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The Smad3 Protein Is Involved in TGF-β Inhibition of Class II Transactivator and Class II MHC Expression

Yuanshu Dong,* Liping Tang,* John J. Letterio,† and Etty N. Benveniste‡*

TGF-β is a immunoregulatory cytokine that inhibits class II MHC expression in a variety of cell types. Previous studies have shown that the class II MHC transactivator (CIITA), a master regulator that controls class II MHC expression, is targeted by TGF-β for repression of IFN-γ-induced class II MHC expression in astrocytes. The mechanism(s) underlying the TGF-β inhibitory effect is not understood. In this study, we demonstrate that TGF-β inhibition of CIITA expression occurs at the transcriptional level, and that both constitutive and IFN-γ-induced human CIITA type IV promoter activity is inhibited by TGF-β. TGF-β does not affect the signaling events that mediate IFN-γ activation of CIITA expression; i.e., TGF-β does not inhibit IFN-γ-induced STAT-1α phosphorylation and/or DNA binding ability, nor is IFN-γ induction of IFN regulatory factor affected. The inhibitory effect of TGF-β on the type IV CIITA promoter is mediated through a promoter region within 80 bp from the transcription start site. Elimination of TGF-β inhibition of class II MHC and CIITA expression in Smad3-deficient astrocytes, as well as restoration of the inhibitory effect by overexpression of the Smad3 protein, demonstrates that Smad3 is essential in mediating TGF-β inhibition of CIITA and class II MHC expression. The Journal of Immunology, 2001, 167: 311–319.

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Abbreviations used in this paper: CIITA, class II MHC transactivator; GAS, IFN-γ activation sequence; IRF, IFN regulatory factor; SBE, Smad binding element; TIE, TGF-β inhibitory element; RLA, relative luciferase activity; RPA, ribonuclease protection assay; JAK, Janus kinase; CBP, CREB-binding protein.
are required for IFN-γ-induced activation of the type IV CIITA promoter (17–19).

TGF-β is a pleiotropic growth factor regulating embryonic development, proliferation, and differentiation of cells, wound healing, and angiogenesis (for review, see Ref. 22). TGF-β is a potent suppressor modulator of immunologic functions by regulating the development and differentiation of immunocompetent cells including B cells, T cells, and monocytes/macrophages (23–25). TGF-β also represses constitutive and IFN-γ-inducible class II MHC expression in a variety of cell types (26, 27). In TGF-β-deficient mice, the levels of class II MHC mRNA are elevated, and the incidence of fatal multifocal inflammatory disease is strikingly high (28), confirming the role of TGF-β as a suppressor of inflammation. In contrast, in mice that are both TGF-β and class II MHC deficient, manifestations of autoimmune diseases are strikingly diminished (29), suggesting that many of the effects of TGF-β on the immune system are mediated through repression of class II MHC gene expression. Based on this, TGF-β has been considered as a candidate to control autoimmune and chronic inflammatory diseases (30).

TGF-β utilizes two transmembrane receptors, the type I and type II receptors, both with serine/threonine kinase activity. Signaling is initiated when TGF-β binds to the type II receptor, which then recruits and phosphorylates the type I receptor, resulting in its activation (for review, see Refs. 31 and 32). The type I receptor phosphorylates a family of transcription factors named Smad proteins. Thus far, eight different Smad proteins have been identified; these include five receptor-activated Smads (R-Smads: Smads 1, 2, 3, 5, and 8), a co-Smad, Smad4, and two inhibitory Smads (I-Smads: Smads 6 and 7). TGF-β stimulation activates Smad2 and Smad3, resulting in their association with the co-Smad, Smad4, and translocation into the nucleus (for review, see Ref. 33). In the nucleus, Smads function to regulate transcriptional responses by directly binding to DNA or interacting with DNA-binding proteins (for review, see Ref. 32). Functional analysis showed that Smad2-deficient mice are embryonic lethal due to the failure of mesoderm formation (34, 35), whereas Smad3-deficient mice are viable, but develop a progressive illness exhibiting leukocytosis with massive inflammation (36). Spleenocytes from Smad3-deficient mice display resistance to TGF-β-mediated repression of activation, proliferation, and cytokine production (36, 37). These data suggest that Smad3 is a potential effector for TGF-β regulation of immune system function.

Previous work from our laboratory demonstrated that TGF-β inhibits IFN-γ-induced class II MHC expression in astrocytes at both the protein and mRNA levels (38, 39). Moreover, IFN-γ-induced CIITA mRNA expression was also inhibited by TGF-β, and this inhibition was not due to destabilization of CIITA mRNA (38). It has been shown that TGF-β suppresses both constitutive and IFN-γ-induced activity of CIITA promoters III and IV in the 2TGH fibrosarcoma cell line (19). In the present study, we wished to identify the mechanism of TGF-β inhibition of class II MHC expression in astrocytes. We show that TGF-β inhibition of CIITA expression in astrocytes occurs at the transcriptional level, and both IFN-γ-inducible and constitutive CIITA type IV promoter activity are inhibited by TGF-β. Analysis of mutated CIITA type IV promoter constructs reveals that the TGF-β inhibition is mediated primarily through a proximal promoter region within 80 bp of the transcription start site. To identify the role of Smad3 in TGF-β inhibition of class II MHC expression in astrocytes, we used astrocytes from Smad3-deficient mice and demonstrate that Smad3 is essential in mediating TGF-β inhibition of CIITA and class II MHC expression.

Materials and Methods

Animals

Pregnant Sprague Dawley rats were purchased from the Charles River Breeding Laboratories (Wilmington, MA). Smad3-deficient mice were generated as described elsewhere (36).

Primary astrocyte cultures

Primary glial cell cultures were established from neonatal rat or mice cerebra as described previously (17). Cells were cultured in DMEM, high glucose formula supplemented with glucose to a final concentration of 6 g/L, 2 mM glutamine, 0.1 mM nonessential amino acid mixture, 0.1% gentamicin, and 10% FBS (HyClone Laboratories, Logan, UT). After 2 wk in primary culture, oligodendrocytes and microglia were removed by mechanical dislodgment. Astrocytes were harvested by trypsinization (0.25% trypsin, 0.02% EDTA) and monitored for purity by immunofluorescence. Astrocyte cultures were routinely >97% positive for glial fibrillary acidic protein, an intracellular Ag unique to astrocytes (40).

Reagents

Recombinant murine IFN-γ was purchased from Genzyme (Boston, MA) and recombinant rat IFN-γ was purchased from Life Technologies (Grand Island, NY). Human recombinant TGF-β2 was purchased from R&D Systems (Minneapolis, MN). PE-conjugated mAb against mouse class II MHC was purchased from Southern Biotechnology Associates (Birmingham, AL).

Plasmids

The construction of the CIITA promoter constructs has been previously described (17). A 1703-bp DNA fragment of the human CIITA type IV promoter was PCR amplified using human genomic DNA as template. The PCR product was ligated into the Smal site of the pGL2-Basic vector which contains the gene for luciferase as reporter. The designated name for this construct is hCIITAp1.7. CIITA promoter deletion constructs hCIITAp-D1, D4, D5, and D6 were prepared as described elsewhere (17) and contain 945, 154, 80, and 54 bp of the CIITA type IV promoter from the transcription start site, respectively (see Fig. 3). The site-directed mutation constructs M1–M8 were generated on the hCIITAp-D5 plasmid backbone using the QuickChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) following the manufacturer’s instructions and were confirmed by sequencing (see Fig. 4). The Smad3 expression construct CS2-Smad3 was a kind gift from J. Massagué (Memorial Sloan-Kettering Cancer Center, New York, NY) (41).

Transient transfection and luciferase assay

Primary rat astrocytes were transfected by electroporation. Ten micromolars of the hCIITA promoter constructs was cotransfected with 1 μg of the pCMV-β-galactosidase construct into 3 × 10^6 astrocytes with a Bio-Rad Gene Pulser (Bio-Rad, Hercules, CA) set at 250 V, 960 μF, as previously described (17). Primary murine astrocytes were transfected using the LipofectAMINE Plus method according to the manufacturer’s directions (42). After transfection, cells were allowed to recover overnight before treatment with TGF-β and/or IFN-γ for various periods of time. Cells were washed with PBS and lysed with 200 μl of lysis buffer containing 25 mM trisphosphate (pH 7.8), 2 mM DTT, 2 mM dianaminoacyclohexane tetracetic acid, 10% glycerol, and 1% Triton X-100. Extracts were assayed in triplicate for luciferase activity in a volume of 130 μl containing 30 μl of cell extract, 20 mM Tricine, 0.1 mM EDTA, 1 mM magnesium carbonate, 2.67 mM MgSO4, 33 mM DTT, 0.27 mM CoA, 0.47 mM luciferin, and 0.53 mM ATP, and light intensity was measured using a luminometer (Promega, Madison, WI). Extracts were also assayed in triplicate for β-galactosidase enzyme activity as previously described (17). The luciferase activity of each sample was normalized to β-galactosidase activity to calculate relative luciferase activity (RLA).

Nuclear extracts and EMSA

Nuclear extracts from astrocytes were prepared as previously described (17). Cells were grown in 100-mm dishes, allowed to adhere overnight, and then were stimulated with TGF-β and/or IFN-γ as indicated. After treatment, cells were washed with cold PBS, harvested by scraping, and pelleted. Cells were resuspended in 1 ml of buffer A (10 mM KCl, 20 mM HEPES, 1 mM MgCl2, 1 mM DTT, 0.4 mM PMSF, 1 mM NaF, 1 mM NaVO4, incubated on ice for 10 min, and pelleted at 1000 × g for 10 min. Pellets were resuspended in 0.5 ml of buffer A plus 0.1% Nonidet P-40, incubated on ice for 10 min, and centrifuged at 3000 × g for 10 min. The nuclear pellet was resuspended in 1 ml of buffer B (10 mM HEPES, 400
mM NaCl, 0.1 mM EDTA, 1 mM MgCl2, 1 mM DTT, 0.4 mM PMSF, 15% glycerol, 1 mM NaF, and 1 mM Na3VO4) and incubated for 30 min at 4°C with constant gentle mixing. Nuclei were then pelleted at 40,000 g for 15 min at 4°C for 30 min in binding buffer, followed by an additional incubation for 12% glycerol, 1 mM KCl, 2.5 mM MgCl2, 1 mM EDTA, 1 mM DTT, 0.4 mM PMSF, 15% glycerol, 1 mM NaF, and 1 mM Na3VO4). Extracts were prepared with 100 mM HEPES, 200 mM KCl, 1 mM MgCl2, 0.1 mM EDTA, 1 mM DTT, 0.4 mM PMSF, 15% glycerol, 1 mM NaF, and 1 mM Na3VO4). We have shown previously that TGF-β due to TGF-β decreases steady-state levels of CIITA mRNA by inhibiting transcription of the CIITA gene, and the inhibition occurs at both the constitutive and IFN-γ-induced expression level.

**TGF-β does not interfere with STAT-1a, USF-1, or IRF-1 binding to the CIITA type IV promoter**

We initially wished to examine the inhibitory influence of TGF-β on IFN-γ-induced CIITA expression. This may be due to interference with IFN-γ signaling pathways. Previous work from our laboratory demonstrated that TGF-β did not affect IFN-γ-induced tyrosine phosphorylation of Janus kinase (JAK) 1, JAK2, and STAT-1a (39). However, it is possible that TGF-β treatment may interfere with downstream events such as the DNA-binding ability of STAT-1a. Our previous work on IFN-γ activation of CIITA gene expression in astrocytes showed that binding of STAT-1a and IRF-1 to the GAS and IRF elements, respectively, in response to IFN-γ stimulation, as well as the constitutive binding of USF-1 to the E box element on the CIITA type IV promoter, are all essential for IFN-γ-induced promoter activity (17). To determine whether TGF-β affects protein binding on these elements in the CIITA type IV promoter, nuclear extracts were prepared from untreated, TGF-β, and/or IFN-γ-stimulated astrocytes, and EMSA was performed with labeled oligonucleotides spanning the proximal GAS and E box elements or the proximal IRF element. Using extracts from unstimulated cells, constitutive DNA-protein complexes formed on the proximal GAS and E box probe, which have been identified as USF-1 binding to the E box (Fig. 2, lane 1).

**RESULTS**

**TGF-β inhibits CIITA gene expression at the transcriptional level**

We have shown previously that TGF-β inhibits IFN-γ-induced CIITA mRNA expression in astrocytes and that inhibition is not due to TGF-β destabilization of CIITA mRNA (38). These data suggest that TGF-β inhibition of IFN-γ-induced CIITA mRNA expression may occur at the transcriptional level. To determine whether TGF-β inhibits CIITA promoter activity, the full-length type IV CIITA promoter construct hCIITAp1.7, which mediates IFN-γ-induced CIITA expression in astrocytes, was used (17). The construct was transfected into astrocytes by electroporation in combination with a β-galactosidase expression vector to monitor transfection efficiency. The cells were incubated in the absence or presence of TGF-β for 24 h, followed by IFN-γ or medium treatment for 10 h, and then RLA was determined. IFN-γ stimulated an ~8.0-fold induction of CIITA promoter activity over the constitutive transcription level, and treatment with TGF-β inhibited ~63% of IFN-γ-induced CIITA promoter activity (Fig. 1). Interestingly, TGF-β also inhibited constitutive CIITA promoter activity to a similar extent (~64%; Fig. 1). These data suggest that TGF-β decreases steady-state levels of CIITA mRNA by inhibiting transcription of the CIITA gene, and the inhibition occurs at both the constitutive and IFN-γ-induced expression level.
constitutive or IFN-γ-induced DNA-protein complex formation (Fig. 2, lanes 2, 4, 6, and 8), demonstrating that TGF-β does not affect the binding of STAT-1α, USF-1, or IRF-1 to elements on the type IV CIITA promoter. These data indicate that TGF-β does not interfere with the signaling pathway that mediates IFN-γ activation of the CIITA gene.

A proximal region within 80 bp of the transcription start site on CIITA type IV promoter is important in mediating TGF-β inhibition

To define the promoter region(s) responsible for TGF-β inhibition of the CIITA gene in astrocytes, the full-length CIITA type IV promoter construct as well as four serial deletion constructs as described in Ref. 17 were transfected into rat astrocytes. Cells were incubated with medium or TGF-β for 24 h, followed by medium or IFN-γ for an additional 10 h. The RLA of each sample was then determined, and the extent of TGF-β inhibition at both the constitutive and IFN-γ induced transcriptional levels were calculated. The percentage of TGF-β inhibition of constitutive and IFN-γ-induced CIITA promoter activity of the deletion constructs was compared with that of the full-length construct, respectively, which was set at 100%. Construct hCIITAp-D1 lacks ∼700 bp at the 5' end of the full-length promoter and was fully susceptible to TGF-β inhibition of either constitutive or IFN-γ-induced CIITA promoter activity (Fig. 3). Deletion of an additional 800 bp in construct hCIITAp-D4 resulted in an ∼23% reduction of TGF-β inhibition of constitutive CIITA promoter activity and ∼26% reduction of TGF-β inhibition of IFN-γ-induced CIITA promoter activity, suggesting that minor TGF-β-responsive element(s) exist in the region from −945 to −154 bp. Deletion from −154 to −80 bp in construct hCIITAp-D5, eliminating the proximal GAS and E box region within 80 bp of the transcription start site on CIITA type IV promoter.
box elements, showed no significant difference in TGF-β inhibition compared with hCIITA-D4. In construct hCIITA-D6, an additional 26 bp from −80 to −54 bp was removed, including the proximal IRF element. This construct is not inducible by IFN-γ due to the lack of the proximal GAS, E box, and proximal IRF elements (17), thus, only constitutive CIITA promoter activity was examined. Deletion from −80 to −54 bp resulted in an ∼46% reduction of TGF-β inhibition of constitutive CIITA promoter activity compared with the D5 construct (Fig. 3). These data show that TGF-β inhibition of constitutive and IFN-γ-induced CIITA type IV promoter activity are similarly affected by the deletion mutations of the CIITA promoter, suggesting that TGF-β inhibition occurs at the level of constitutive CIITA transcription.

To further characterize the proximal region of the CIITA promoter for TGF-β inhibitory effects, we systematically mutated the fragment from −80 to −4 bp using the hCIITA-D5 construct as the backbone. Eight different mutant constructs, designated as M1 through M8, were transfected into rat astrocytes and then assayed for luciferase activity (Fig. 4). Several of these constructs are non-responsive to IFN-γ due to mutations in the IRF element, thus we focused on TGF-β inhibition at the constitutive level in this set of experiments. The percentage of TGF-β inhibition of constitutive promoter activity of each mutant construct was compared with that of hCIITA-D5, which was set at 100%. The M2 construct, with a mutation from −70 to −60 bp, showed an ∼50% reduction in TGF-β inhibition, and the M3 construct, mutated from −60 to −50 bp, reduced TGF-β inhibition by ∼34% (Fig. 4). The other mutations had no effect on TGF-β inhibition. A construct with a mutation from −70 to −50 bp did not further reduce TGF-β inhibition compared with the M2 construct (data not shown), suggesting that the M2 and M3 mutations affect the same element(s) mediating the TGF-β inhibitory effect. These results indicate that the region from −70 to −50 bp is critical for TGF-β inhibition of CIITA promoter activity, consistent with that of the deletion constructs (Fig. 3). It should be taken into consideration that the −945 to −154 bp region, which mediates a minor component of TGF-β inhibition, is lacking from the −80-bp construct and the derived mutants. This may impact on the extent of TGF-β inhibition observed in this system.

TGF-β signaling is mediated by the Smad family of proteins, and a consensus sequence for Smad binding, the Smad binding element (SBE), CAGAC, has been described previously (44). Close examination of the CIITA promoter sequence from −70 to −50 bp identified no putative SBE. It is possible that other transcription factor(s) activated or induced by Smads mediate TGF-β inhibition of CIITA promoter activity. To attempt to identify the protein(s) involved in TGF-β inhibition within this critical region, EMSA was performed with nuclear extracts from medium or TGF-β-treated astrocytes. Nuclear extracts from untreated cells formed a constitutive complex, and TGF-β stimulation did not affect complex formation nor induce any new complexes (data not shown). Computer analysis of the sequence of the probe identified two potential cytokine-responsive elements, the IRF element and a C/EBPβ-like element. Since we have shown that TGF-β treatment does not affect the binding pattern on the IRF element (Fig. 2), we investigated whether the C/EBP element was involved in complex formation. An ICAM-1 C/EBP oligonucleotide and C/EBP consensus sequence oligonucleotide were used as competitors in EMSA, but did not affect complex formation (data not shown). Currently, we have not identified the protein(s) contained in this constitutively expressed complex and do not know whether the

![FIGURE 4](http://www.jimmunol.org/)

The CIITA type IV proximal promoter region −70 to −50 bp is important in mediating TGF-β inhibition. Scanning mutations of the CIITA type IV proximal promoter region are indicated. Mutated sequences are depicted as boxed lower case letters with numbers indicating the 5′ starting position of the mutations. Each of the mutant constructs was cotransfected with the reference plasmid β-galactosidase into rat astrocytes. The transfected cells were treated with medium or TGF-β2 (10 ng/ml) for 24 h, then analyzed for luciferase and β-galactosidase activity. The percentage of TGF-β inhibition was calculated by comparing the RLA of the TGF-β-treated samples with the RLA of the medium-treated sample. Values for each construct are plotted as the percentage of the hCIITA-D5 promoter, in which the extent of TGF-β inhibition was set at 100%. Data shown are the mean ± SEM of two experiments.
complex is involved in mediating TGF-β inhibition of CIITA gene expression in astrocytes.

Smad3 plays an important role in mediating TGF-β inhibition of class II MHC and CIITA gene expression

To decipher another component of TGF-β-mediated inhibition of CIITA and class II MHC expression, we investigated the involvement of Smad proteins in this response because there is evidence that Smad3 is an effector for TGF-β regulation of immune system function (36, 37). To examine the role of Smad3 in mediating TGF-β inhibition of class II MHC and CIITA gene expression, primary astrocytes from Smad3-deficient mice were prepared, and their responsiveness to TGF-β was compared with that of wild-type astrocytes. First, the effect of TGF-β on IFN-γ-induced class II MHC protein expression was analyzed. Cells were treated with medium or TGF-β for 24 h, then incubated with medium or IFN-γ for an additional 48 h, and class II MHC Ag expression on cells was assessed by flow cytometry. Constitutive expression of class II MHC is extremely low on astrocytes from both wild-type and Smad3-deficient mice and is up-regulated in response to IFN-γ stimulation to a similar extent (Fig. 5). TGF-β treatment inhibited IFN-γ-induced class II MHC protein expression by ~60% in wild-type astrocytes (Fig. 5). However, Smad3-deficient astrocytes were not susceptible to the inhibitory effect of TGF-β as evidenced by the lack of inhibition of IFN-γ-induced class II MHC expression (Fig. 5). This result indicates that Smad3 is necessary for mediating TGF-β inhibition of IFN-γ-induced class II MHC protein expression in astrocytes.

Next, the role of Smad3 in TGF-β inhibition of IFN-γ-induced CIITA mRNA expression was examined. Astrocytes from wild-type or Smad3-deficient mice were incubated with medium or TGF-β for 24 h and then stimulated with medium or IFN-γ for an additional 7 h. Total RNA was isolated and CIITA mRNA levels were analyzed by RPA. CIITA mRNA expression was not detected from cells incubated in medium or TGF-β from either Smad3-deficient or wild-type mice (Fig. 6, lanes 1, 2, 5, and 6), whereas IFN-γ treatment induced strong expression of CIITA mRNA in astrocytes from Smad3-deficient and wild-type mice (lanes 3 and 7). TGF-β inhibited ~40% of IFN-γ-induced CIITA mRNA expression in wild-type astrocytes (lane 8); however, it did not affect IFN-γ-induced CIITA mRNA levels in Smad3-deficient astrocytes (compare lanes 3 and 4). These results indicate that TGF-β inhibition of IFN-γ-induced CIITA mRNA expression involves Smad3.

Finally, to confirm the importance of Smad3 in mediating TGF-β inhibition of CIITA gene expression, the Smad3 protein was transfected into Smad3-deficient astrocytes to determine whether it could restore the inhibitory effect of TGF-β on IFN-γ-induced CIITA type IV promoter activity. Wild-type or Smad3-deficient astrocytes were transfected with a mixture of the hCIITAp1.7 plasmid, CMV-β-galactosidase construct, and the CS2-Smad3 expression vector or pCDNA3 empty vector to normalize for amounts of DNA, as indicated in Fig. 7. Cells were incubated in the absence or presence of TGF-β for 24 h, followed by IFN-γ stimulation for an additional 10 h. The RLA of each sample was calculated, and the percentage of TGF-β inhibition of IFN-γ-induced CIITA promoter activity was determined. TGF-β treatment resulted in an ~30% inhibition of IFN-γ-induced CIITA promoter activity in wild-type murine astrocytes. It should be noted that the extent of TGF-β inhibition of CIITA type IV promoter activity in murine astrocytes (~30%) is lower than that in rat astrocytes (~60%; Fig. 1). Overexpression of Smad3 in wild-type astrocytes increased the extent of TGF-β inhibition to ~50%.

FIGURE 5. TGF-β does not inhibit IFN-γ-induced class II MHC Ag expression in Smad3-deficient astrocytes. Wild-type or Smad3-deficient astrocytes were incubated with medium alone for 72 h, or medium or TGF-β (50 ng/ml) for 24 h, followed by IFN-γ (1 U/ml) for an additional 48 h. Surface expression of class II MHC was assessed by flow cytometry. In each histogram, the dotted line represents the medium-treated samples, the gray line is the IFN-γ-treated samples, and the black line is the TGF-β/IFN-γ-treated samples. Data shown are representative of six experiments.

FIGURE 6. TGF-β does not inhibit IFN-γ-induced CIITA mRNA expression in Smad3-deficient astrocytes. A, Smad3-deficient or wild-type astrocytes were incubated with medium or TGF-β (50 ng/ml) for 31 h, or medium or TGF-β (50 ng/ml) for 24 h, followed by IFN-γ (1 U/ml) for an additional 7 h. Fifteen micrograms of total RNA was subjected to RPA for expression of CIITA and GAPDH mRNA. B, Quantification of the above experiment after normalization of CIITA/GAPDH levels for each experimental condition. Data shown are representative of three experiments.
indicates that the Smad3 protein potentiated the inhibitory effect of TGF-β in wild-type astrocytes. TGF-β treatment of Smad3-deficient astrocytes resulted in ~6% inhibition of IFN-γ-induced CIITA promoter activity. Overexpression of Smad3 in Smad3-deficient astrocytes resulted in an ~50% inhibition by TGF-β, comparable to that in wild-type astrocytes. These data demonstrate that Smad3 is responsible for mediating the inhibitory effect of TGF-β on IFN-γ-induced CIITA type IV promoter activity.

**Discussion**

Previous studies have shown that IFN-γ-induced *class II MHC* and CIITA gene expression in astrocytes is inhibited by TGF-β. However, the mechanism(s) underlying this inhibitory effect is poorly understood. In this study, we demonstrate that TGF-β inhibition of CIITA gene expression occurs at the transcriptional level in astrocytes, and both constitutive and IFN-γ-induced human CIITA type IV promoter activity is inhibited by TGF-β. We demonstrate that the inhibitory effect of TGF-β on type IV CIITA promoter is mediated through a proximal promoter region within 80 bp from the TSS. In addition, our results indicate that Smad3 is necessary in mediating TGF-β inhibition of IFN-γ-induced *class II MHC* and CIITA gene expression.

Expression of the CIITA gene is regulated by four distinct promoters; each direct CIITA expression in different cell types (16). We show here that TGF-β inhibits transcription of the CIITA type IV promoter at both the constitutive and IFN-γ-induced transcriptional levels. A 7-kb 5′ flanking sequence of the CIITA gene was isolated by the Ting laboratory (20), which contains predominantly the type III CIITA promoter. This group previously demonstrated that both constitutive and IFN-γ-induced transcription in 2TGH fibrosarcoma cells directed by this 5′ flanking region were inhibited by TGF-β (20), similar to what we have described for the type IV CIITA promoter. Further analysis of the type III and type IV CIITA promoters in 2TGH cells revealed that TGF-β completely inhibited IFN-γ-induced type III promoter activity, whereas type IV promoter activity induced by IFN-γ was inhibited by ~50% (19). Again, TGF-β inhibited the basal activity of both promoters, indicating that TGF-β interferes with CIITA promoter activity in the absence of IFN-γ, a result we have confirmed in this study. These results suggest that a similar mechanism may be utilized by TGF-β to inhibit transcription from the type III or type IV CIITA promoters.

Inhibition of IFN-γ-induced CIITA transcription upon exposure to TGF-β in astrocytes can result from a number of cellular modifications, including alterations in the number and/or affinity of IFN-γ receptors and/or interference with IFN-γ-induced signaling events. Previous work from our laboratory demonstrated that IFN-γ-induced tyrosine phosphorylation of Jak1, Jak2, and STAT-1α is not affected by TGF-β treatment (39). Because the transactivation ability of STAT-1α depends not only on tyrosine phosphorylation, but also on serine phosphorylation of residue 727 (45), TGF-β may inhibit IFN-γ-induced transcriptional activation by affecting the serine phosphorylation of STAT-1α. However, the fact that TGF-β does not inhibit IFN-γ-induced IRF-1 expression in astrocytes suggests that TGF-β does not affect the transactivation ability of STAT-1α. It still remained a possibility that TGF-β may interfere with the binding of STAT-1α, IRF-1, and USF-1 to their respective elements on the type IV CIITA promoter. Our results from EMSA, however, revealed no influence of TGF-β on complex formation over the GAS, E box, and IRF elements of the CIITA promoter. These data demonstrate that TGF-β does not affect the activation of IFN-γ signaling molecules in astrocytes nor their binding ability, suggesting that TGF-β inhibition of IFN-γ-induced CIITA transcription is due to inhibition of constitutive CIITA transcription levels. This is supported by our observation as well as that of Piskurich et al. (19) that the extent of TGF-β inhibition of IFN-γ-induced CIITA promoter activity is comparable to that of constitutive CIITA promoter activity.

In this study, we also demonstrated that inhibition of CIITA gene expression by TGF-β requires a minimal promoter region within 80 bp from TSS, as well as the region from −945 to −154 bp. Given that the inhibitory effect mediated by the −945 to −154 bp region was quite modest (23–26%), we did not perform further analysis on this region. However, within the minimal promoter region, we identified a DNA sequence from −50 to −70 bp that partially mediates the inhibitory effect of TGF-β on CIITA promoter activity. Analysis of this DNA sequence identified an IRF element and a potential C/EBP element within the region. Examination of protein binding to this fragment by EMSA showed that TGF-β did not induce new protein-DNA complex formation and did not affect the binding of a constitutive DNA-protein complex formed on the fragment (data not shown). The complex does not contain IRF-1 or C/EBP proteins as determined by competition and supershift analysis (data not shown). We currently have no evidence to determine whether this constitutive complex is involved in TGF-β inhibition of CIITA expression. A 10-bp TGF-β inhibitory element (TIE) identified in the transin gene promoter has been shown to be required for TGF-β inhibition of growth factor-induced transin expression. This element specifically binds a nuclear protein complex containing Fos (46). The TIE is also conserved in several other TGF-β-inhibited genes such as elastase and collagenase (46). In addition, TGF-β-activated Smads have been shown to recruit transcription corepressors and strongly inhibit transcription from TGF-β-responsive promoters by binding to a specific SBE (47–49). However, careful examination of the
whether this is pertinent to TGF-b 270- to 270 we will investigate in the future whether CBP and/or p300 are TGF-b promoter activity in the Smad3-deficient astrocytes, indicating that proteins are transcriptional coactivators such as CBP/p300. A factors binding on the CIITA promoter. One group of candidate regulation of splenocyte activation and mucosal immunity (36, the inhibitory effect of TGF-b constitutive complex which will enable us to delineate its involvement in mediating the inhibitory effect of TGF-b on CIITA gene expression.

It is also a possibility that TGF-b inhibition of CIITA expression may be mediated by protein(s) that interacts with transcription factors binding on the CIITA promoter. One group of candidate proteins are transcriptional coactivators such as CBP/p300. A number of reports have shown that TGF-b-activated Smads interact with CBP/p300 (for review, see Refs. 32 and 33). This may sequester the limited amount of CBP/p300 within the nucleus and inhibit transcription from genes that require these coactivator proteins. Such a scenario has been shown to occur in TGF-b inhibition of TNF-3-activated E-selectin gene expression in endothelial cells in which TGF-b-activated Smad proteins compete with TNF-3-activated NF-3xB for the coactivator CBP (50). To determine whether this is pertinent to TGF-b inhibition of CIITA expression, we will investigate in the future whether CBP and/or p300 are involved in CIITA gene transcription.

In this study, we also analyzed another aspect of TGF-b inhibition, that being the components of the TGF-b signal transduction pathway that mediate the inhibitory effect of TGF-b on class II MHC and CIITA expression. For this purpose, we used Smad3-deficient mice to specifically determine the role of Smad3 in this response. Our results indicate that Smad3 is essential for TGF-b inhibition of CIITA and class II MHC expression. In Smad3-deficient astrocytes, TGF-b did not inhibit IFN-g-induced class II MHC protein expression or CIITA mRNA expression, compared with astrocytes from wild-type mice. Interestingly, TGF-b did cause a slight inhibition (~6%) of IFN-g-induced CIITA type IV promoter activity in the Smad3-deficient astrocytes, indicating that TGF-b modulation of the IFN-g-induced endogenous CIITA gene and exogenously introduced CIITA promoter may have subtle differences. However, overexpression of the Smad3 protein enhanced the inhibitory effect of TGF-b in wild-type murine astrocytes and restored TGF-b inhibition of IFN-g-induced CIITA promoter activity in Smad3-deficient astrocytes to levels comparable to that of wild-type astrocytes. These data demonstrate that Smad3 is the factor that mediates the inhibitory effect of TGF-b on IFN-g-induced class II MHC and CIITA gene expression and that this function of Smad3 is not redundant with that of Smad2. Given that the CIITA type IV promoter does not contain SBEs, our results further indicate that Smad3 does not directly inhibit CIITA transcription by binding to its promoter. Smad3 is involved in TGF-b-mediated regulation of splenocyte activation and mucosal immunity (36, 37). Smad3 is required for TGF-b inhibition of TCR-stimulated T cell activation, proliferation, and cytokine production (36, 37). Our results add another aspect of immune reactivity stimulated by Smad3, that being CIITA and class II MHC gene expression. Given the important physiological role of TGF-b in suppressing in vivo expression of class II MHC (28, 29), our findings highlight the importance of Smad3 in this response.

In conclusion, the results from this study provide a foundation for further analysis into the mechanism by which TGF-b inhibits both constitutive and IFN-g-induced CIITA gene expression. The 70- to 50-bp region of the type IV CIITA promoter will be subject to a vigorous analysis to determine its involvement in TGF-b inhibition of CIITA expression. As well, the importance of Smad3 in mediating TGF-b inhibition of CIITA and class II MHC expression provides a basis for the development of strategies for down-regulation of class II MHC genes, which has therapeutic potential for the control of autoimmune diseases.

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References


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References


