Cross-talk between IL-6 and TGF-β signaling in hepatoma cells

Tetsuya Yamamoto[a,1], Tadashi Matsuda[a,1,*], Atsushi Muraguchi[a], Kohei Miyazono[b], Masahiro Kawabata[b]

[a] *Department of Immunology, Faculty of Medicine, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930-0194, Japan*
[b] *Department of Biochemistry, The Cancer Institute of Japanese Foundation for Cancer Research (JFCR), 1-37-1 Kami-ikebukuro, Toshima-ku, Tokyo 170-8455, Japan*

Received 1 February 2001; accepted 20 February 2001

First published online 2 March 2001

Edited by Marco Baggiolini

Abstract Interleukin-6 (IL-6) is a multifunctional cytokine that plays important roles in the immune system, hematopoiesis, and acute phase reactions. Transforming growth factor-β (TGF-β) also has pleiotropy including the production of acute phase proteins in hepatocytes. To elucidate the cross-talk between IL-6 and TGF-β signaling pathways in hepatic cells, we investigated the effects of TGF-β on IL-6-induced signal transducer and activator of transcription-3 (STAT3) activation in a human hepatoma cell line, Hep3B. IL-6-induced activation of STAT3 activity and STAT3-mediated gene expression were augmented by TGF-β in Hep3B cells. We provide evidence that these activities were due to physical interactions between STAT3 and Sma- and MAD-related protein-3, bridged by p300. These results demonstrate a molecular mechanism of a cross-talk between STAT3 and TGF-β signaling pathways in hepatocytes.

Key words: Interleukin-6; Signal transducer and activator of transcription-3; Transforming growth factor-β; Sma- and MAD-related protein; p300; Cross-talk; Transcription

1. Introduction

Interleukin-6 (IL-6) is a pleiotropic cytokine that regulates immune and inflammatory responses [1,2]. The receptors for the IL-6 family of cytokines share the gp130 molecule through which signals are generated, although the cytoplasmic region of gp130 does not contain any catalytic domain. Instead, the Janus kinase (Jak) family of protein kinases constitutively associate with gp130 and are activated by the IL-6 family of cytokines [3], leading to the tyrosine phosphorylation and activation of the signal transducer and activator of transcription (STAT) family of transcription factors.

One member of the STAT family of proteins is STAT3 which is mainly activated by the IL-6 family of cytokines, epidermal growth factor, and leptin [2,3]. Like other members of the STAT family, STAT3 is tyrosine-phosphorylated by Jak kinases, upon which it dimerizes, and translocates into the nucleus to activate target genes [4,5].

The members of the Smad (Sma- and MAD-related protein) family are signal transducers of the transforming growth factor-β (TGF-β) superfamily. Smad2 and Smad3 transduce signals for TGF-β. Smad4 acts as a common partner for these Smad proteins. When TGF-β receptors are activated by the binding of cognate ligands, Smads are phosphorylated by the type I receptor (TβR-I) serine-threonine kinases. Phosphorylated Smads form stable complexes with Smad4, and these complexes translocate into the nucleus where they activate transcription as a coactivator of DNA-binding transcription factors. Smad7, one of the inhibitory Smads, also stably interacts with activated TβR-I and inhibits the TGF-β signal [6,7].

IL-6 has been shown to be the principal regulator of most acute phase proteins [2,8] although other inflammation-associated cytokines also contribute to this process. IL-1 and tumor necrosis factor-α have been found to participate in induction of a broad subset of acute phase proteins, and both TGF-β and interferon-γ can induce limited subsets of acute phase proteins [9]. TGF-β has also been shown to have an effect on IL-6-regulated acute phase proteins [10–12]. IL-6 has been shown to activate members of the CCAAT/enhancer binding protein (C/EBP) family of transcription factors in hepatoma cell lines [13]. It has recently been found that STAT3 may also play a major role in mediating IL-6 effects in hepatocytes [3–5].

Recently, an IL-6 family cytokine, leukemia inhibitory factor (LIF), and a TGF-β family cytokine, bone morphogenetic protein-2 (BMP-2), were shown to act in synergy on primary fetal neural progenitor cells to induce astrocytes [14]. It was also demonstrated that the formation of a complex between STAT3 and Smad1, bridged by p300, is involved in the cooperative signaling of LIF and BMP-2 and the subsequent induction of astrocytes from neural progenitors.

In this study, we showed that IL-6-induced gene expression in hepatoma cells was enhanced by TGF-β signal and the cross-talk between the IL-6 and TGF-β signaling cascades occurs by physical and functional interactions between STAT3 and Smad3, bridged by p300 in a hepatoma cell line.

2. Materials and methods

2.1. Reagents and antibodies

Human recombinant IL-6 was a kind gift from Ajinomoto (Tokyo,

*Corresponding author. Fax: (81)-76-434 5019.
E-mail: tmatsuda@ms.toyama-mpu.ac.jp

[1] These authors contributed equally.

Abbreviations: IL, interleukin; TGF, transforming growth factor; STAT, signal transducer and activator of transcription; LUC, luciferase; LIF, leukemia inhibitory factor; TβR-I, TGF-β type I receptor; Smad, Sma- and MAD-related protein; BMP, bone morphogenetic protein

0014-5793/01/$20.00
PII: S0014-5793(01)02258-X

Japan). Human recombinant TGF-β1 was purchased from Strathmann Biotech GmbH (Germany). Human recombinant LIF was purchased from Intergen (Purchase, NY, USA). Expression vectors, Smad2, Smad3, Smad4, Smad7, Smad3DE, 6×Myc-tagged Smad3, and Flag-tagged p300 were described previously [15]. Flag-tagged STAT3-C [16], wild-type TβR-I, TβR-I (T204D), p3TP-LUC [17], C/EBPδ [18], a dominant-negative form of STAT3 (DN-STAT3) [19], and STAT3-LUC [20] were kindly provided by Dr. J.F. Bromberg (Rockefeller University, New York, NY, USA), Dr. J. Massague (Memorial Sloan-Kettering Cancer Center, New York, NY, USA), Dr. S. Akira (Osaka University, Osaka, Japan) and Dr. T. Hirano (Osaka University, Osaka, Japan), respectively. Anti-HA, anti-Myc, anti-STAT3, anti-Smad3, anti-p300 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phospho-STAT3 (Ser727) was purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-Flag M2 antibody was purchased from Upstate Biotechnology (Lake Placid, NY, USA).

2.2. Cell culture, transfections, and luciferase assays

The human hepatoma cell line Hep3B was maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS). Before stimulation, the cells were cultured for 24 h in DMEM without FCS, followed by treatment with IL-6 and/or TGF-β1. Hep3B cells ($2–2.5\times10^5$ in a 6-cm dish) were transfected using FuGENE6 (Roche Molecular Biochemicals) following the manufac-

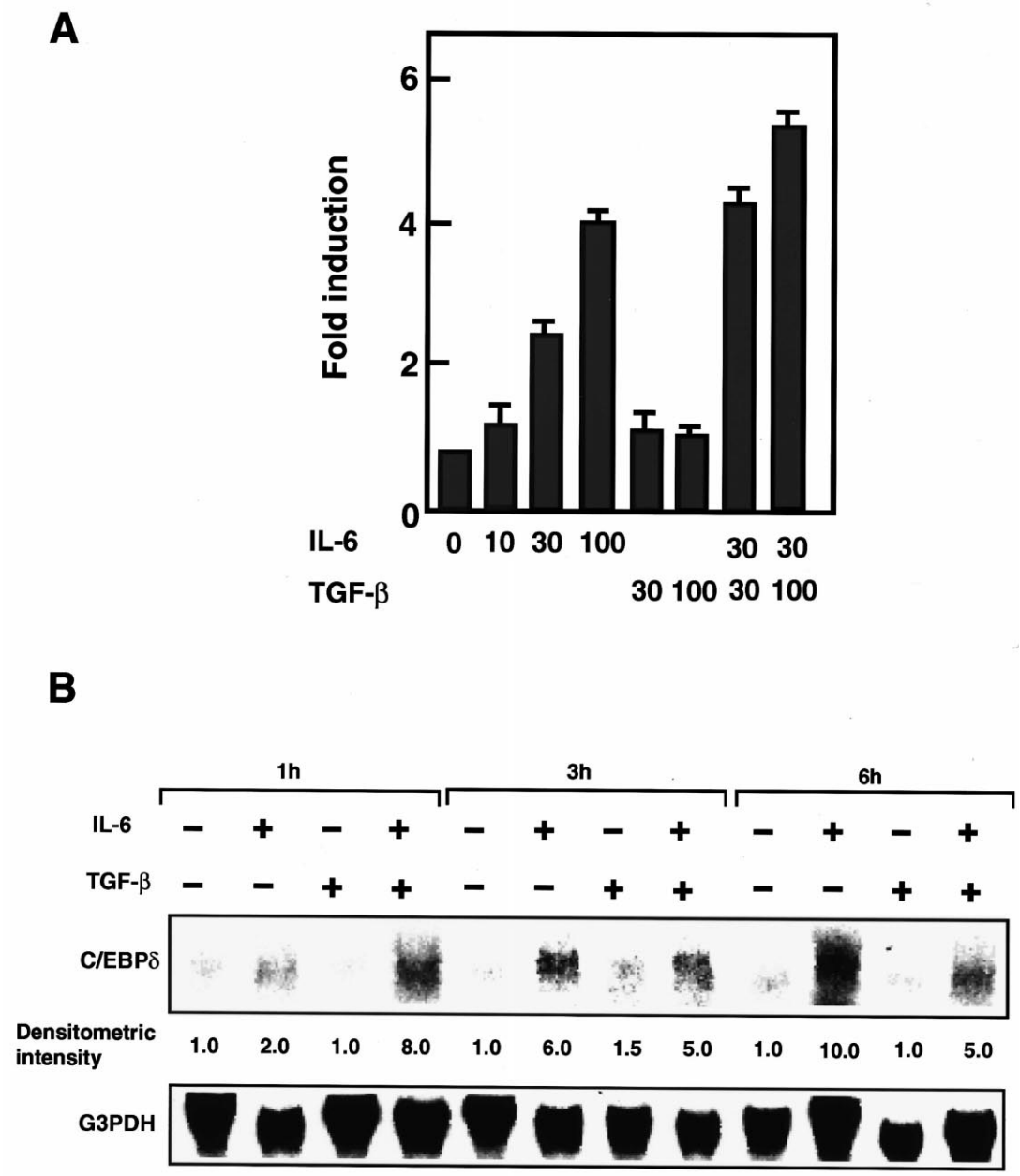

Fig. 1. TGF-β potentiates IL-6-induced STAT3 activation. A: Hep3B cells were transfected with STAT3-LUC reporter (1 μg). 48 h after transfection, cells were stimulated with various concentrations of IL-6 and/or TGF-β1 and cells were harvested and relative luciferase activities were measured. The results are presented as fold induction of luciferase activity from triplicate experiments. The error bars represent the standard deviations. B: Effect of IL-6 and/or TGF-β on C/EBPδ expression in Hep3B cells. 20 μg of total RNA from Hep3B cells treated with IL-6 (100 ng/ml) and/or TGF-β1 (30 ng/ml) for 1, 3, or 6 h was used for Northern blot analysis. The fold induction of C/EBPδ expression is shown as the densitometric intensity. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA is included as a loading control (lower panel).

turer's instructions. 293T cells were maintained in DMEM containing 10% FCS and transfected in DMEM containing 1% FCS by the standard calcium precipitation protocol. Luciferase assay was performed as described [21]. The cells were harvested 48 h after transfection and lysed in 100 μl of PicaGene Reporter Lysis Buffer (Toyo Ink, Tokyo, Japan) and assayed for luciferase and β-galactosidase activities according to the manufacturer's instructions. Luciferase activities were normalized to the β-galactosidase activities. Three or more independent experiments were carried out.

2.3. Northern blot analysis

Hep3B cells were maintained as described above. After serum starvation, cells (1×10^7) were treated with IL-6 (100 ng/ml) and/or TGF-β1 (30 ng/ml) for 1, 3, or 6 h. Total RNAs were prepared using IsoGen (Nippon Gene) and used in Northern analysis according to established procedures. A nylon membrane (Hybond N^+, Amersham Pharmacia Biotech) and radiolabelled cDNA probes, as indicated, were used.

2.4. Immunoprecipitation and immunoblotting

The immunoprecipitation and Western blotting were performed as described previously [22]. 293T cells were harvested and lysed in lysis buffer (50 mM Tris–HCl, pH 7.4, 0.15 M NaCl, containing 0.5% NP-40, 1 μM sodium orthovanadate, 1 μM phenylmethylsulfonyl fluoride, and 10 μg/ml each of aprotinin, pepstatin, and leupeptin). Hep3B cells were stimulated with IL-6 (100 ng/ml) and/or TGF-β1 (100 ng/ml) for 15 min. Nuclear extracts were prepared as described previously [23]. Nuclear extracts in the above lysis buffer were immunoprecipitated with anti-p300 antibody. The immunoprecipitates from cell lysates were resolved on 5–20% SDS–PAGE and transferred to Immobilon filters (Millipore, Bedford, MA, USA). The filters were then immunoblotted with each antibody. Immunoreactive proteins were visualized using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech).

3. Results and discussion

3.1. TGF-β potentiates IL-6-induced STAT3 activation

To examine the molecular basis of the cross-talk between IL-6 and TGF-β signaling pathways, we utilized an IL-6-responsive human hepatoma cell line, Hep3B, and the transient transfection assay. The STAT3-mediated transcriptional responses were measured using STAT3-LUC, in which the α2-macroglobulin promoter [20] drives expression of the luciferase (LUC) reporter gene. Hep3B cells were transfected with STAT3-LUC and treated with IL-6 and/or TGF-β and LUC activities were determined. As shown in Fig. 1A, IL-6 stimulated STAT3-LUC activity in a dose-dependent manner, whereas TGF-β alone did not have an effect. When cells were treated with both IL-6 and TGF-β1, STAT3-LUC activity increased two-fold compared with the activation by IL-6 alone.

To further examine whether TGF-β has any effects on IL-6-induced transcriptional activation of cellular genes, we carried out Northern analysis on RNA samples prepared from Hep3B cells which had been stimulated with IL-6 and/or TGF-β. As a cellular target for IL-6/STAT3, we analyzed the expression of C/EBPδ which is a regulator of acute phase response genes in hepatocytes and is upregulated by IL-6 treatment [18]. As shown in Fig. 1B, C/EBPδ expression was induced at 1 h after treatment with IL-6 alone in Hep3B cells. This IL-6-induced C/EBPδ expression was markedly enhanced by the addition of TGF-β, whereas TGF-β alone did not affect C/EBPδ expression. This enhancement was not observed at 3 h after treatment, and suppressed at 6 h after treatment. These data show that TGF-β potentiates IL-6-induced STAT3-LUC transcription activity as well as IL-6-induced early transcription of C/EBPδ in Hep3B cells.

3.2. Reconstitution of the cross-talk between STAT3 and TGF-β signaling pathways in 293T cells

Previous studies have shown that p300/CBP is involved in STAT3- or Smad-mediated transcriptional activation [15,24]. Recently, it has been shown that p300 interacts physically with STAT3 at its amino-terminus, and with Smad1 at its carboxy-terminus in a ligand-dependent manner. Furthermore, it was demonstrated that the formation of a complex between STAT3 and Smad1, bridged by p300, is involved in the cooperative signaling of LIF and BMP-2 in neural progenitor cells [14].

To further delineate the details of the cross-talk between IL-6 and TGF-β signaling pathways via p300/CBP, we carried out transient transfection experiments in 293T cells. 293T cells were transfected with STAT3-LUC, and cells were stimulated with increasing amounts of LIF. We utilized LIF instead of IL-6 to activate STAT3-LUC in 293T cells, because LIF stimulated STAT3-LUC more effectively than IL-6 in these cells (data not shown). As shown in Fig. 2A, STAT3-LUC activity was induced by LIF in a dose-dependent manner. As expected from a previous report [24], additional expression of p300 augmented LIF-induced STAT3 activation in 293T cells. To assess whether these effects were mediated through STAT3 or some other intermediary factors, we used DN-STAT3 [19]. As expected, DN-STAT3 significantly inhibited LIF-induced STAT3-LUC expression in a dose-dependent fashion (Fig. 2A).

We next assessed the transcriptional activity by TGF-β signal in 293T cells using p3TP-LUC as reporter gene (Fig. 2B). 293T cells were transfected with p3TP-LUC together with or without an expression vector for wild-type TβR-I, and cells were stimulated with increasing amounts of TGF-β. p3TP-LUC activity was augmented by increasing amounts of TGF-β1 in 293T cells. When we co-transfected increasing amounts of the constitutively active form of TβR-I (T204D), with p3TP-LUC into 293T cells, p3TP-LUC activity increased in a dose-dependent fashion. TβR-I (T204D) stimulated p3TP-LUC more effectively than TGF-β/wild-type TβR-I in these cells. In addition, TβR-I (T204D) did not affect STAT3-LUC activity.

We then assessed the effect of TGF-β signal on STAT3 activity in 293T cells using TβR-I (T204D). 293T cells were transfected with increasing amounts of TβR-I (T204D) and STAT3-LUC in the presence of p300, and cells were stimulated with LIF. As shown in Fig. 2C, TβR-I (T204D) enhanced LIF-induced STAT3-LUC activity in a dose-dependent fashion. This enhancement of STAT3 activity by TβR-I (T204D) was observed in the absence of p300, but it was less effective compared with that observed in the presence of p300 (data not shown). These results indicate that the enhanced effect of TGF-β signal on STAT3 transcriptional activity can be reconstituted in 293T cells similar to those observed in Hep3B cells.

We next examined whether this effect was due to the Smad protein family, downstream signal transducers of the TGF-β superfamily. When either receptor-regulated Smads, Smad2 and Smad3, or a common-partner Smad, Smad4, was expressed in 293T cells, enhancement of LIF-induced STAT3-LUC activity by TGF-β signal was increased in a dose-dependent fashion (Fig. 2D). Conversely, no enhancement of LIF-induced STAT3-LUC activity by TGF-β signal was observed by expression of Smad7, an inhibitory Smad in 293T cells.

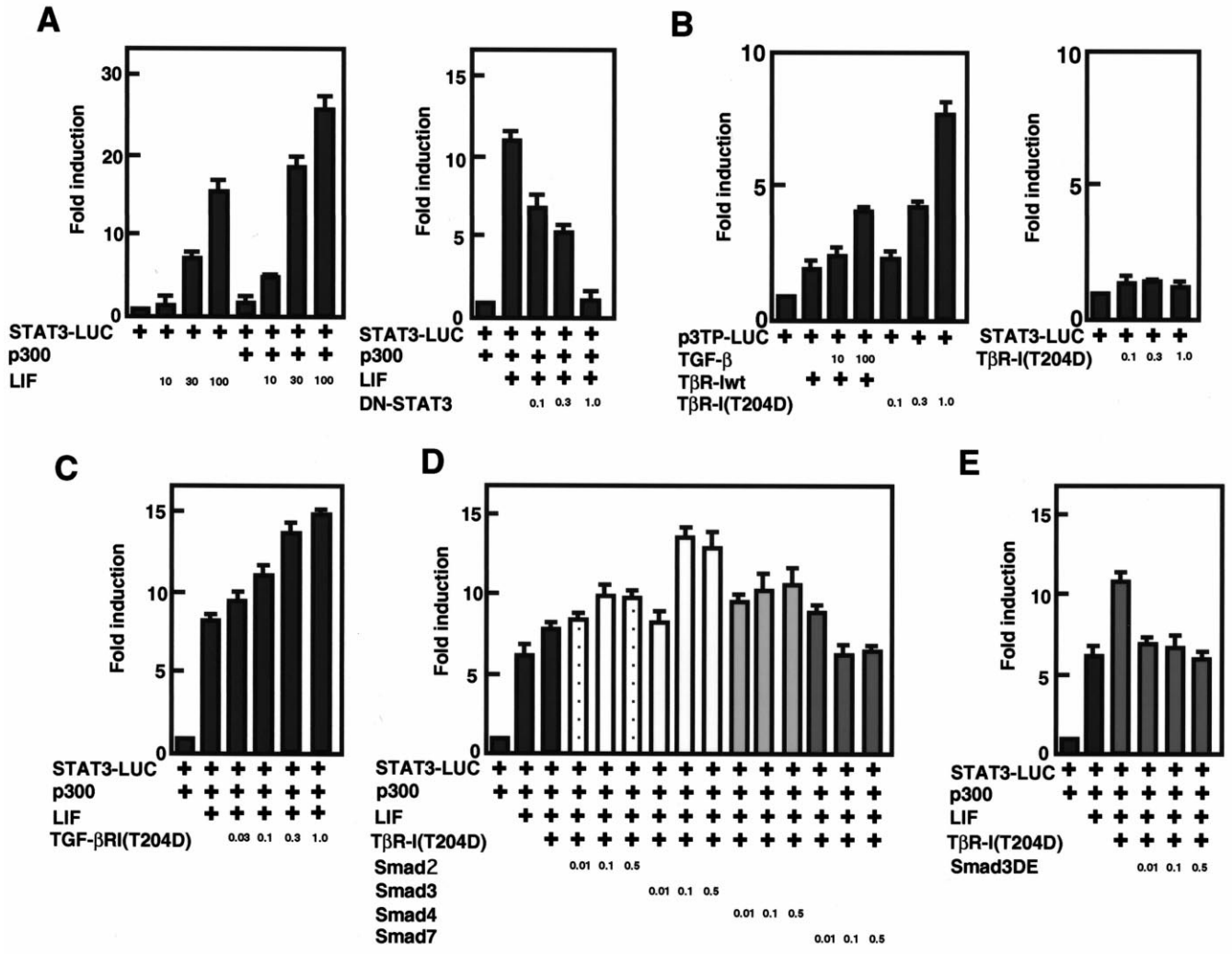

Fig. 2. Reconstitution of the cross-talk between STAT3 and TGF-β signaling pathways in 293T cells. A: 293T cells were transfected with STAT3-LUC (1 μg), and/or p300 expression construct (0.1 μg), and/or various doses (0.1–1.0 μg) of DN-STAT3. 48 h after transfection, cells were stimulated for an additional 12 h with LIF (10–100 ng/ml) as indicated and cells were harvested and relative luciferase activities were measured. B: 293T cells were transfected with p3TP-LUC (1 μg), and/or wild-type TβR-I (1 μg), various doses (0.1–1.0 μg) of TβR-I (T204D) expression construct. 48 h after transfection, cells were stimulated for an additional 12 h with TGF-β (100 ng/ml) and cells were harvested and relative luciferase activities were measured. C: 293T cells were transfected with STAT3-LUC (1 μg), and p300 expression construct (0.1 μg), and/or various doses (0.03–1.0 μg) of TβR-I (T204D). 48 h after transfection, cells were stimulated for an additional 12 h with LIF (30 ng/ml) and cells were harvested and relative luciferase activities were measured. D: 293T cells were transfected with STAT3-LUC (1 μg), and p300 expression construct (0.1 μg), and/or various doses (0.01–0.5 μg) of Smad2, Smad3, Smad4, Smad7 as indicated, together with TβR-I (T204D) (0.1 μg). 48 h after transfection, cells were stimulated for 12 h with LIF (30 ng/ml) and cells were harvested and relative luciferase activities were measured. E: 293T cells were transfected with STAT3-LUC (1 μg), and p300 expression construct (0.1 μg), and/or various doses (0.01–0.5 μg) of Smad3DE as indicated, together with TβR-I (T204D) (0.3 μg). 48 h after transfection, cells were stimulated for 12 h with LIF (30 ng/ml) and cells were harvested and relative luciferase activities were measured. The results are presented as fold induction of luciferase activity from triplicate experiments. The error bars represent the standard deviations.

Furthermore, a dominant-negative form of Smad3, Smad3DE, also suppressed the enhancement of LIF-induced STAT3-LUC activity by TGF-β signal (Fig. 2E). These data suggest that enhancement of STAT3 activation by the TGF-β1 signal in 293T cells is mediated through the Smad protein family in the presence of p300.

3.3. TGF-β signal enhances STAT3 activation by an active form of STAT3

To examine the direct cross-talk between STAT3 and the TGF-β signal, we used a constitutively active form of STAT3, STAT3-C [16]. 293T cells were transfected with STAT3-LUC, expression vectors for STAT3-C and/or increasing amounts of TβR-I (T204D), and the LUC activity was measured. As shown in Fig. 3, high STAT3-LUC activity was induced by STAT3-C, whereas TβR-I (T204D) alone did not show any effect (data not shown). Expression of TβR-I (T204D) resulted in enhancement of STAT3-C-induced STAT3-LUC activation and further expression of Smad3 but not Smad3DE showed the increase of STAT3-LUC activation. Additional expression of p300 showed the marked increase of STAT3 activation induced by STAT3-C with or without TβR-I (T204) and Smad3. These results clearly show the existence of cross-talk between the TGF-β signal and STAT3 in 293T cells.

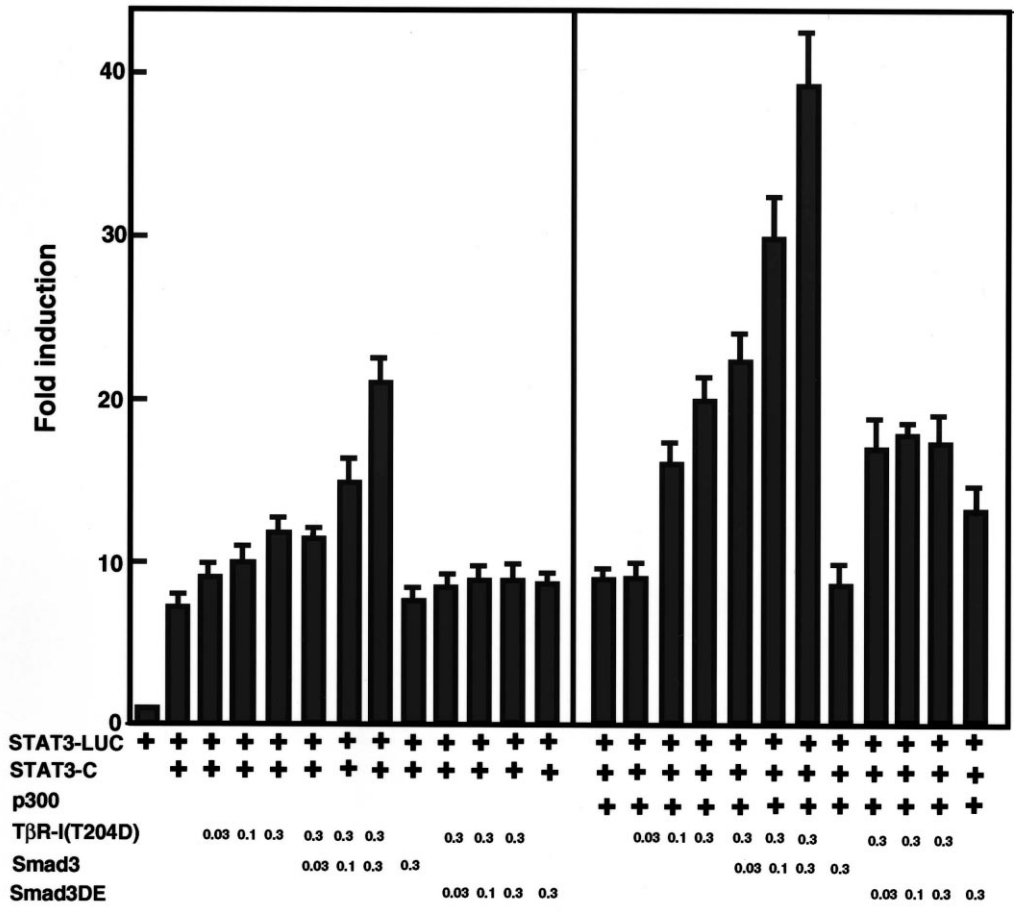

Fig. 3. TGF-β signal enhances STAT3 activation by an active form of STAT3. 293T cells were transfected with STAT3-LUC (1 μg) together with STAT3-C expression construct (1 μg), and/or various doses (0.03–0.3 μg) of TβR-I (T204D), Smad3 or Smad3DE, with or without p300 (0.1 μg). 48 h after transfection, cells were harvested and relative luciferase activities were measured. The results are presented as fold induction of luciferase activity from triplicate experiments. The error bars represent the standard deviations.

3.4. STAT3 and Smad3 physically interact via p300 in vivo

One of the mechanisms that are consistent with the data described above is direct modification of STAT3 by TβR-I (T204D), such as phosphorylation of STAT3, which trigger its activation. Therefore, we assessed changes in tyrosine phosphorylation of STAT3 in 293T cells. As shown in Fig. 4A, tyrosine phosphorylation of STAT3 did not show any change by expression of TβR-I (T204D), whereas phosphorylation of STAT3 on Ser727 was enhanced two-fold by the densitometric intensity. Although it remains unclear how phosphorylation of STAT3 on Ser727 is linked to transcriptional activation, it has been demonstrated in many cellular settings that this residue is essential for maximal STAT3 transcriptional activation [25]. In addition, a direct physical interaction between STAT3 and TβR-I (T204D) was observed in 293T cells (Fig. 4B). We tested another possibility, whether there are direct physical interactions between STAT3 and Smads. Expression vectors encoding 6×Myc-tagged Smad3 and/or HA-tagged STAT3 together with Jak1 were transiently transfected into 293T cells. However, we could not find a direct interaction between STAT3 and Smad3 in 293T cells (data not shown). A recent study showed that the formation of a complex between STAT3 and Smad1 bridged by p300 is involved in the synergistic signaling in fetal brain [14]. Therefore, we next examined this possibility. We transfected those expression constructs together with Flag-p300 into 293T cells. As shown in Fig. 4C, both STAT3 and Smad3 physically associated with p300. In parallel, similar co-immunoprecipitation experiments were performed using nuclear extracts obtained from Hep3B cells that had been either left untreated or treated with IL-6 and TGF-β. As shown in Fig. 4D, anti-p300 immunoprecipitate from Hep3B cells contained both STAT3 and Smad3 protein. These data indicate that STAT3 and Smad3 physically interact via p300 in vivo.

3.5. Concluding remarks

We have shown here that the TGF-β signal potentiates IL-6 signaling mediated by STAT3 in hepatoma cells and that active Smad interacts with STAT3 via p300. TGF-β treatment augmented endogenous early STAT3-mediated C/EBPδ gene expression in Hep3B cells as well as STAT3-dependent reporter activity in Hep3B and 293T cells. On the other hand, endogenous late STAT3-mediated C/EBPδ gene expression in Hep3B cells was suppressed by the treatment of TGF-β. This result coincides with previous data that TGF-β abolishes expression of several genes expressed in basal conditions in Hep3B cells and inhibits some IL-6-induced liver genes [11]. The mechanism of this suppressive effect of TGF-β in Hep3B cells is unclear at the present time and further studies are required to understand this event.

It is noteworthy that phosphorylation of STAT3 on Ser727 was enhanced by overexpression of TβR-I (T204D) and that a

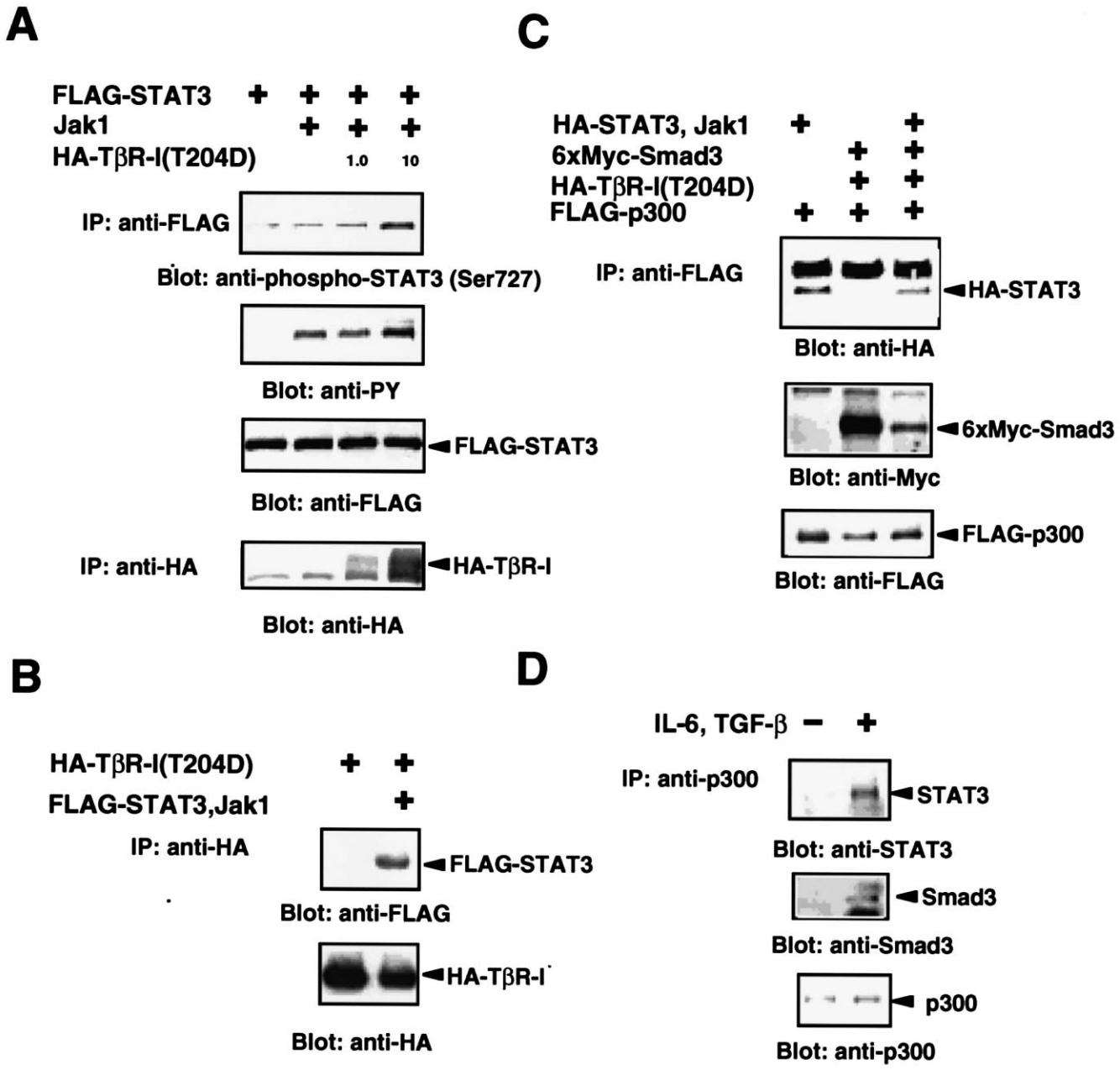

Fig. 4. STAT3 and Smad3 physically interact via p300 in vivo. A: 293T cells (1×10^7) were transfected with Flag-tagged STAT3 (5 μg) and/or various doses of TβR-I (T204D), together with Jak1 (1 μg). 48 h after transfection, cells were lysed, immunoprecipitated with anti-Flag or anti-HA as indicated. The immunoprecipitate was probed with anti-phosphotyrosine, anti-phospho-STAT3 (Ser727), anti-Flag, or anti-HA antibody as indicated. B: 293T cells (1×10^7) were transfected with Flag-tagged STAT3 (5 μg) and HA-tagged TβR-I (T204D) (10 μg), together with Jak1 (1 μg). 48 h after transfection, cells were lysed, immunoprecipitated with anti-HA. The immunoprecipitate was probed with anti-Flag or anti-HA antibody as indicated. C: 293T cells (2×10^7) were transfected with Flag-p300 (10 μg) with or without HA-tagged STAT3 (5 μg), 6×Myc-Smad3 (5 μg), HA-TβR-I (T204D) (1 μg) together with Jak1 (1 μg). 48 h after transfection, cells were lysed, immunoprecipitated with anti-Flag. The immunoprecipitate was probed with anti-HA, anti-Myc, or anti-Flag antibody as indicated. D: Hep3B cells (5×10^7) were unstimulated or stimulated with IL-6 (100 ng/ml) and TGF-β1 (100 ng/ml) for 15 min, and cells were lysed, immunoprecipitated, and immunoblotted with anti-STAT3, anti-Samd3, or anti-p300 antibody as indicated.

direct physical interaction between STAT3 and TβR-I (T204D) was observed in 293T cells. This implies that TβR-I (T204D) may be a receptor serine kinase for STAT3. However, no enhancement of phosphorylation of STAT3 on Ser727 was observed in Hep3B cells after stimulation of both IL-6 and TGF-β, indicating that overexpression of TβR-I (T204D) in 293T cells may be responsible for the enhancement.

It was reported that the formation of a complex between STAT3 and Smad1 bridged by p300 is involved in the synergistic signaling in fetal neural cells [14].

In this study, we have demonstrated a similar mechanism in a hepatoma cell line. The reconstituted system in 293T cells described here may not be physiological, but may be a good tool to explain synergistic actions of distinct types of cytokines in various biological signaling pathways and

thus provide a clue to develop new drugs for IL-6-related diseases.

Acknowledgements: We thank Dr. J.F. Bromberg, Dr. J. Massague, Dr. S. Akira, and Dr. T. Hirano for their kind gifts of reagents and Akira Junicho for technical help. This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture in Japan.

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