Transforming growth factor β-1 enhances Smad transcriptional activity through activation of p8 gene expression

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We report that exposure of mouse embryonic fibroblasts to transforming growth factor β-1 (TGFβ-1) (5 ng/ml) results in a strong activation of p8 mRNA expression that precedes the induction of cell growth. Involvement of the p8 promoter in the regulation was demonstrated by using a p8–chloramphenicol acetyltransferase construct. We therefore speculated that p8 might be a mediator of TGFβ-1 in these cells. The incorporation of [^{3}H]thymidine on treatment with TGFβ-1 was indeed significantly higher in $p8^{+/+}$ fibroblasts than in $p8^{-/-}$ fibroblasts. Smad transcriptional activity was used as marker of the TGFβ-1 signalling pathway, to probe the lower $p8^{-/-}$ response to TGFβ-1. Two Smad-binding elements (SBEs)–luciferase constructs were transfected into $p8^{-/-}$ and $p8^{+/+}$ embryonic fibroblasts before treatment with TGFβ-1. A lower level of Smad transactivation was observed in $p8^{-/-}$ embryonic fibroblasts, under basal conditions and after stimulation with TGFβ-1. To test whether Smad underexpression in $p8^{-/-}$ cells was actually due to p8 depletion, $p8^{-/-}$ embryonic fibroblasts were transfected with a human p8 expression plasmid together with an SBE–luciferase construct. The expression of p8 restored Smad transactivation in unstimulated and TGFβ-1-treated cells to the level found in $p8^{+/+}$ cells. We concluded that TGFβ-1 activates p8 expression, which in turn enhances the Smad-transactivating function responsible for TGFβ-1 activity.

Key words: fibroblasts, mouse, TGFβ-1.

INTRODUCTION

We have previously identified a new gene, called p8, whose expression is strongly induced in acinar cells of the pancreas during the acute phase of pancreatitis, pancreatic regeneration and pancreatic development [1]. Further experiments have shown that p8 activation is not restricted to pancreatic cells because p8 mRNA is also activated in brain and kidney after transient ischaemic injury (G. V. Mallo and J. L. Iovanna, unpublished work), and in several tissues in response to systemic lipopolysaccharide [2]. Studies *in vitro* have shown that almost all tested cells induced transient p8 mRNA expression in response to pro-apoptotic agents [1]. In contrast, Ree et al. [3] identified a gene named *candidate of metastasis 1* (*Com 1*) that is identical with human *p8* [4] and seems to mediate the growth of tumour cells after metastatic establishment in a secondary organ. Theoretical studies of the p8 primary structure suggested that the 80-residue polypeptide contains a helix–loop–helix motif, as found in some transcription factors, and a potential nuclear targeting signal [1]. This was confirmed recently with the use of specific antibodies and transient transfection of an expression plasmid [4]. More recent structural and thermodynamic studies have shown strong conformational changes of p8 after serine/threonine phosphorylation *in vitro*, associated with increased DNA-binding capacity. Importantly, binding is independent of the DNA sequence, as observed previously in high-mobility-group (HMG)-I/Y proteins, leading to the hypothesis that p8 is an HMG-I/Y-like protein [5].

While studying various cytokines and growth factors as potential mediators of *p8* gene activation, we observed that p8 mRNA expression was strongly induced in NIH 3T3 cells on treatment with transforming growth factor β-1 (TGFβ-1). TGFβ-1 family members are secreted cytokines, produced by many cell types, that regulate a wide variety of cellular processes including cell proliferation, cell differentiation and extracellular matrix production [6–8]. Control of cell growth by TGFβ-1 is complex and depends very markedly on the target cell type, the cell density and the presence of other growth factors in the culture medium. TGFβ-1 acts as either a positive or a negative regulator of cell division. It stimulates the growth of certain mesenchyme-derived cells such as fibroblasts and osteoblasts, but acts as a potent growth inhibitor of many other cell types, such as epithelial and endothelial cells [6,9]. TGFβ-1 family members exert their cellular effects through binding to transmembrane receptors with serine/threonine kinase activity. On the binding of ligand, a heteromeric receptor complex consisting of two type II and two type I receptors is formed. Within the complex, the constitutively active kinase of the type II receptor phosphorylates and activates the type I receptor, which subsequently propagates signals through the Smad pathway. Smad proteins are a conserved family of receptor substrates that act as transcriptional factors. After the activation of TGFβ receptor I, Smad2 and Smad3 are phosphorylated, form heteromeric complexes with the common mediator Smad4 and translocate to the nucleus to regulate gene transcription. The accumulation of the active Smad complex into the nucleus leads to specific DNA

Abbreviations used: CAT, chloramphenicol acetyltransferase; C/EBP, CCAAT-enhancer-binding protein; CMV, cytomegalovirus; Com 1, candidate of metastasis 1; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; HMG, high-mobility group; MEFs, mouse embryonic fibroblasts; PAP I, pancreatitis-associated protein I; SBE, Smad-binding element; TGFβ-1, transforming growth factor β-1.

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binding and the formation of transcriptional complex by the recruitment of DNA-binding and transcriptional cofactors, resulting in the expression of key target genes (reviewed in [10–12]). Disruption of this signalling pathway has been associated with various human diseases including cancers [10].

Here we report that TGFβ-1 induces p8 mRNA expression in fibroblasts and that p8 is necessary for optimal TGFβ-1 growth promotion in primary embryonic fibroblasts. We also show that p8 expression enhances the transcriptional activity of Smad.

MATERIALS AND METHODS

Cell culture and Northern blot analysis of p8 mRNA expression

NIH 3T3 cells (10^6) were cultivated in 100 mm Petri dishes at 37 °C in an air/CO_2 (19:1) atmosphere in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL)/50 i.u./ml penicillin/50 μg/ml streptomycin, supplemented with 5% (v/v) fetal calf serum (FCS) (Gibco BRL) for 24 h before TGFβ-1 treatment. Human recombinant TGFβ-1 (5 ng/ml) (Sigma) was added to the cells, without changing the culture medium; cells were collected after 0, 1.5, 3, 6, 9 or 12 h. Total RNA was prepared from the cells with TRIzol reagent in accordance with the manufacturer's instructions (Gibco BRL). RNA integrity and loading amounts were assessed by examining 18 S and 28 S ribosomal RNA banding of samples after electrophoresis under non-denaturing conditions and staining with ethidium bromide. For analysis of mRNA levels, 20 μg of total RNA was resolved by denaturing 1% (w/v) agarose-gel electrophoresis, transferred to GeneScreen Hybridation Transfer Membrane (NEN Life Science), and cross-linked by UV irradiation. The filters were prehybridized at 65 °C in a solution containing 5 × SSPE [1 × SSPE is 180 mM NaCl/1 mM EDTA/10 mM NaH_2PO_4 (pH 7.5)]/5 × Denhardt solution/0.5% SDS/100 μg/ml single-stranded herring sperm DNA. The prehybridization buffer was replaced with fresh buffer containing the corresponding ^{32}P-labelled p8 cDNA probe and left for 16 h at 65 °C. Filters were then washed twice for 15 min at room temperature in 2 × SSC/0.1% SDS and once for 15 min at 65 °C in 0.2 × SSC, then autoradiographed on XAR film (Kodak) for 1–3 days at −80 °C. The mouse p8 cDNA used as probe has been previously described [13].

Cell transfection and chloramphenicol acetyltransferase (CAT) gene reporter assays

NIH 3T3 cells (10^5) were cultivated in 30 mm diameter culture dishes for 36 h and then transiently transfected with 1 μg of p8-CAT reporter plasmid and 1 μg of pCMV/βgal (in which CMV stands for cytomegalovirus) plasmid (to control the transfection efficiency) by using the Fugene reagent in accordance with the manufacturer's protocol (Roche Molecular Biochemicals). This method of transfection was chosen in these cells because, in contrast with the calcium phosphate transfection method, it does not result in the activation of p8 gene transcription [13]. The p-1471/+37p8-CAT promoter construct [13] was used in CAT reporter assays. CAT activity was measured as described previously [13] in the NIH 3T3 cells after treatment with TGFβ-1 (TGFβ-1 was added 7 h after transfection). Cell extracts were prepared with the reporter lysis buffer (Promega) 24 h after transfection. CAT activity was determined by using the phase extraction procedure [14]; β-galactosidase assay was performed essentially as described previously [15]. CAT activity was normalized to β-galactosidase activity. Experiments were carried out in triplicate.

Targeted disruption of the mouse p8 gene and production of $p8^{-/-}$ and $p8^{+/+}$ embryonic fibroblasts

Inactivation of the p8 gene was obtained by homologous recombination in embryonic stem cells by following published procedures [16]. (Details of the construction are available from authors upon request.) Embryonic stem cells from three heterozygous clones for the mutated p8 allele were injected into blastocysts of C57BL/6 mice. We obtained highly chimaeric seven male mice in the littermates. These were intercrossed with wild-type C57BL/6 females; four showed germline transmission. Genotype identification was done by PCR and/or Southern blot with genomic DNA prepared from tail biopsies of 10-day-old pups. Heterozygous mice for the mutated p8 allele were thus generated. Heterozygous mice were used in breeding experiments to generate mice that were homozygous (−/−) for the disruption of p8. A phenotypic characterization of these mice will be published elsewhere. Primary embryo fibroblasts were isolated from 14.5-day-old mouse embryos by following standard protocols [17]. p8 genotypes of cultured cells were determined by PCR. Cells were grown in DMEM supplemented with 10% (v/v) FCS and used after a maximum of six passages.

Incorporation of [^{3}H]thymidine in $p8^{-/-}$ and $p8^{+/+}$ embryonic fibroblasts

$p8^{-/-}$ or $p8^{+/+}$ embryo fibroblasts (10^4 per well) were cultivated in a 96-well plate for 12 h in DMEM with 10% (v/v) FCS. The medium was removed and the cells were incubated for 24 h in DMEM with only 1% (v/v) FCS. TGFβ-1 was then added to the medium and the treatment was maintained for 12, 24 or 36 h before measurement of [^{3}H]thymidine incorporation: 1 μCi of [^{3}H]thymidine was added to each well and 6 h later the incorporation of tritium was measured with a scintillation counter. As control, we measured incorporation of [^{3}H]thymidine in $p8^{-/-}$ or $p8^{+/+}$ fibroblasts cultivated for 24 h in DMEM/1% (v/v) FCS without TGFβ-1.

Cell transfection and luciferase gene reporter assays

To test the Smad activity we used two plasmids containing sequences specific for Smad binding, plasmid pGL3ti-$(SBE)_4$ (in which SBE stands for Smad-binding element), which contains four 5′-CAGACA-3′ sites, and plasmid pGL3-$(CAGA)_{12}$, which contains twelve 5′-CAGA-3′ sequences [18]. Embryonic fibroblasts (7.5×10^5 per well) were cultivated in a six-well plate for 36 h. Then, cells were transfected with pGL3ti-$(SBE)_4$ or pGL3-$(CAGA)_{12}$ plasmids in combination with the pCMV/βgal (to control the transfection efficiency). At 7 h after transfection, TGFβ-1 (2 ng/ml) was added to the medium and cells were collected 24 h later to perform a luciferase assay with the Luciferase Assay System (Promega) in accordance with the instructions of the manufacturer. The β-galactosidase assay was performed as described previously; luciferase activity was normalized to β-galactosidase activity. As a control, we used the p-1256/+10PAPI-CAT construct [15] in which the promoter region (from nt −1256 to +10) of the pancreatitis-associated protein I (PAP I) gene drives the CAT reporter gene. This promoter was chosen because PAP I does not respond to TGFβ-1. Finally, we measured the Smad activity in embryonic $p8^{-/-}$ fibroblasts after re-expression of p8 by co-transfection of pGL3-$(CAGA)_{12}$ with pCMV-p8hu [4], a vector expressing the human p8, in the presence or absence of TGFβ-1 (2 ng/ml).

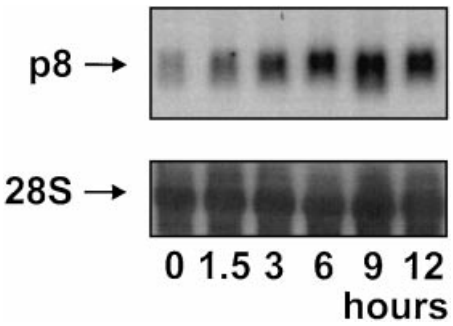

Figure 1 p8 transcriptional activation after treatment with TGFβ-1

NIH 3T3 cells (10^6) were cultivated in 100 mm Petri dishes in DMEM with 5% (v/v) FBS for 24 h before treatment with TGFβ-1. TGFβ-1 (5 ng/ml) was added to the medium and cells were collected after 1.5, 3, 6, 9 or 12 h for the preparation of total RNA. p8 mRNA induction was analysed by Northern blotting with a mouse p8 cDNA probe.

RESULTS AND DISCUSSION

TGFβ-1 activates p8 gene expression

While testing different growth factors and cytokines as possible inductors of p8 gene expression *in vitro*, we found that TGFβ-1 (5 ng/ml) resulted in a strong activation of p8 mRNA expression in mouse fibroblasts (Figure 1) and also in mouse embryonic fibroblasts (MEFs) (results not shown). The kinetics of p8 gene expression after TGFβ-1 treatment, monitored by measuring p8 mRNA levels, showed a maximum after 9 h. Interestingly, we found three putative SBE sequences (CAGACA) [18] in the mouse p8 gene promoter. Importantly, one of three SBEs partly overlaps with the CCAAT-enhancer-binding protein (C/EBP) *cis*-acting element present at position -111 (attgcatCAGACA; the C/EBP-binding site is underlined and the SBE site is in capital letters). This common C/EBP-SBE sequence was shown to have a key role in the transcriptional activation of p8 [13]. It is therefore possible that C/EBP and Smad proteins co-operate to form the transcriptional complex necessary for DNA binding and the facilitation of Smad transactivation. Smad and C/EBP seem to co-operate in the activation of other genes, as previously suggested [19,20]. The two other SBE sites are organized in tandem, separated by 2 T bases (CAGACAttCAGACA), at position -21 of the p8 promoter. Involvement of the p8 promoter in the response to TGFβ-1 treatment was demonstrated by using a construct in which the CAT reporter gene was under the control of the p8 promoter (p-1471/+36p8-CAT construct) [13]. As expected, treatment of the transfected NIH 3T3 cells with TGFβ-1 (5 ng/ml) yielded a 4.41 ± 0.98-fold induction in CAT activity (mean $\pm$ S.D.).

Decreased response of the p8$^{-/-}$ embryonic fibroblasts to the TGFβ-1 treatment

Because TGFβ-1 induces p8 mRNA expression in fibroblasts, we speculated that p8 could act as a mediator of TGFβ-1 in these cells. We tested that hypothesis by comparing [^{3}H]thymidine incorporation in p8$^{-/-}$ and p8$^{+/+}$ fibroblasts, because the modulation of cellular proliferation is one of the most significant effects of TGFβ-1. p8$^{+/+}$ and p8$^{-/-}$ MEFs were obtained from p8$^{+/+}$ and p8$^{-/-}$ mice respectively. p8 deficiency was verified by Western blotting and immunohistochemistry of the pancreas with acute pancreatitis by using a p8 polyclonal antibody (results not shown) because p8 is induced in pancreas with acute

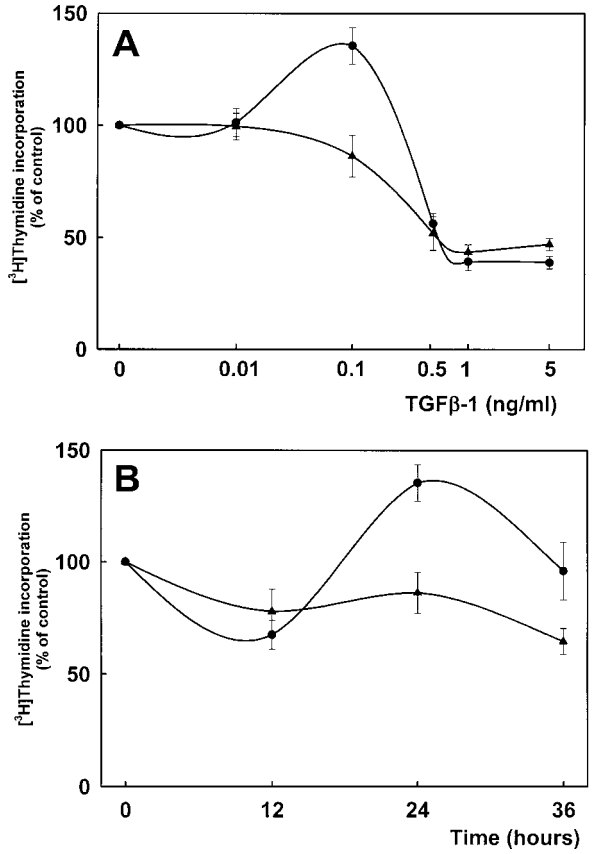

Figure 2 Effects of TGFβ-1 treatment on [^{3}H]thymidine incorporation in MEFs

p8$^{+/+}$ (●) or p8$^{-/-}$ (▲) MEFs were plated at 10^4 cells per well in a 96-well plate in the presence of 1% (v/v) FBS for 24 h. (**A**) Cells were treated with the indicated concentrations of TGFβ-1 for 24 h. (**B**) Cells were treated with 0.1 ng/ml TGFβ-1 for 12, 24 or 36 h. After treatment, cells were incubated with [^{3}H]thymidine (1 μCi) for a further 6 h; the incorporation of radioactivity was measured by liquid-scintillation counting. [^{3}H]Thymidine incorporation is expressed as a percentage of control (no TGFβ-1); results are means $\pm$ S.D. for four separate experiments.

pancreatitis [1]. As shown in Figure 2(A), low doses of TGFβ-1 (0.1 ng/ml) stimulated [^{3}H]thymidine incorporation in p8$^{+/+}$ embryonic fibroblasts, whereas higher doses (0.5 ng/ml or more) strongly inhibited cell proliferation. This biphasic effect of TGFβ-1, with a transient mitogenic action at low concentrations and growth inhibition at higher doses, has already been observed in human smooth-muscle cells [21]. It is important to note that, after treatment of p8$^{+/+}$ fibroblasts with TGFβ-1, the induction of p8 mRNA was already maximal after 9 h, whereas maximal [^{3}H]thymidine incorporation occurred only after 24–36 h. This sequence indicates that p8 could mediate the effects of TGFβ-1. This is supported by the observation that, in contrast with p8$^{+/+}$ cells, p8$^{-/-}$ cells did not show a proliferative response with low doses of TGFβ-1, and growth inhibition with higher doses was less important (Figure 2). Therefore growth regulation by TGFβ-1 in embryonic fibroblasts seems to be dependent, at least in part, on p8 expression. Finally, participation of p8 in the proliferative effect of the TGFβ-1 seems quite specific because the mitogenic stimulation of the p8$^{-/-}$ or p8$^{+/+}$ fibroblasts with insulin growth factor I was similar (results not shown) and because p8 transcriptional activation was not observed after

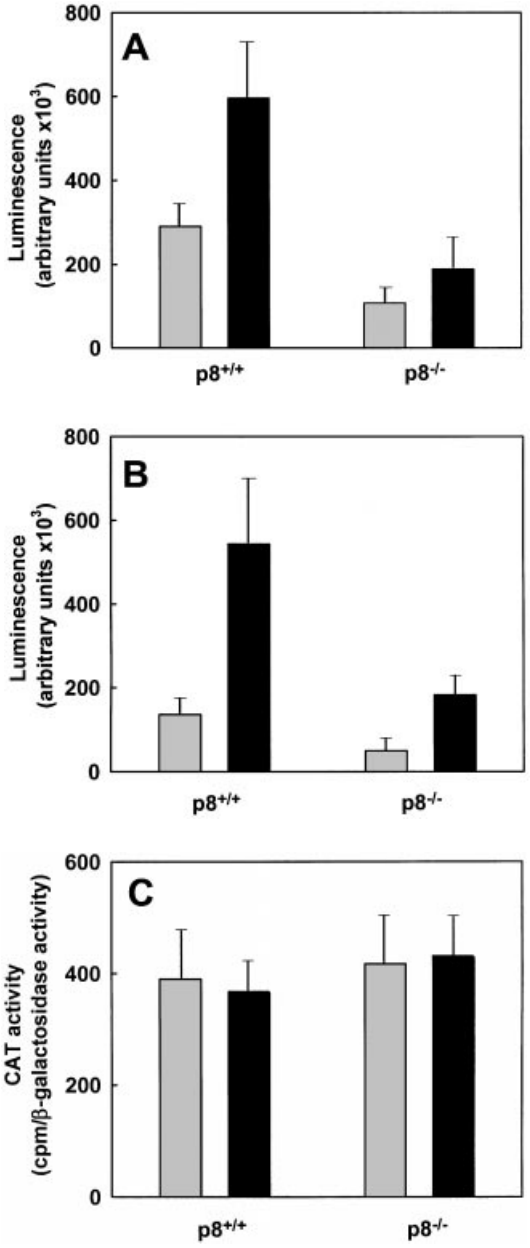

Figure 3 Smad activity in $p8^{+/+}$ and $p8^{-/-}$ MEFs

$p8^{+/+}$ and $p8^{-/-}$ MEFs were obtained as described in the Materials and methods section. Fibroblasts (7.5×10^4 per well) were cultivated in a six-well plate for 36 h. Cells were then transfected with 3 μg of pGL3ti-$(SBE)_4$ (**A**), pGL3-$(CAGA)_{12}$ (**B**) or p-1256/+10PAPI-CAT (control) (**C**) and 1 μg of pCMV/βgal (to control transfection efficiency). At 7 h after transfection, TGFβ-1 (2 ng/ml) (black columns) was added to the medium; cells were collected 24 h later for luciferase and β-galactosidase assays. Grey columns represent the results obtained from untreated cells. Results represent luciferase activity related to β-galactosidase activity and are means $\pm$ S.D. for experiments in triplicate.

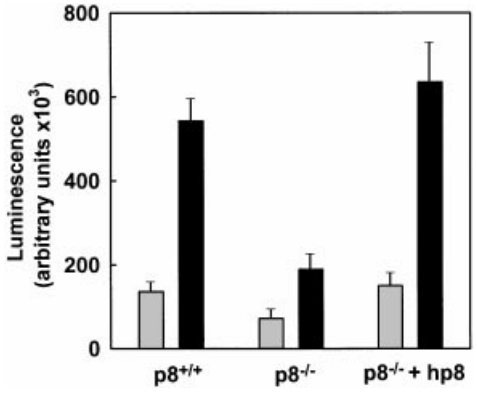

Figure 4 p8 expression influences Smad transactivation activity

$p8^{+/+}$ and $p8^{-/-}$ MEFs were obtained as described in the Materials and methods section. Fibroblasts (7.5×10^4 per well) were cultivated in a six-well plate for 36 h. Cells were then transfected with 3 μg of pGL3-$(CAGA)_{12}$ plasmid and 0.5 μg of pCMV-hp8 or 0.5 μg of pCMV-CAT (to normalize the amount of transfected DNA). pCMV/βgal (1 μg) was used to control the transfection efficiency. TGFβ-1 (2 ng/ml) (black columns) was added and cells were collected 24 h later for luciferase and β-galactosidase assays. Grey columns represent the results obtained from untreated cells. Results represent luciferase activity related to β-galactosidase activity and are means $\pm$ S.D. for experiments in triplicate.

treatment of NIH 3T3 cells with insulin growth factor I or epidermal growth factor (results not shown).

p8 enhances transcriptional activity of Smad

So far, the Smad proteins are the only TGFβ-1 receptor substrates with a demonstrated ability to propagate signals [10]. We have studied, in $p8^{-/-}$ and $p8^{+/+}$ embryonic fibroblasts, the Smad transcriptional activity as a direct marker of the TGFβ-1 signalling pathway, to probe the lower $p8^{-/-}$ response of embryonic fibroblasts to TGFβ-1 stimulation. To this end we transfected two SBE-containing constructs driving the luciferase gene reporter into $p8^{-/-}$ and $p8^{+/+}$ embryonic fibroblasts and then treated the cells with TGFβ-1. As expected, a lower Smad transactivating activity in the $p8^{-/-}$ embryonic fibroblasts, under both basal and TGFβ-1 stimulation conditions, was observed with both pGL3ti-$(SBE)_4$ and pGL3-$(CAGA)_{12}$ plasmids (Figure 3). However, a lower Smad activity in $p8^{-/-}$ MEFs could merely reflect a non-specific activity of p8 on gene transcription. Control experiments were conducted in which the CAT reporter gene was driven by the PAP I promoter, a TGFβ-1-unresponsive promoter. A similar transcriptional activity was observed in $p8^{+/+}$ and $p8^{-/-}$ MEFs (see Figure 3C), arguing against a non-specific activity of p8 on gene transcription. It is therefore likely that p8 has a role in Smad transactivation.

To test whether Smad underexpression in $p8^{-/-}$ cells was actually due to p8 depletion we designed a new approach in which the $p8^{-/-}$ embryonic fibroblasts were transfected with a human p8 expression plasmid (CMV-hp8) [4] or a control (CMV-CAT) plasmid together with the SBE-containing pGL3-$(CAGA)_{12}$ plasmid. As shown in Figure 4, transfection of $p8^{-/-}$ embryonic fibroblasts with CMV-hp8 restored the Smad-transactivating activity to the level found in $p8^{+/+}$ cells, in basal as well as in TGFβ-1-stimulated conditions. It was therefore concluded that p8 expression enhances the Smad-transactivating activity. The mechanism of enhancement of the Smad activity by p8 remains to be elucidated. However, p8 could be one of the necessary DNA-binding cofactors that facilitate Smad binding to DNA and the eventual transcriptional regulation of specific genes [11,22]. This hypothesis is supported by the recent finding that p8 is a DNA-binding protein whose affinity is strongly increased on Ser/Thr phosphorylation [5].

In conclusion, expression of the gene encoding p8 in mouse fibroblasts is transcriptionally activated by TGFβ-1 and participates in the intracellular TGFβ-1 pathway, one of its targets

being the Smad proteins. These findings shed new light on previous results linking p8 to cancer. TGFβ-1 is implicated in the metastasis of several cancers [10] and particularly of breast cancer [23,24]. In contrast, *p8* was called *Com 1* by Ree et al. [3] because they found that its expression was required for the metastasis of breast carcinoma cells. We have observed in tumour sections that staining with anti-p8 antibody was very strong in transformed cells and was only at background levels in adjacent normal cells. Staining outlined the tumour perfectly, indicating specific p8 gene expression after malignant transformation (S. Garcia and J. L. Iovanna, unpublished work). It is therefore possible that the mechanism by which TGFβ-1 favours the metastatic process involves p8 overexpression, p8 helping in the adaptation of cancer cells to the new, potentially stressful, environment of the target tissue, thereby facilitating establishment of the metastasis. However, it is important to note that TGFβ-1 action can be proliferative or anti-proliferative, depending on the cellular context. For example, in mammary epithelial cells, TGFβ-1 causes growth arrest or induces metastases, depending on the level of oncogenic Ras activity present in the cells [25]. The expression of p8 therefore seems to be only one of the regulators of the complex system mediated by TGFβ-1. In fact, a very attractive hypothesis is that p8 might regulate Smad transcriptional activity as an architectural transcription factor, as has previously been suggested for some HMG proteins [26–31].

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REFERENCES

1 Mallo, G. V., Fiedler, F., Calvo, E. L., Ortiz, E. M., Vasseur, S., Keim, V., Morisset, J. and Iovanna, J. L. (1997) Cloning and expression of the rat p8 cDNA, a new gene activated in pancreas during the acute phase of pancreatitis, pancreatic development, and regeneration, and which promotes cellular growth. J. Biol. Chem. **272**, 32360–32369

2 Jiang, Y. F., Vaccaro, M. I., Fiedler, F., Calvo, E. L. and Iovanna, J. L. (1999) Lipopolysaccharides induce p8 mRNA expression *in vivo* and *in vitro*. Biochem. Biophys. Res. Commun. **260**, 686–690

3 Ree, A. H., Tvermyr, M., Engebraaten, O., Rooman, M., Rosok, O., Hovig, E., Meza-Zepeda, L. A., Bruland, O. S. and Fodstad, O. (1999) Expression of a novel factor in human breast cancer cells with metastatic potential. Cancer Res. **59**, 4675–4680

4 Vasseur, S., Mallo, G. V., Fiedler, F., Bödeker, H., Canepa, E., Moreno, S. and Iovanna, J. L. (1999) Cloning and expression of the human p8, a nuclear protein with mitogenic activity. Eur. J. Biochem. **259**, 670–675

5 Encinar, J. A., Mallo, G. V., Mizyrycki, C., Giono, L., Gonzalez-Ros, J. M., Rico, M., Canepa, E., Moreno, S., Neira, J. L. and Iovanna, J. L. (2001) Human p8 is a HMG-I/Y-like protein with DNA binding activity enhanced by phosphorylation. J. Biol. Chem. **276**, 2742–2751

6 Massagué, J. (1990) The transforming growth factor-beta family. Annu. Rev. Cell Biol. **6**, 597–641

7 Moses, H. L., Yang, E. Y. and Pietenpol, J. A. (1990) TGF-beta stimulation and inhibition of cell proliferation: new mechanistic insights. Cell **63**, 245–257

8 Alexandrow, M. G. and Moses, H. L. (1995) Transforming growth factor beta 1 inhibits mouse keratinocytes late in G1 independent of effects on gene transcription. Cancer Res. **55**, 1452–1457

9 Roberts, A. B. and Sporn, M. B. (1993) Physiological actions and clinical applications of transforming growth factor-beta (TGF-beta). Growth Factors **8**, 1–9

10 Massagué, J. (1998) TGF-beta signal transduction. Annu. Rev. Biochem. **67**, 753–791

11 Massagué, J. and Wotton, D. (2000) Transcriptional control by the TGF-beta/Smad signaling system. EMBO J. **19**, 1745–1754

12 Wrana, J. L. (2000) Regulation of Smad activity. Cell **100**, 189–192

13 Vasseur, S., Mallo, G. V., Garcia-Montero, A., Ortiz, E., Fiedler, F., Canepa, E., Moreno, S. and Iovanna, J. L. (1999) Structural and functional characterization of the mouse p8 gene: promotion of transcription by the CAAT-enhancer binding protein α (C/EBPα) and C/EBPβ *trans*-acting factors involves a C/EBP *cis*-acting element and other regions of the promoter. Biochem. J. **343**, 377–383

14 Seed, B. and Sheen, J. Y. (1988) A simple phase-extraction assay for chloramphenicol acyltransferase activity. Gene **67**, 271–277

15 Dusetti, N. J., Ortiz, E. M., Mallo, G. V., Dagorn, J. C. and Iovanna, J. L. (1995) Pancreatitis-associated protein I (PAP I), an acute phase protein induced by cytokines. Identification of two functional interleukin-6 response elements in the rat PAP I promoter region. J. Biol. Chem. **270**, 22417–22421

16 Biel, M., Seeliger, M., Pfeifer, A., Kohler, K., Gerstner, A., Ludwig, A., Jaissle, G., Fauser, S., Zrenner, E. and Hofmann, F. (1999) Selective loss of cone function in mice lacking the cyclic nucleotide-gated channel CNG3. Proc. Natl. Acad. Sci. U.S.A. **96**, 7553–7557

17 Harvey, M., Sands, A. T., Weiss, R. S., Hegi, M. E., Wiseman, R. W., Pantazis, P., Giovanella, B. C., Tainsky, M. A., Bradley, A. and Donehower, L. A. (1993) *In vitro* growth characteristics of embryo fibroblasts isolated from p53-deficient mice. Oncogene **8**, 2457–2467

18 Jonk, L. J. C., Itoh, S., Heldin, C. H., Dijke, P. ten and Kruijer, W. (1998). Identification and functional characterization of a Smad binding element (SBE) in the *JunB* promoter that acts as a transforming growth factor-β, activin, and bone morphogenetic protein-inducible enhancer. J. Biol. Chem. **273**, 21145–21152

19 Buck, M., Poli, V., van der Geer, P., Chojkier, M. and Hunter, T. (1999) Phosphorylation of rat serine 105 or mouse threonine 217 in C/EBP β is required for hepatocyte proliferation induced by TGF α. Mol. Cell. **4**, 1087–1092

20 Pan, Z., Hetherington, C. J. and Zhang, D. E. (1999) CCAAT/enhancer-binding protein activates the CD14 promoter and mediates transforming growth factor β signaling in monocyte development. J. Biol. Chem. **274**, 23242–23248

21 Battegay, E. J., Raines, E. W., Seifert, R. A., Bowen-Pope, D. F. and Ross, R. (1990) TGF-beta induces bimodal proliferation of connective tissue cells via complex control of an autocrine PDGF loop. Cell **63**, 515–524

22 Heldin, C. H., Miyazono, K. and Dijke, P. (1997) TGF-β signalling from cell membrane to nucleus through SMAD proteins. Nature (London) **390**, 465–471

23 Welch, D. R., Fabra, A. and Nakajima, M. (1990) Transforming growth factor beta stimulates mammary adenocarcinoma cell invasion and metastatic potential. Proc. Natl. Acad. Sci. U.S.A. **87**, 7678–7682

24 Bandyopadhyay, A., Zhu, Y., Cibull, M. L., Bao, L., Chen, C. and Sun, L. (1999) A soluble transforming growth factor beta type III receptor suppresses tumorigenicity and metastasis of human breast cancer MDA-MB-231 cells. Cancer Res. **59**, 5041–5046

25 Oft, M., Peli, J., Rudaz, C., Schwarz, H., Beug, H. and Reichmann, E. (1996) TGF-beta1 and Ha-Ras collaborate in modulating the phenotypic plasticity and invasiveness of epithelial tumor cells. Genes Dev. **10**, 2462–2477

26 Himes, S. R., Reeves, R., Attema, J., Nissen, M., Li, Y. and Shannon, M. F. (2000) The role of high-mobility group I(Y) proteins in expression of IL-2 and T cell proliferation. J. Immunol. **164**, 3157–3168

27 Bonnefoy, E., Bandu, M. T. and Doly, J. (1999) Specific binding of high-mobility-group I (HMGI) protein and histone H1 to the upstream AT-rich region of the murine beta interferon promoter: HMGI protein acts as a potential antirepressor of the promoter. Mol. Cell. Biol. **19**, 2803–2816

28 Shannon, M. F., Himes, S. R. and Attema, J. (1998) A role for the architectural transcription factors HMGI(Y) in cytokine gene transcription in T cells. Immunol. Cell. Biol. **76**, 461–466

29 Mantovani, F., Covaceuszach, S., Rustighi, A., Sgarra, R., Heath, C., Goodwin, G. H. and Manfioletti, G. (1998) NF-κB mediated transcriptional activation is enhanced by the architectural factor HMGI-C. Nucleic Acids Res. **26**, 1433–1439

30 Arlotta, P., Rustighi, A., Mantovani, F., Manfioletti, G., Giancotti, V., Tell, G. and Damante, G. (1997) High mobility group I proteins interfere with the homeodomains binding to DNA. J. Biol. Chem. **272**, 29904–29910

31 Wood, L. D., Farmer, A. A. and Richmond, A. (1995) HMGI(Y) and Sp1 in addition to NF-κB regulate transcription of the MGSA/GRO α gene. Nucleic Acids Res. **23**, 4210–4219

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