-p38). The total level of p38 in cell lysates is also indicated (p38). (B) TGF- β -induced delayed activation of p38. Primary cultures of human dermal fibroblasts (HDFs) and an immortalized human keratinocyte cell line, HaCaT cells, were treated with TGF- β (10 ng/ml) and subjected to immunoblot analyses as in (A).

observation that overexpression of Smad proteins can induce gene expression even in the absence of exogenous TGF- β (Zhang *et al.*, 1996; Nakao *et al.*, 1997). HeLa cells were transiently transfected with expression plasmids for Smad3 and Smad4 together with Flag-tagged p38. Some cells were also transfected with either wild-type T β RI or constitutively active T β RI(T204D). The Flag-p38 protein was immunoprecipitated from cell lysates, and its kinase activity was measured *in vitro* using GST-ATF2 as a specific substrate. Figure 2A, lane 1 shows the basal Flag-p38 activity when neither T β RI nor Smad vector was co-transfected. When cells were co-transfected with expression plasmids for Smad3 and Smad4, a significant increase in p38 kinase activity was observed (lane 2). Expression of the constitutively active T β RI(T204D), but not wild-type T β RI, also increased p38 activity (lanes 3 and 5). Finally, co-expression of the Smad3 and Smad4 proteins with the constitutively active T β RI(T204D) synergistically activated p38 (lane 6).

We also examined the role of the Smad proteins in TGF- β -induced p38 activation using a dominant-negative Smad4 mutant (Smad4 Δ C) (Zhang *et al.*, 1996). For this experiment, HeLa cells were co-transfected with T β RI(T204D) and Flag-p38 expression plasmids, together with increasing amounts of the dominant-negative Smad4 Δ C plasmid, and the activity of immunoprecipitated Flag-p38 was assayed *in vitro*. As seen in Figure 2B,

Fig. 2. Smad-dependent activation of p38 MAPK. (A) Overexpression of Smad proteins activates p38 MAPK. HeLa cells were co-transfected with expression vectors for Flag-p38 (0.05 μ g/plate) and, where indicated, HA-T β RI (T β RI; 0.2 μ g/plate), constitutively active HA-T β RI [T β RI(T204D); 0.2 μ g/plate], Flag-Smad3 (0.1 μ g/plate) and Flag-Smad4 (0.1 μ g/plate). The total amount of transfected plasmid DNA was kept constant by including appropriate amounts of pcDNA3. After 36 h, kinase activity of immunoprecipitated Flag-p38 was measured *in vitro* using an exogenous substrate GST-ATF2 (upper panel). The expression levels of Flag-p38 (middle panel) and Flag-Smad3/4 (lower panel) proteins in the total cell lysates were monitored by immunoblotting using an anti-Flag antibody. (B) Dominant-negative Smad4 (Smad4 Δ C) inhibits p38 activation induced by the constitutively active T β RI(T204D). HeLa cells were transfected with expression vectors for Flag-p38 (0.05 μ g/plate), together with HA-T β RI(T204D) (0.1 μ g/plate) and Smad4 Δ C as indicated. Kinase activity of Flag-p38 was assayed *in vitro* as in (A). The middle and lower panels show the amounts of the Flag-p38 and HA-T β RI(T204D) proteins in the total cell lysates. (C) Reintroduction of Smad4 into Smad4-null cells restores TGF- β -induced p38 activation. Smad4-deficient BxPc-3 and AsPc-1 cells were infected with a recombinant adenovirus expressing Smad4 or a control GFP adenovirus (Control). At 24 h post-infection, the cells were treated with TGF- β (10 ng/ml) for 2 h and cell extracts were prepared. The phosphorylation status of endogenous p38 was analyzed by immunoblotting using an antibody specific to phosphorylated p38 (upper panel). The total level of p38 (middle panel) and Smad4 (lower panel) in cell lysates is also shown. These experiments (A–C) were repeated at least three times with similar results. (D) Rapid and sustained activation of p38 MAPK by TGF- β in C2C12 cells. TGF- β -induced p38 activation in a mouse myoblast cell line, C2C12, was analyzed by immunoblotting as in Figure 1. (E) Smad4 Δ C selectively inhibits delayed activation of p38 by TGF- β . C2C12 cells were transfected with expression vectors for Flag-p38 (0.1 μ g/plate), together with 0.3 μ g of either the pSmad4 Δ C expression vector (+) or the empty vector pcDNA3 (–). After 36 h, cells were treated with TGF- β (10 ng/ml). Kinase activity of Flag-p38 was assayed *in vitro* as in (A). The fold activation of p38 was determined by FLA-3000 imageanalyzer (Fujifilm) from three independent experiments and is indicated below each lane. Error bars indicate the SEM.

Smad4 Δ C suppressed T β RI(T204D)-induced p38 activity in a dose-dependent manner. These results corroborate the notion that Smad proteins are required for the TGF- β -induced p38 activation.

We next employed gene transfer experiments to test whether the lack of the Smad4 protein alone accounts for the unresponsiveness of BxPc-3 and AsPc-1 (Figure 2C). An efficient gene transfer into these cell lines was achieved by a recombinant adenovirus encoding wild-type Smad4 (Fujii *et al.*, 1999). A recombinant virus encoding green fluorescent protein (GFP) was used as a control. The expression of Smad4 in infected cells was confirmed by western blot analysis (bottom panels). Consistent with earlier results, TGF- β did not activate p38 in the control GFP-expressing cells (lanes 2 and 6). In contrast, re-establishment of Smad4 expression in the same cell lines restored TGF- β -induced activation of p38 (lanes 4 and 8), thus proving that a lack of Smad4 protein is the cause of unresponsiveness in these cell lines.

To test whether Smad proteins are also involved in the early activation of p38 by TGF- β , we assessed the effect of

the dominant-negative Smad4 Δ C in C2C12 cells, a mouse myoblast cell line. As reported (Hanafusa *et al.*, 1999), TGF- β induced p38 activation in C2C12 cells by as early as 15 min, which was sustained for at least 2 h (Figure 2D). Expression of Smad4 Δ C significantly suppressed p38 activation at 2 h following TGF- β stimulation, but not at 15 min (Figure 2E). These findings suggest that Smad-dependent signaling mediates TGF- β -induced delayed p38 activation, while the rapid p38 activation occurs independently of Smad. We thus conclude that delayed, but not early, p38 activation induced by TGF- β involves Smad in different cell types.

TGF- β induces GADD45 β expression in a Smad-dependent manner

Because the Smad proteins function primarily in transcriptional regulation, it is likely that TGF- β activates p38 in these cells through Smad-induced gene expression. We previously found that the GADD45 family proteins (GADD45 α , β and γ) directly bind to the N-terminal regulatory domain of MTK1, a human MAPKKK involved

Fig. 3. Expression of the GADD45-family genes in various cell lines. Panc-1 (A), MiaPaCa-2 (B), human dermal fibroblasts (HDFs) (C; left panel) or HaCaT cells (C; right panel) were treated with TGF-β or MMS for various times, as indicated. Total RNA (20 μg/lane) was separated by agarose gel electrophoresis and hybridized with either a ³²P-labeled GADD45α or a β cDNA probe. Ethidium bromide (EtBr)-stained gels of the same samples are shown at the bottom to indicate the amounts of applied RNA.

in the p38 pathway, and thereby activate its kinase domain (Takekawa and Saito, 1998; Mita *et al.*, 2002). Expression of GADD45 family genes is induced by environmental stresses (e.g. UV, MMS and high osmolarity), as well as by certain cytokines [e.g. interleukin (IL)-18 and IL-6] (Zhan *et al.*, 1994; Takekawa and Saito, 1998; Lu *et al.*, 2001; Yang *et al.*, 2001). To test the possible involvement of the GADD45 proteins in Smad-mediated p38 activation, we analyzed GADD45 expression following TGF-β stimulation. MMS, a potent GADD45 inducer, was used as a positive control. As shown in Figure 3, TGF-β induced GADD45β mRNA in the TGF-β-responsive Panc-1 cells (Figure 3A), but not in the TβRII-deficient MiaPaCa-2 cells (Figure 3B). Because MMS induced GADD45β mRNA expression in both cell lines, there is no intrinsic defect in the GADD45β gene itself. It should be noted that the time course of the GADD45β mRNA expression in Panc-1 cells approximates that of TGF-β-induced p38 activation. TGF-β-induced neither GADD45α nor GADD45γ in these cells (Figure 3, and data not shown). TGF-β induction of GADD45β mRNA expression was also observed in human dermal fibroblasts and immortal-

Fig. 4. TGF-β-induced GADD45β expression requires Smad4. (A) The absence of TGF-β-stimulated GADD45β mRNA induction in Smad4-deficient BxPc-3 and AsPc-1 cells. (B) Re-expression of Smad4 in the same Smad4-null cell lines restores TGF-β-induced GADD45β expression. Cells were infected with a recombinant adenovirus expressing Smad4 or a control GFP adenovirus (Control). At 24 h post-infection, the cells were treated for 2 h with TGF-β, and total RNA was prepared for northern blot analysis, as in Figure 3.

ized human keratinocytes, HaCaT cells (Figure 3C). Thus, TGF-β-induced expression of GADD45β was observed in a number of different cell types we examined.

If Smad is responsible for the TGF-β-induced GADD45β expression, no induction of GADD45β mRNA expression would be expected in Smad4-null cell lines, such as BxPc-3 and AsPc-1. Indeed, in these cell lines, no significant GADD45β expression was induced by TGF-β (Figure 4A). To exclude the possibility that these cell lines harbor additional mutation(s) that are in fact responsible for their failure to induce GADD45β expression, these cells were infected with a recombinant adenovirus encoding wild-type Smad4. While the control, GFP virus-infected cells did not respond to TGF-β at all, the Smad4 virus-infected cells induced strong GADD45β expression upon TGF-β stimulation (Figure 4B). Thus, in these Smad4-deficient cells, expression of Smad4 restores both TGF-β-induced p38 activation (Figure 2C) and GADD45β expression (Figure 4B).

TGF-β-induced GADD45β expression activates the p38 MAPK via MTK1

The results in the previous section are consistent with either of the following two models. Model 1: the activated Smad complex induces GADD45β expression and, concomitantly but independently, activates p38 MAPK. Model 2: the activated Smad complex induces GADD45β expression, which subsequently activates p38 MAPK. Model 2 is more attractive in the light of our previous observation that GADD45β activates MTK1, a MAPKKK upstream in the p38 MAPK pathway

Fig. 5. Antisense GADD45 β inhibition suppresses TGF- β -induced p38 activation. (A) RT-PCR detection of GADD45 β sense transcripts in the parental Panc-1 cells, and the Panc-1 cells stably transfected with either an empty vector (Vector) or an antisense GADD45 β expression vector (AS1 and AS2). Following 2 h incubation of the cells with (+) or without (–) TGF- β , total RNA was prepared and subjected to RT-PCR using primers specific to the GADD45 β sense transcript. PCR products were analyzed by agarose gel electrophoresis. (B) GADD45 β antisense RNA suppresses p38 activation in response to TGF- β . After 2 h incubation of the same cells as in (A) with (+) or without (–) TGF- β , the phosphorylation status of the endogenous p38 was analyzed by immunoblotting using the anti-phospho p38 antibody (upper panel). The level of p38 in cell lysates is also shown (lower panel). (C) Effects of antisense GADD45 β on TGF- β -induced p38 activation in HaCaT cells. HaCaT cells were transiently transfected with Flag-p38 expression plasmid together with empty vector pcDNA3 (vector) or antisense GADD45 β expression vector (pAS). After 36 h, cells were treated with TGF- β (+) or left untreated (–). After 2 h, the kinase activity of Flag-p38 was determined as in Figure 2A.

(Takekawa and Saito, 1998; Mita *et al.*, 2002). In order to determine which is the correct model, we first tested whether GADD45 β expression is required for TGF- β -induced p38 activation. For this purpose, TGF- β -responsive Panc-1 cells were stably transfected with antisense (AS) GADD45 β cDNA, thereby blocking expression of the endogenous GADD45 β gene. As a control, the same cells were transfected with the empty vector. In each transfectant, GADD45 β mRNA levels, before and after TGF- β stimulation, were assayed by RT-PCR. The antisense GADD45 β effectively suppressed the endogenous GADD45 β expression, as seen in Figure 5A. We then assessed the p38 activity in these cells using anti-phospho-p38 immunoblot analysis. Figure 5B shows that GADD45 β antisense RNA significantly suppressed p38 activation in response to TGF- β stimulation, indicating a requirement for GADD45 β expression in TGF- β -induced p38 activation. We further tested the effect of reduced GADD45 β expression on p38 activation in the TGF- β -responsive HaCaT cells. A Flag-p38 expression plasmid

was transiently co-transfected with either an empty vector or an antisense GADD45 β expression vector into HaCaT cells. Kinase activity of immunoprecipitated Flag-p38 was then assayed *in vitro*. As shown in Figure 5C, expression of GADD45 β antisense RNA suppressed p38 activation induced by TGF- β . We conclude, therefore, that model 2 is correct.

A corollary of this model is that ectopic expression of GADD45 β should activate p38 even in Smad4-null cells. To test this prediction, Smad4-deficient cells (AsPc-1 and BxPc-3) were transiently transfected with a GADD45 β expression plasmid together with a Flag-tagged p38 construct. Kinase activity of immunoprecipitated Flag-p38 was assayed *in vitro*. In both the Smad4-null cell lines, p38 MAPK was activated by expression of GADD45 β (Figure 6A), as predicted by the model. We then tested another prediction of the model, namely that the MTK1 MAPKKK is involved in TGF- β -induced activation of p38. Previously it was shown that a C-terminally truncated MTK1 mutant (MTK1 Δ K) has a dominant-inhibitory effect on p38 activation induced by the GADD45 family proteins (Takekawa and Saito, 1998; Yang *et al.*, 2001). Using the dominant-inhibitory MTK1 Δ K, we tested whether the activation of p38 by the constitutively active T β RI(T204D) requires MTK1 activity. Thus HeLa cells were co-transfected with a T β RI(T204D) and a Flag-p38 expression plasmid together with increasing amounts of the MTK1 Δ K expression plasmid. As seen in Figure 6B, expression of MTK1 Δ K suppressed T β RI(T204D)-induced p38 activity in a dose-dependent manner, supporting the notion that GADD45 β -MTK1 signaling mediates the activation of p38 in response to TGF- β .

To test whether other MAPKKKs that have been implicated in p38 activation are involved in the GADD45 β response, we transfected COS-7 cells with various MAPKKK constructs (Flag-MTK1, Myc-TAK1 or HA-ASK1) together with increasing amounts of the GADD45 β expression vector (Yamaguchi *et al.*, 1995; Ichijo *et al.*, 1997). The epitope-tagged MAPKKKs were immunoprecipitated from cell extracts, and their kinase activities assayed *in vitro* using a common substrate [GST-MKK6(K/A)]. As shown in Figure 6C, the catalytic activity of MTK1, but not those of TAK1 and ASK1, was stimulated by GADD45 β in a dose-dependent manner.

Smad-dependent activation of p38 is required for TSP-1 expression

Finally, we examined the role of p38 MAPK in the cellular response to TGF- β . TGF- β affects the expression of numerous genes via Smad activation. TGF- β may also influence gene expression through activation of p38, which modifies a number of transcription factors. In order to identify the subset of genes whose expression is regulated by TGF- β via p38 activation, we examined the effect of SB203580, a specific inhibitor of p38 (Lee *et al.*, 1994). Panc-1 cells were treated with TGF- β in the presence or absence of SB203580, and the gene expression pattern at three time points (0, 2 and 12 h) was profiled by differential cDNA array hybridization. Initially we identified genes whose expression is regulated (either up or down) by TGF- β . Among these TGF- β -responsive genes, we then identified genes whose TGF- β response is diminished by SB203580. From ~40 genes that met these

Fig. 6. GADD45 β mediates TGF- β -induced p38 activation via MTK1. (A) Ectopic expression of GADD45 β activates the p38 pathway in Smad4-null cell lines. The Smad4-defective BxPc-3 and AsPc-1 cells were transiently transfected with 10 μ g of pFlag-p38, together with 20 μ g of either pGADD45 β or control pcDNA3. After 24 h, the kinase activity of Flag-p38 was determined as described in Figure 2. The middle and bottom panels show the amounts of the Flag-p38 and GADD45 β proteins in the total cell lysates, respectively. (B) Dominant-inhibitory MTK1 Δ K suppresses p38 activation induced by the constitutively active T β RI(T204D). HeLa cells were transfected with expression vectors for Flag-p38 (0.05 μ g/plate), HA-T β RI(T204D) (0.1 μ g/plate) and MTK1 Δ K as indicated. The total amount of transfected plasmid DNA was kept constant by including appropriate amounts of pcDNA3. After 36 h, the kinase activity of immunoprecipitated Flag-p38 was measured by *in vitro* kinase assay. (C) GADD45 β selectively activates the MTK1 MAPKKK. COS-7 cells were transfected with 0.1 μ g of expression vectors encoding Flag-MTK1, Myc-TAK1 or HA-ASK1, together with the indicated amounts of pGADD45 β . Activities of the epitope-tagged MAPKKKs were measured in immune complex kinase assays using a specific exogenous substrate GST-MKK6(K/A). The expression levels of the MAPKKKs in cell lysates were monitored by immunoblotting. These experiments (A–C) were repeated at least three times with similar results.

criteria, we chose TSP-1, a potent inhibitor of tumor cell growth and angiogenesis (Lawler, 2000; Bornstein, 2001), for further studies. It has been reported recently that Smad4-mediated suppression of tumorigenicity in human pancreatic adenocarcinoma cells is due primarily to repression of tumor angiogenesis (Schwarte-Waldhoff *et al.*, 2000).

Northern blot analysis of the total RNA isolated from Panc-1 cells confirmed the cDNA array data that TSP-1

mRNA was strongly induced by TGF- β (Figure 7A). Furthermore, SB203580 reduced the TGF- β -induced TSP-1 expression in a dose-dependent manner (Figure 7B). The TSP-1 protein level is regulated similarly, as determined by western blotting with an anti-TSP-1 monoclonal antibody. The amounts of the TSP-1 protein (which is secreted) in cell culture supernatants significantly increased following TGF- β stimulation, and this increase was suppressed by SB203580 (Figure 7D). Thus, p38 activity is required for TSP-1 expression in response to TGF- β . To evaluate whether TGF- β -stimulated Panc-1 cells secrete a significant amount of active TSP-1, we examined the anti-angiogenic activity of cell culture supernatants using an *in vitro* endothelial cell migration assay (Figure 7F). Conditioned medium of TGF- β -stimulated Panc-1 cells, which contains secreted TSP-1, strongly inhibited the basic fibroblast growth factor (bFGF)-induced endothelial cell migration. This inhibition was abolished when a neutralizing antibody to TSP-1 was included in the assay, indicating that TSP-1 secreted by TGF- β -stimulated Panc-1 cells inhibits angiogenesis.

Because TGF- β -induced activation of p38 is mediated by Smad-dependent GADD45 β expression in these cell lines, TSP-1 expression should also be dependent on Smad and GADD45 β . To test this prediction, we first analyzed TSP-1 mRNA levels in the Smad4-null BxPc-3 cells. TSP-1 mRNA expression was strongly induced by TGF- β in BxPc-3 cells infected with Smad4 recombinant adenovirus, but not in the control (GFP virus-infected) cells. In the Smad4-transfected cells, TGF- β -mediated TSP-1 expression was completely inhibited by SB203580 (Figure 7C). We also found that antisense suppression of GADD45 β expression inhibits TGF- β -induced TSP-1 expression in Panc-1 cells (Figure 7E). This is consistent with the reduced p38 activity by GADD45 β antisense inhibition (Figure 5B). Finally, conditioned medium from TGF- β -stimulated Panc-1 cells expressing GADD45 β antisense RNA did not significantly inhibit the bFGF-induced endothelial cell migration *in vitro* (Figure 7G). Thus we conclude that TGF- β induces TSP-1 expression through the p38 activation mediated by Smad-dependent GADD45 β expression. We further suggest that the p38 pathway plays an important role in tumor inhibition by inducing TSP-1 expression.

Discussion

In this study, we explored the mechanism of TGF- β -induced p38 activation using several types of cells including pancreatic carcinoma cells. The pancreas, whose cancer has a high incidence of mutations in the TGF- β signaling pathway, is one of the most important tissues in which to define this pathway. Our results provide evidence that TGF- β activates p38 through Smad-mediated induction of GADD45 β gene expression, at least in pancreatic cells.

We found that TGF- β activates p38 in TGF- β -responsive pancreatic cells, but not in Smad4-deficient cells. In parallel, we also found that TGF- β induces expression of GADD45 β in TGF- β -responsive cells, but not in Smad4-deficient cells. Reestablishment of Smad4 expression in the Smad4-deficient cells restores both p38 activation and

Fig. 7. TGF- β -induced thrombospondin 1 expression is regulated by Smad-mediated p38 activation. (A–C) Northern blot analyses of TGF- β -induced TSP-1 expression. Total RNA samples extracted from Panc-1 cells at the indicated times following TGF- β treatment were analyzed in (A). Ethidium bromide (EtBr)-stained gels of the same samples are also shown to indicate the amounts of RNA applied. In (B), the p38-specific inhibitor SB203580 was added to Panc-1 cells at the indicated concentrations (μ M) 1 h before TGF- β stimulation. Total RNA was prepared 12 h later. In (C), Smad4-deficient BxPc-3 cells were infected with recombinant adenovirus expressing Smad4, or with GFP (Control). At 24 h post-infection, cells were treated for an additional 12 h with TGF- β in the presence or absence of 10 μ M SB203580, before preparation of total RNA. (D and E) Western blot analyses of TSP-1 expression in cell culture supernatants. Proteins in conditioned media (8 μ g/lane) of parental Panc-1 cells treated with (+) or without (–) TGF- β in the presence or absence of SB203580 (10 μ M) (D), or Panc-1 cells stably transfected with either an empty vector (Vector) or an antisense GADD45 β expression vector (AS1 and AS2) treated with (+) or without (–) TGF- β (E), were separated by SDS-PAGE, and TSP-1 was detected using a specific monoclonal antibody. A non-specific background band of immunoblotting was shown as a loading control (asterisk). (F and G) Anti-angiogenic activity of conditioned media. In (F), conditioned media (CM) from Panc-1 cells treated with or without TGF- β were tested in the presence or absence of neutralizing anti-TSP-1 antibody (20 μ g/ml) for their ability to block the migration of microvascular endothelial cells induced by bFGF (20 ng/ml). All samples were tested in quadruplicate. Data are reported as percentage of maximum migration. In (G), conditioned media were prepared from TGF- β -stimulated Panc-1 cells transfected with either an empty vector (Vector) or an antisense GADD45 β expression vector (AS1 and AS2).

GADD45 β expression in response to TGF- β . We also showed that inhibition of GADD45 β expression by antisense RNA significantly suppresses p38 activation by TGF- β , whereas ectopic expression of GADD45 β is sufficient to activate the p38 pathway even in Smad4-null cells. GADD45 β expression selectively activates MTK1, but not other MAPKKs such as TAK1 and ASK1. These observations, together with previous findings, indicate the following scenario for TGF- β -induced p38 activation (Figure 8). TGF- β activates its receptor which phosphorylates Smad3 (or Smad2), resulting in the formation of active Smad3/4 or Smad2/4 complexes. The active Smad complexes translocate to the nucleus and induce GADD45 β expression. GADD45 β then activates the MTK1 MAPKK. Activated MTK1 activates its cognate MAPKKs (MKK3 or MKK6), which eventually activate p38 MAPK.

It has been reported that TGF- β activates the p38 pathway through the TAK1 MAPKKK, which associates with T β RI (Sano *et al.*, 1999; Yamaguchi *et al.*, 1999; Reffey *et al.*, 2001). Unlike our current observations, the TAK1 model does not involve the Smad proteins. This apparent discrepancy is probably due to cell- or tissue-specific differences. It has been shown that TGF- β activates the p38 MAPK pathway with different time

courses in different cells. TAK1 is activated very rapidly, within 10 min, by TGF- β through T β RI (Yamaguchi *et al.*, 1995; Zhang *et al.*, 2000). In certain cell types (e.g. human neutrophils, HEK-293 cells and C2C12 cells), TGF- β activates p38 with very rapid kinetics that are consistent with the TAK1 mechanism (Hannigan *et al.*, 1998; Hanafusa *et al.*, 1999; Sano *et al.*, 1999). In other cell types (e.g. pancreatic acinar cells, keratinocytes, osteoblasts, gingival fibroblasts, podocytes and hepatocytes), however, a significant delay is observed between exposure to TGF- β and p38 activation (Ravanti *et al.*, 1999; Johansson *et al.*, 2000; Herrera *et al.*, 2001; Karsdal *et al.*, 2001; Schiffer *et al.*, 2001; Schrantz *et al.*, 2001; Simeone *et al.*, 2001). Indeed, TGF- β takes as much as 1 h to stimulate p38 activity in pancreatic carcinoma cells, HaCaT cells and human dermal fibroblasts (see Figure 1). It seems likely that the rapid activation of p38 in certain cells is mediated primarily by TAK1, while the delayed-type activation of p38 in other cells is mediated mainly by Smad-dependent GADD45 β -MTK1 signaling. Indeed, in C2C12 cells, the dominant-negative Smad4 Δ C mutant suppressed only delayed p38 activation, but not the early activation phase (Figure 2E).

We have demonstrated previously that the GADD45 family proteins bind to and activate the MTK1 MAPKKK,

Fig. 8. Model of signal flow from TGF- β to p38-dependent gene expression. Upon TGF- β binding, T β RII phosphorylates and activates T β RI, which subsequently phosphorylates the cytoplasmic Smad2 or Smad3, resulting in formation of a stable heterodimer with Smad4. The active Smad2/4 or Smad3/4 complex translocates to the nucleus and induces GADD45 β expression. The GADD45 β protein binds and activates the MTK1 MAPKKK, which leads to activation of the p38 MAPK. Active p38 translocates into the nucleus and regulates genes (including TSP-1) via phosphorylation of transcription factors.

leading to activation of p38 MAPK (Takekawa and Saito, 1998; Mita *et al.*, 2002). Interestingly, recent studies have reported that, in Th1 (T helper type 1) cells, cytokine- and T-cell receptor (TCR)-induced activation of p38 MAPK, which is essential for interferon- γ production, is mediated by GADD45 β and GADD45 γ , respectively (Lu *et al.*, 2001; Yang *et al.*, 2001), showing functional distinction among the GADD45 isoforms. Our current study suggests that GADD45 β is more important than the other isoforms in TGF- β signaling.

To investigate the role of p38 activation in TGF- β -mediated gene regulation, we searched, by cDNA array analysis, for genes that are induced by TGF- β in a p38-dependent manner. One such gene encodes TSP-1, a potent inhibitor of tumor cell growth and angiogenesis (Lawler, 2000; Bornstein, 2001). Interestingly, it has been reported recently that restoration of Smad4 to Smad4-deficient pancreatic carcinoma cells increased the steady-state mRNA levels of TSP-1, and suppressed tumor formation *in vivo* by repressing tumor angiogenesis (Schwarte-Waldhoff *et al.*, 2000). Nonetheless, TSP-1 mRNA expression is unlikely to be mediated directly by the Smad proteins, because the TSP-1 promoter region is devoid of any known Smad-binding element (Schwarte-Waldhoff *et al.*, 2000). Our finding that TSP-1 expression requires Smad- and GADD45 β -dependent p38 activation offers a mechanism for TGF- β -induced TSP-1 expression.

It is likely that TSP-1 expression is only indirectly dependent on the Smad proteins, and that direct gene induction is mediated by transcription factors that are activated by p38, such as ATF2. We can speculate further that TSP-1 expression, in normal pancreatic cells, might contribute to the tumor-suppressive effect of TGF- β . Indeed, the TSP-1 protein secreted by TGF- β -stimulated cells showed an anti-angiogenic effect *in vitro* (Figure 7F and G). If so, inefficient activation of p38 in Smad4-deficient tumor cells, and a resulting deficit of TSP-1, might contribute to the etiology of invasive pancreatic cancer. We should note, however, that the role of p38 in the cellular response to TGF- β is by no means limited to TSP-1 expression.

Materials and methods

Media and buffers

Lysis buffer, kinase buffer and SDS loading buffer have been described previously (Takekawa *et al.*, 2000).

Expression constructs

Mammalian expression plasmids pCMV-Flag-p38, pFlag-MTK1, pMTK1 Δ K and pGADD45 β , and the GST fusion constructs GST-ATF2 and GST-MKK6(K/A) have been described previously (Takekawa and Saito, 1998). pSmad4 Δ C was constructed by deletion of the coding sequence of human Smad4 from Lys515 to the C-terminus of the human Smad4-coding sequence. To generate an antisense GADD45 β expression vector (pBCMG-AS-GADD45 β), GADD45 β cDNA containing the complete coding sequence was subcloned in the antisense orientation into pBCMG-hygro (Karasuyama *et al.*, 1990). Expression vectors for Flag-Smad3 and Flag-Smad4 were provided by M. Kurokawa. Myc-TAK1 and HA-ASK1 expression vectors were provided by K. Matsumoto and H. Ichijo, respectively (Yamaguchi *et al.*, 1995; Ichijo *et al.*, 1997). Mammalian expression vectors for wild-type T β RI and constitutively activated T β RI(T204D) were from J. Massagué (Wieser *et al.*, 1995). Recombinant adenovirus expressing Smad4 was provided by K. Miyazono (Fujii *et al.*, 1999).

Tissue culture and gene transfer

For transient transfection assays, HeLa, COS-7 and C2C12 cells, grown in 35 mm dishes, were transfected with the appropriate expression plasmids using the Effectene Transfection Reagent (Qiagen). The total amount of plasmid DNA was adjusted to 0.4 μ g per plate with vector DNA (pcDNA3). AsPc-1, BxPc-3 and HaCaT (Boukamp *et al.*, 1988) cells were suspended at a density of 2×10^7 cells/ml and transfected by electroporation using the Bio-Rad Gene Pulser II at 0.25 kV and 975 μ F. For adenovirus-mediated transient transfection, cells in serum-free RPMI-1640 were infected with recombinant adenovirus at a multiplicity of infection (m.o.i.) of 20 p.f.u./cell as described (Fujii *et al.*, 1999) and, after 1 h, were refed with medium containing 10% serum. For both plasmid and viral transfection experiments, cells were harvested 36 h after transfection and, where indicated, treated with TGF- β 1 (10 ng/ml; Sigma) or MMS (100 μ g/ml). To establish transfectants stably expressing antisense GADD45 β , Panc-1 cells were transfected using the Effectene Reagent, selected with hygromycin (0.2 mg/ml), and stable transfectants were isolated by ring cloning.

Immunoblotting

Immunoblot analyses were performed as described previously (Takekawa and Saito, 1998). For TSP-1 immunoblots, cells were incubated with TGF- β (10 ng/ml) in the presence or absence of 10 μ M SB203580 for 24 h in serum-free Dulbecco's modified Eagle's medium (DMEM), cell culture supernatants were concentrated with Centricon-100 tubes, and 8 μ g of total protein per lane was electrophoresed under reducing conditions as described (Schwarte-Waldhoff *et al.*, 2000). The following antibodies were used: anti-Flag monoclonal antibody (mAb) M2 (Sigma); anti-HA mAb 12CA5 (Roche Molecular Biochemicals); anti-Myc mAb 9E10 (Santa Cruz); anti-Smad4 mAb (Transduction Laboratories); anti-TSP-1 mAb A6.1 (Oncogene Research Products); rabbit polyclonal antibodies to p38 (Santa Cruz); rabbit polyclonal antisera specific to

phosphorylated p38 (Cell Signaling); and goat polyclonal antisera to GADD45 β (Santa Cruz).

Immune-complex protein kinase assay

To assess the kinase activity of epitope-tagged MAPKKs, transfected cells were lysed in lysis buffer plus 0.5% deoxycholate. Cell lysates were incubated with an appropriate antibody for 2 h at 4°C. Immunocomplexes were recovered with the aid of protein G–Sepharose beads, washed twice with lysis buffer containing 500 mM NaCl, twice with lysis buffer, and twice again with kinase buffer. Immunoprecipitates were resuspended in 30 μ l of kinase buffer containing 3 μ g of GST–MKK6(K/A). The kinase reaction was initiated by addition of 20 μ Ci of [γ -³²P]ATP and 20 μ M ATP. Following 15 min incubation at 30°C, reactions were terminated by the addition of SDS loading buffer. Samples were boiled, separated by SDS–PAGE, dried and visualized by autoradiography. The kinase activity of precipitated Flag-p38 was measured in a similar *in vitro* kinase assay using the specific substrate GST–ATF2 as described previously (Takekawa and Saito, 1998).

Northern blot analysis and RT–PCR

Cells were treated with either TGF- β 1 (10 ng/ml) or MMS (100 μ g/ml). A 20 μ g aliquot per lane of total RNA, prepared using Trizol (Gibco-BRL), was fractionated by denaturing agarose gel electrophoresis, and transferred onto a nylon membrane. The blot was hybridized with a probe specific to either GADD45 α , β , γ or TSP-1, as described (Takekawa *et al.*, 2000). To assess the presence of sense transcripts in antisense transfectants, one-step RT–PCR was carried out with total RNA (1.0 μ g) and primers specific to the sense transcript, using the Platinum Quantitative RT–PCR ThermoScript One-Step System (Life Technologies).

cDNA expression array

The Gene Navigator cDNA cancer array (Toyobo) was used to examine differential gene expression in Panc-1 cells stimulated with TGF- β (10 ng/ml) in the presence or absence of 10 μ M SB203580. The array comprises sequences of 550 annotated cancer-associated genes and 11 housekeeping genes. Isolation of poly(A) RNA, cDNA synthesis, labeling of cDNA with chemiluminescence and hybridization of cDNA probes to the array filters were performed according to the manufacturer's protocol. Following hybridization and washing, the array filters were scanned in a FluorImager (Amersham Pharmacia Biotech), and were data-processed with Array-Pro Analyzer software (Toyobo). As the arrays were double-spotted, the intensity value for each gene was calculated as the average of the two spots, and normalized to hybridization signals from the 11 housekeeping genes.

Endothelial cell migration assays

Endothelial cell migration was assayed in a modified Boyden chamber as described (Schwarte-Waldhoff *et al.*, 2000). Briefly, human microvascular endothelial cells (HMVECs) were attached to the undersurface of a gelatinized membrane (8- μ m pore size) in the lower wells. Concentrated serum-free conditioned medium (CM) (20 μ g/ml) in DMEM with 0.1% bovine serum albumin (BSA) and bFGF (20 ng/ml; Sigma) was added to the upper wells. Where noted, neutralizing antibody to TSP-1 (A4.1; Oncogene Research Products) was added at 20 μ g/ml. After 4 h, the filters were stained and the number of cells migrating to the top of the membrane per 10 high-power fields was counted. Results are reported as the percentage of the maximum migration induced by bFGF (20 ng/ml) after subtraction of background migration toward the vehicle (0.1% BSA). All samples were tested in quadruplicate. All assays contained neutralizing antibody to TGF- β (15 μ g/ml; Genzyme-Techne) to eliminate the effects of exogenous TGF- β .

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