Up-regulation of Transforming Growth Factor (TGF)-β Receptors by TGF-β1 in COLO-357 Cells

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In the present study we investigated the actions of transforming growth factor (TGF)-β1 on gene induction and cyclin-dependent kinase inhibitors in relation to TGF-β receptor modulation in COLO-357 pancreatic cancer cells. TGF-β1 inhibited the growth of COLO-357 cells in a time- and dose-dependent manner and caused a rapid but transient increase in plasminogen activator inhibitor-1 and insulin-like growth factor binding protein-3 mRNA levels. TGF-β1 caused a delayed but sustained increase in the protein levels of the cyclin-dependent kinase inhibitors p15\(^{INK4B}\), p21\(^{CIP1}\), and p27\(^{KIP1}\) and a sustained increase in type I and II TGF-β receptors (T\(\beta\)RI and T\(\beta\)RII) mRNA and protein levels. The protein synthesis inhibitor cycloheximide (10 μg/ml) completely blocked the TGF-β1-mediated increase in T\(\beta\)RI and T\(\beta\)RII expression. Furthermore, a nuclear runoff transcription assay revealed that the increase in receptor mRNA levels was due to newly transcribed RNA. There was a significant increase in T\(\beta\)RI and T\(\beta\)RII mRNA levels in confluent cells in comparison to subconfluent (≤80% confluent) controls, as well as in serum-starved cells when compared with cells incubated in medium containing 10% fetal bovine serum. COLO-357 cells expressed a normal SMAD4 gene as determined by Northern blot analysis and sequencing. These results indicate that TGF-β1 modulates a variety of functions in COLO-357 cells and up-regulates TGF-β receptor expression via a transcriptional mechanism, which has the potential to maximize TGF-β1-dependent antiproliferative responses.

Transforming growth factor (TGF)\(^{1,β1}\) is a multifunctional cytokine that plays an important role in regulating cellular growth and differentiation in many biological systems (1). TGF-β1 induces growth inhibitory or stimulatory responses, depending on the cell type and growth conditions (2, 3). In most epithelial cells, TGF-β1 inhibits growth while enhancing the production of extracellular matrix proteins (4).

TGF-β1 signals through a family of transmembrane receptors that have intrinsic serine/threonine kinase activity (1). The type II TGF-β receptor (T\(\beta\)RII) binds TGF-β1 and then forms a heteromeric complex with the type I TGF-β receptor (T\(\β\)RI). This complex most likely includes both type I and type II receptors (1). Activated T\(\beta\)RII transphosphorylates the glycine- and serine-rich domain of the type I receptor kinase, thereby activating T\(\beta\)RI, which then transiently associates with and phosphorylates SMAD2 and/or SMAD3. These proteins belong to a recently discovered family of intracellular signaling molecules (1). Phosphorylated SMAD2 and/or SMAD3 form a heteromeric complex with SMAD4, which is required for the translocation of both proteins into the nucleus, where they can act as transcriptional activators (1, 5–7). TGF-β1 is thus able to induce the expression of growth inhibitory proteins that suppress cell cycle progression, such as the cyclin-dependent kinase (Cdk) inhibitors p15\(^{INK4B}\), p21\(^{CIP1}\), and p27\(^{KIP1}\) (8–11) or proteins that interfere with mitogenic signaling, such as insulin-like growth factor binding protein-3 (IGFBP-3) (12, 13). However, depending on the cell type, TGF-β1 activates or inhibits IGFBP-3 transcription (14, 15).

In some cell types growth inhibition may be mediated via a pathway that is distinct from the signaling pathway regulating expression of genes that modulate the extracellular matrix. For example, TGF-β1 is able to induce the expression of a number of genes such as PAI-I (16), which inhibits both tissue-type and urokinase-type plasminogen activators (17). In mink lung epithelial cells, expression of a truncated T\(\beta\)RII does not attenuate TGF-β1-mediated induction of PAI-I. In contrast, expression of the truncated T\(\beta\)RII renders these cells resistant to the antiproliferative effects of TGF-β1 (18).

Cultured human pancreatic cancer cell lines are usually resistant to the growth inhibitory effects of TGF-β1 (19). This resistance may be the consequence of a number of alterations, including the presence of mutated TGF-β receptors (20), decreased expression of T\(\beta\)RII (19) or T\(\beta\)RI (21), and mutations in the SMAD4/DPC4 gene (22). Although, COLO-357 pancreatic cancer cells are relatively sensitive to TGF-β1-mediated growth inhibition (23), the mechanisms underlying this sensitivity are not known. Furthermore, it has not been established whether TGF-β1 modulates the expression of growth-regulating genes in these cells. Therefore, in the present study we characterized the effects of TGF-β1 on the expression of PAI-I, IGFBP-3, p15\(^{INK4B}\), p21\(^{CIP1}\), and p27\(^{KIP1}\) in relation to its effects on the expression of T\(\β\)RI and T\(\β\)RII. We now report that TGF-β1 induces rapid but transient up-regulation of PAI-I and IGFBP-3 mRNA in COLO-357 cells and enhances p15\(^{INK4B}\), p21\(^{CIP1}\), and p27\(^{KIP1}\) and T\(\β\)RII expression in a sustained manner in these cells.

EXPERIMENTAL PROCEDURES

Materials—The following materials were purchased: FBS, Dulbecco’s modified Eagle’s medium, trypsin solution, and penicillin-streptomycin...
solution from Irvine Scientific (Santa Ana, CA); Genescreen membranes from NEN Life Science Products; Immobilon P membranes from Millipore (Bedford, MA); restriction enzymes and random primed labeling kit from Boehringer Mannheim; Sequenase Version 1.0 DNA sequencing kit from U.S. Biochemicals (Cleveland, OH); \( \alpha^{32P} \)-labeled dCTP, \( \alpha^{35S} \)-labeled dATP, ECL blotting kit, and leupeptin from U.S. Biochemicals (Cleveland, OH); PCR primers from Bio Synthesis, Inc. (Lewisville, TX); reverse transcriptase kit from Life Technologies, Inc. All other reagents were from Sigma.

TGF-\( \beta \)-1 was a gift from Genentech, Inc. (South San Francisco, CA); COLO-357 cells were a gift from Dr. R. S. Metzger (Durham, NC).

**Cell Culture—**COLO-357 were routinely grown in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS, 100 units/ml penicillin, and 100 \( \mu \)g/ml streptomycin (complete medium). For TGF-\( \beta \)-1 experiments, subconfluent cells were incubated overnight in serum-free medium in the absence or presence of TGF-\( \beta \)-1. Incubations were continued for the indicated time prior to adding 3-(4,5-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 62.5 \( \mu \)g/well) for 4 h (19). Cellular MTT was solubilized with acidic isopropyl alcohol and optical density was measured at 570 nm with an enzyme-linked immunosorbent assay plate reader ( Molecular Devices, Menlo Park, CA). In pancreatic cancer cells the results of the MTT assay correlate with results obtained by cell counting with a hemocytometer and by monitoring \(^{3} \)H-thymidine incorporation (23, 24).

**RNA Extraction and Northern Blot Analysis—**Total RNA was extracted by the single step acid guanidium thiocyanate phenol chloroform method. RNA was size fractionated on 1.2% agarose/1.8 M formaldehyde gels, electrotransferred onto nylon membranes and cross-linked by UV irradiation (25). Blots were prehybridized and hybridized with cDNA probes (TbRI, PAI-1, IGFBP-3, 7S) or a TpRI ribonuclease and washed under high stringency conditions as previously reported (25). Blots were then exposed at -80 °C to Kodak XAR-5 films using intensifying screens. A 1.7-kilobase pair fragment of PAI-I (obtained from Dr. J. Massague, Memorial Sloan-Kettering Cancer Center, New York) was labeled with \(^{32} \)P-dCTP to the recommended protocol. To measure specific gene transcription, COLO-357 cells (5 \times 10^6 cells/sample) were trypsinized and suspended in complete medium. After centrifugation at 500 \( \times \) g for 5 min, the cell pellet was resuspended and washed in phosphate-buffered saline (4 °C) and centrifuged at 500 \( \times \) g for 5 min. Cells were then lysed in 4 ml of lysis buffer containing 10 mM Tris (pH 7.4), 10 mM NaCl, 3 mM MgCl\(_2\), and 0.5% Nonident P-40. After a second 500 \( \times \) g (5 min) centrifugation step, the nuclear pellet was resuspended in 4 ml of lysis buffer and centrifuged again. The pellet was then resuspended in 200 \( \mu \)l of storage buffer containing 50 mM Tris (pH 8.3), 5 mM MgCl\(_2\), 0.1 mM EDTA, and 40% glycerol and stored at -80 °C for subsequent transcription.

Nuclear runoff assays were initiated by incubating 200 \( \mu \)l of the frozen nuclei with 200 \( \mu \)l of reaction buffer containing 5 \( \mu \)l of 1 mM ATP, CTP, GTP, and 10 \( \mu \)l of 10 mM Tris (pH 7.4) centrifuged at 500 \( \times \) g for 5 min. After incubating samples for 30 min (42 °C) with 10 \( \mu \)l of proteinase K in 200 \( \mu \)l of SDS/Tris buffer consisting of 5% SDS, 0.5 mM Tris, pH 7.4, and 0.125 mM EDTA, nuclear RNA was extracted as described previously (25) and dissolved in 0.5 ml of diethyl pyrocarbonate-H\(_2\)O. When necessary, samples were diluted in diethyl pyrocarbonate-H\(_2\)O to adjust \(^{32} \)P-labeled RNA equally for each sample to 5 \( \times \) 10^6 cpm/ml. Linearized TpRI and TbRI cDNA (100 \( \mu \)g) was denatured by incubating samples for 30 min (23 °C) in 0.2 mM NaOH and neutralized with 6X SSC, cDNA samples (5 \( \mu \)g) were slot blotted onto nylon membranes and UV cross-linked. Membranes were preincubated at 42 °C as described earlier and incubated at 42 °C with \(^{32} \)P-labeled RNA samples for 24 h. Membranes were washed twice in 2X SSC at 65 °C for 30 min, incubated for 30 min at 37 °C with 10 mg/ml RNase A in 2X SSC to remove unbound \(^{32} \)P-labeled RNA, washed again in 2X SSC at 37 °C for 1 h, and exposed at -80 °C to Kodak XAR-5 films using intensifying screens.

**RESULTS**

**Effects of TGF-\( \beta \)-1 on COLO-357 Pancreatic Cancer Cell Growth—**To confirm that COLO-357 cells are sensitive to TGF-\( \beta \)-1-mediated growth inhibition (19), cells were incubated in the absence (control) or presence of TGF-\( \beta \)-1 (Fig. 1). TGF-\( \beta \)-1 significantly inhibited growth after a 48-h incubation, maximal effects occurring at 100 pM TGF-\( \beta \)-1 (35.8% ± 6.3%, \( p < 0.01 \)).
To investigate the effects of TGF-β on PAI-I and IGFBP-3 mRNA levels, COLO-357 cells were serum starved overnight and incubated with 200 pM TGF-β1 for the indicated times. RNA was extracted as described under “Experimental Procedures,” and 20 μg of total RNA were size fractionated on 1.2% agarose, 1.8 M formaldehyde gels. Blots were hybridized with 32P-labeled cDNA probes for PAI-I (5 x 10⁶ cpm/ml), IGFBP-3 (5 x 10⁶ cpm/ml), and 7S (5 x 10⁶ cpm/ml). Exposure times were 24 h (PAI-I, IGFBP-3) and 4 h (7S).

Following a more prolonged incubation (72 h), 100 pM TGF-β1 inhibited the growth of COLO-357 cells by 47.7% ± 4.5% (p < 0.01).

Effects of TGF-β1 on PAI-I and IGFBP-3 Expression—To study the effects of TGF-β1 on the expression of PAI-I and IGFBP-3 mRNA, Northern blot analysis was performed using total RNA extracted from control and TGF-β1-treated (200 pM) COLO-357 cells. TGF-β1 caused a time-dependent increase in PAI-I and IGFBP-3 mRNA levels (Fig. 2). Densitometric analysis revealed a rapid increase in PAI-I mRNA levels, with maximal stimulation (11-fold) occurring within 3 h. PAI-I mRNA levels then decreased gradually but were still elevated above baseline levels (5-fold) after 48 h of incubation. IGFBP-3 mRNA levels also increased maximally (4-fold) after 3 h of TGF-β1 stimulation. In contrast to PAI-I mRNA levels, IGFBP-3 mRNA returned to control levels after 6 h.

Effects of TGF-β1 on p15INK4B, p21Cip1, and p27Kip1 Levels—To investigate the effects of TGF-β1 on the levels of the Cdk inhibitors p15INK4B, p21Cip1, and p27Kip1, 60% confluent COLO-357 cells were initially incubated in serum-free medium for 12 h. Cells were then incubated in serum-free medium for an additional 48 h in the absence or presence of 200 pM TGF-β1, which was present for 12, 24, and 48 h prior to analysis. Lysates were then analyzed by immunoblotting with specific antibodies. Initially, TGF-β1 did not increase p15INK4B, p21Cip1, and p27Kip1 protein levels. The mild decrease observed after 12 h (Fig. 3) was inconsistent. In contrast, after 24 h, TGF-β1 caused a marked and consistent increase in all three cyclin-dependent kinase inhibitors, and this effect was consistently sustained for at least 48 h (Fig. 3).

Effects of TGF-β1 on TβRI/II Expression—To investigate the effects of TGF-β1 on the expression of TGF-β receptors, we first sought to determine the effects of serum and cell confluency on TβRI and TβRII mRNA expression. COLO-357 cells were incubated in complete medium until they were 70% confluent and then incubated an additional 12 h in serum-free or complete medium. Densitometric analysis revealed that following 12 h of serum starvation there was a 5- and 6-fold increase in TβRI and TβRII mRNA levels, respectively, compared with control cells incubated with medium containing 10% FBS (Fig. 4).

Prolonged incubation (24, 48, and 72 h) of COLO-357 cells in serum-free medium did not significantly alter TβRII mRNA levels by comparison with the 12-h incubation (data not shown), suggesting that a steady state of TβRII mRNA was achieved within the first 12 h of serum starvation. Next, COLO-357 cells were incubated in complete medium and grown to 60, 80, and 100% confluent. There was a 6- and 2-fold increase in TβRI and TβRII mRNA levels, respectively, in 100% confluent cells compared with that of the 60% confluent cells (Fig. 4). In contrast, receptor expression was comparable in 60 and 80% confluent cells.

To assure that serum starvation did not contribute to any ligand-induced change in receptor expression, all subsequent experiments were carried out after 12 h of serum starvation, so that the effects of serum starvation were already maximal prior to ligand addition. Conversely, to minimize the effects of cell confluency and serum on TβRII expression, all subsequent experiments were carried with cell confluency not exceeding 80% during the entire course of the experiments. Accordingly,
COLO-357 cells were grown to 60% confluency and initially incubated in serum-free medium for 12 h. Cells were subsequently incubated in serum-free medium for 48 h in the absence or presence of 200 μM TGF-β1, which was added 3, 6, 12, 24, and 48 h prior to RNA extraction. Under these experimental conditions, TGF-β1 caused a 4-fold increase in TβRI mRNA after 24 h and a further increase (6-fold) after 48 h in comparison to the unstimulated control (Fig. 5A). Similarly, TβRII mRNA levels exhibited a 3-fold increase after 24 h of incubation with 200 μM TGF-β1, and this increase was sustained for at least 48 h (4-fold).

To determine whether the TGF-β1-induced increase in steady-state TβRI and TβRII mRNA levels was associated with an increased in the corresponding protein levels, COLO-357 cells were incubated in the absence or presence of 200 μM TGF-β1 under the same conditions, and protein lysates were analyzed by SDS-polyacrylamide gel electrophoresis. TβRI protein levels were slightly elevated after 24 h and markedly increased after 48 h in comparison with control cells. In contrast, TβRII protein up-regulation was evident within 12 h of TGF-β1 addition and maximal after 48 h (Fig. 5B). Thus, the effects of TGF-β1 on TβRI/RII protein levels paralleled its effects on the corresponding mRNA levels.

Mechanisms of TGF-β1-mediated TβRII Up-regulation—To determine whether TGF-β1-mediated receptor up-regulation requires protein synthesis, COLO-357 cells were incubated 24–48 h in the presence or absence of 10 μg/ml cycloheximide or 1 nM TGF-β1. Cycloheximide did not cause cell death and did not significantly alter basal TβRI or TβRII mRNA levels (Fig. 6A and B). In contrast, after both 24 h (Fig. 6A) and 48 h (Fig. 6B), cycloheximide completely blocked the TGF-β1-mediated increase in TβRI/II mRNA levels. Cycloheximide also markedly attenuated the TGF-β1-induced increase in TβRI/II protein levels (Fig. 6C). Thus, TGF-β1-mediated up-regulation of TβRI/II is dependent on new protein synthesis.

To determine whether up-regulation of TβRII expression was due to increased RNA synthesis or enhanced stability of the mRNA moieties, the effects of TGF-β1 on newly transcribed RNA were examined next with the nuclear runoff transcription assay. COLO-357 cells were grown to 60% confluency, serum-starved for 12 h, and incubated in serum-free medium for 48 h in the absence or presence of 1 nM TGF-β1 for 3, 6, 12, 24, and 48 h prior to analysis. No increase of newly transcribed TβRI/II mRNA was observed during the first 12 h of stimulation. However, there was a marked increase in newly transcribed TβRI and TβRII mRNA after a 24- or 48-h incubation with TGF-β1 (Fig. 7).

SMAD4 Sequencing—COLO-357 cells were recently reported to harbor a homozygous deletion involving exons 1–4 of SMAD4 (27). In view of the importance of SMAD4 in TGF-β1-dependent signaling, we examined next the status of SMAD4 in our COLO 357 cells. Northern blot analysis of total RNA from COLO-357 cells clearly demonstrated a SMAD4 transcript in these cells that had the same size (approximately 4.5 kilobase pairs) as the SMAD4 transcript in human placenta (Fig. 8). Next, three reverse transcriptase-PCR fragments of the SMAD4 gene covering its entire coding region were sequenced, revealing that COLO-357 cells did not harbor deletions or mutations in the SMAD4 gene (data not shown).

DISCUSSION

An important mechanism for regulating the cellular response to cytokines and hormones resides at the level of receptor expression. It has been shown by Northern blot analysis and binding studies that 1,25-dihydroxyvitamin D₃ and prostaglandin E₂ down-regulate TβRI mRNA levels compared to the unstimulated control (31, 32). Conversely, in human lung fibroblasts (31) and human corpus carvernosum smooth muscle cells (32), TGF-β1 increases steady-state levels of TβRI mRNA, potentially by increasing TβRI promotor activity (31). However, little is known about the regulation of TβRII by TGF-β1. In the present study we determined that TGF-β1 enhances both TβRI and TβRII expression levels in COLO-357 pancreatic cancer cells. This effect occurred in a time-dependent manner with a marked increase after 24 and 48 h. This increase in mRNA levels was associated with enhanced protein synthesis of both receptors. Two lines of evidence indicate that the TGF-β1-induced increase in TβRI and TβRII mRNA levels was effected at the level of transcription. First, blocking protein synthesis with cycloheximide completely abrogated the TGF-β1-induced TβRI or TβRII up-regulation. Second, the nuclear runoff transcription assay demonstrated that TGF-β1 acted to enhance transcription of both receptors.

In the present study we also determined that TβRII is up-regulated following serum starvation, reaching a new steady-state level within 12 h. Moreover confluent COLO-357 cells also exhibited increased TβRII mRNA levels compared with subconfluent control cells. It has been shown for the...
up-regulation. COLO-357 cells were serum starved overnight and incubated in the absence (−) or presence (+) of 10 μg/ml cycloheximide or 1 nm TGF-β1 for 24 h (A) or 48 h (B and C). A and B, total RNA (20 μg/sample) was hybridized with a 32P-labeled TβRI riboprobe (2 × 10⁶ cpm/ml) and 32P-labeled TβRII (5 × 10⁵ cpm/ml) and 7S cDNA (5 × 10⁴ cpm/ml) probes. Exposure times were 48 h (TβRI and TβRII) and 4 h (7S). C, cell lysates (30 μg of protein/lane) following the 48-h incubation were subjected to immunoblotting with anti-TβRI and anti-TβRII antibodies.

epidermal growth factor receptor, that serum starvation leads to its down-regulation (33). Similarly, cell density-dependent down-regulation of several growth factor receptors, such as vascular endothelial growth factor receptor or hepatocyte growth factor receptor, has been demonstrated in a variety of cell types (34, 35). Ostensibly, this down-regulation is an important component of the signaling mechanism that leads to suppression of growth when cells are either deprived of nutrients or approaching confluence. In this context, up-regulation of TβRI and TβRII may serve to enhance growth inhibitory pathways under the same culture conditions (serum-free medium, confluent cells). While the mechanisms that contribute to cell density-dependent TGF-β receptor up-regulation are not known, it has been recently shown that activation of focal adhesion kinase by α5β1 integrins causes decreased surface expression of TβRI and TβRII (36). Inasmuch as α5β1 integrins and focal adhesion kinase participate in extracellular matrix-initiated intracellular signaling, our findings raise the possibility that cell-cell contact may also act to activate pathways that modulate TβRI/II gene expression.

The mammalian Cdk inhibitors p21Cip1, and p27Kip1 inhibit the activities of cyclin D-Cdk4, cyclin D-Cdk6, cyclin E-Cdk2, and cyclin A-Cdk2, whereas p15Ink4b interferes specifically with cyclin D binding to Cdk4 and Cdk6 (8–11, 37). Previous studies in a variety of epithelial cells have demonstrated that TGF-β1 can markedly up-regulate the expression of p21Cip1 and p15Ink4b, but exerts only a small stimulatory effect on p27Kip1 levels (11). In cell lines that are highly sensitive to TGF-β1-mediated growth inhibition, the effect of TGF-β1 on p21Cip1 and p15Ink4b up-regulation are relatively rapid (11, 37). Subsequently, TGF-β1 acts via the increase in p15Ink4b levels to displace p27Kip1 from Cdk4 and Cdk6 (11). However, in normal mouse B lymphocytes, TGF-β1 increases p27Kip1 protein levels (38), and such an effect may be due in part to decreased degradation of the protein (37). In the present study, we determined that TGF-β1 causes a delayed but sustained increase in p15Ink4b, p21Cip1, and p27Kip1 protein levels, which was readily and consistently evident only after 24 h. While the molecular mechanisms that lead to this increase of three different Cdk inhibitors in COLO-357 cells are not known, the relatively slow kinetics of this up-regulation underscore our observation that these cells are insensitive to the TGF-β1-mediated growth inhibition during the initial 24 h incubation period (Fig. 1). Conversely, the marked increase in p15Ink4b, p21Cip1, and p27Kip1 protein levels that occurs 24 and 48 h after the addition of TGF-β1 suggests that these Cdk inhibitors are then able to contribute to the inhibitory effect of TGF-β1 on cell growth. These observations also raise the possibility that the delayed up-regulation of the Cdk inhibitors is dependent on the TGF-β1-induced increase in TβRI/II expression.
TGF-β1 caused a rapid increase in PAI-1 mRNA levels in COLO-357 cells followed by a less pronounced but sustained increase during the subsequent 48 h. PAI-I is the main inhibitor of the urokinase plasminogen activator system, which is thought to play an important role in cancer cell invasion (40–42). Our observation that TGF-β1 up-regulates PAI-I expression in COLO-357 cells suggests that TGF-β1 derived from the cells may act via PAI-I to enhance their metastatic potential.

TGF-β1 also caused a rapid increase in IGFBP-3 mRNA in COLO-357 cells with a maximal response occurring within 3 h of TGF-β1 addition. Growth inhibitory effects of IGFBP-3 may be caused by inhibition of IGF-1-dependent mitogenesis (43) or by IGF-1-independent mechanisms (12, 44). TGF-β1 is known to induce divergent effects on IGFBP-3 expression. In endothe-

SMAD4

lial cells, TGF-β1 reduces IGFBP-3 mRNA levels (14), whereas it causes a dose-dependent increase of IGFBP-3 in porcine myogenic cells (15). In human breast cancer cells TGF-β1 stimu-

lates IGFBP-3 production and induces binding of IGFBP-3 to the cell surface (44). Irrespective of its role in other cell lines, the transient nature of IGFBP-3 induction observed in the present study suggests that IGFBP-3 does not play a major role in the TGF-β1-induced antiproliferative response in COLO-357 cells.

SMAD4 is an important signaling molecule that is downstream of the TβRI and TβRII signaling pathway. It is crucial in mediating TGF-β1 responses (5). Pancreatic cancers and cultured pancreatic cancer cell lines often harbor SMAD4 muta-

tions, which lead to loss of TGF-β1-dependent growth sup-

pression (22, 27, 45). Although COLO-357 cells have been re-

ported to harbor a homozygous deletion in the SMAD4 gene (27), four lines of evidence indicate that COLO-357 cells used in the present study express functional SMAD4. First, COLO-357 cells exhibited a SMAD4 transcript by Northern blot analysis, which was the same size as the SMAD4 transcript in human placenta. Second, the SMAD4 transcript was readily demonstrated by reverse transcriptase-PCR analysis, using specific SMAD4 primers (27). Third, complete sequencing of the SMAD4 gene in COLO-357 cells did not reveal any mutation. Fourth, COLO-357 cells exhibited rapid, intermediate, and delayed responses to TGF-β1, including our previous finding of autoinduction of autodownregulation of TGF-β1 (23) and the present results demonstrat-

ing increased expression of IGFBP-3, PAI-1, TβRI, and TβRII. Taken together, these observations suggest that up-

regulation of TβRI and TβRII may be part of an overall gene response in certain cells that serves to maximize the antiprolifera-

tive actions of TGF-β1.

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