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Physical and Functional Interaction between GATA-3 and Smad3 Allows TGF-β Regulation of GATA Target Genes

Andries Blokzijl,[1] Peter ten Dijke,[2] and Carlos F. Ibáñez[1,3]
[1] Division of Molecular Neurobiology
Department of Neuroscience
Karolinska Institute
171 77 Stockholm
Sweden
[2] Division of Cellular Biochemistry
The Netherlands Cancer Institute
Amsterdam
The Netherlands

Summary

Background: Members of the GATA family of zinc finger transcription factors are genetically controlled "master" regulators of development in the hematopoietic and nervous systems. Whether GATA factors also serve to integrate epigenetic signals on target promoters is, however, unknown. The transforming growth factor-β (TGF-β) superfamily is a large group of phylogenetically conserved secreted factors controlling cell proliferation, differentiation, migration, and survival in multiple tissues.

Results: GATA-3, a key regulator of T helper cell development, was found to directly interact with Smad3, an intracellular signal transducer of TGF-β. Complex formation required a central region in GATA-3 and the N-terminal domain of Smad3. GATA-3 mediated recruitment of Smad3 to GATA binding sites independently of Smad3 binding to DNA, and the two factors cooperated synergistically to regulate transcription from the *IL-5* promoter in a TGF-β-dependent manner. Treatment of T helper cells with TGF-β promoted the formation of an endogenous Smad3/GATA-3 nuclear complex and stimulated production of the Th2 cytokine IL-10 in a Smad3- and GATA-3-dependent manner.

Conclusions: Although Smad proteins are known to interact with a number of general transcription factors, these are insufficient to explain the tissue-specific biology of TGF-β proteins. Through its interaction with Smad3, GATA-3 is able to integrate a genetic program of cell differentiation with an extracellular signal, providing a molecular framework for the effects of TGF-β on the development and function of specific subsets of immune cells and possibly other cell types.

Introduction

In many cell lineages, cell fate decisions and phenotypic differentiation are determined by intrinsic transcriptional programs coordinated by extracellular signals provided by local organizer centers. The understanding of how genetic and epigenetic mechanisms are integrated at the molecular level remains a major challenge. Signal integration may take place at target promoters through the modular arrangement of DNA binding sites for different transcription factors. These may include signal-activated factors, regulated by extracellular signals, and tissue-restricted factors, the expression of which can determine specific cellular phenotypes. Another possibility for integration of genetic and epigenetic inputs involves the direct recruitment of signal-activated factors to target promoters by tissue-restricted factors.

GATA-3 is a member of the GATA family of zinc finger-containing transcription factors, best known for their important roles in the control of cell fate decisions in different hematopoietic cell lineages [1–3]. Targeted disruption of the GATA-3 gene in mice results in embryonic death on day 12, with failure of fetal liver hematopoiesis and defects in the central and peripheral nervous systems [4, 5]. In mouse chimeras, *GATA-3*$^{-/-}$ ES cells fail to give rise to thymocytes or mature peripheral T cells, indicating that GATA-3 is an essential and specific regulator of early thymocyte development [6]. In addition to its role in T cell development, GATA-3 is an indispensable differentiation factor for a subtype of T helper cells (Th2), characterized by their ability to produce a specific set of cytokines, including IL-4, IL-5, and IL-10, and to promote humoral immunity. GATA-3 DNA binding sites have been found in the IL-4 and IL-5 gene promoters, and GATA-3 is necessary for the expression of all Th2 cytokine genes [7, 8]. Cytokine gene expression can be regulated by a vast number of extracellular signals, including antigen stimulation, membrane-bound cofactors, soluble hormones, and other cytokines and growth factors. It is, however, unknown whether GATA proteins are able to directly integrate signals from extracellular stimuli at cytokine gene promoters.

Members of the TGF-β superfamily are pleiotropic-secreted polypeptides regulating proliferation, cell fate, differentiation, migration, adhesion, and apoptosis in numerous cell types. The TGF-β is one of the largest families of growth factors known and includes TGF-βs, bone-morphogenetic proteins (BMPs), activins, nodals, and growth and differentiation factors (GDFs), altogether comprising more than 30 different proteins. TGF-β family members are expressed in complex spatio-temporal patterns and play important roles in development, homeostasis, and repair in practically all tissues, from flies to humans. In comparison to this wide functional diversity, the signal transduction mechanism of TGF-β proteins appears deceptively simple. Two receptor serine-threonine kinases cooperate to bind ligand, one of the kinases phosphorylates the other, and the latter phosphorylates specific members of a family of signal transducers (i.e., Smad proteins) that move into the nucleus, where they participate in DNA binding complexes [9–11]. Remarkably, only a few Smads, i.e., Smads 2 and 3 for TGF-βs and activins/nodals and Smads 1, 5, and 8 for BMPs and certain GDFs, are in charge of mediating the activities of all TGF-β proteins known. Although some Smads (Smad3 and Smad4, a

[3] Correspondence: carlos.ibanez@neuro.ki.se

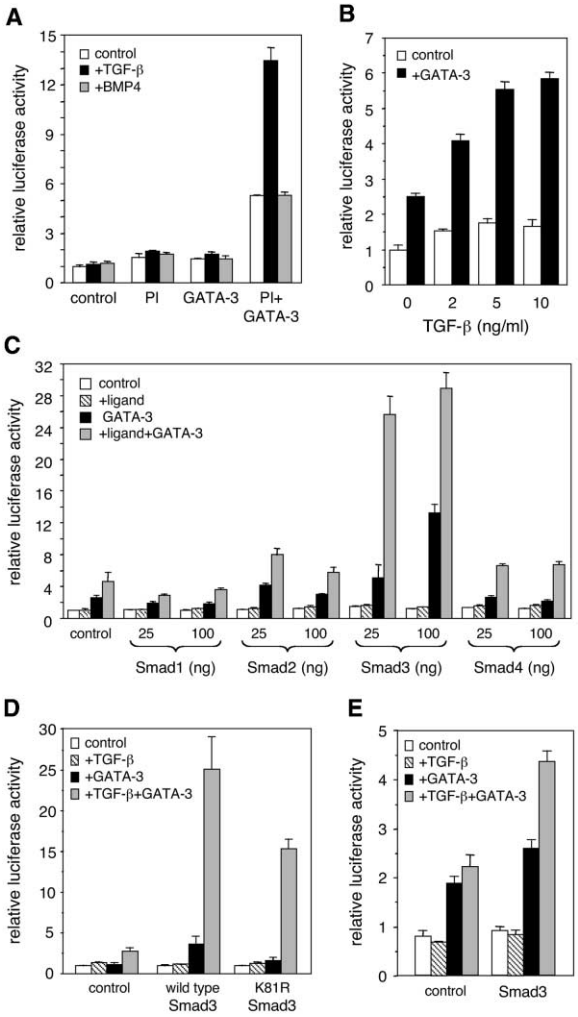

Figure 1. Synergistic Interaction between Smad3 and GATA-3 in the Regulation of the *IL-5* Promoter

(A) Luciferase activity in lysates of HepG2 cells transfected with a 1.2-kb *IL-5* promoter construct in the presence or absence of GATA-3 and PMA/ionomycin (PI) treatment as indicated. Cells were left untreated (open bars) or were treated overnight with 10 ng/ml TGF-β (solid bars) or BMP4 (gray bars). Normalized results are expressed relative to control as the average ± SD of triplicate determinations.

(B) Luciferase activity in lysates of HepG2 cells transfected with the *IL-5* promoter construct in the absence (open bars) or presence (solid bars) of GATA-3 and increasing amounts of TGF-β (overnight treatment). Cells were also treated with PMA/ionomycin. Normalized results are expressed relative to control as the average ± SD of triplicate determinations.

(C) Role of individual Smad proteins in the regulation of *IL-5* promoter activity by TGF-β ligands. Different amounts of Smad expression plasmids, as indicated, were transfected along with the *IL-5* reporter construct in HepG2 cells in the presence or absence of GATA-3 and TGF-β ligands (TGF-β1 for Smads 2, 3, and 4 and BMP-4 for Smad1) at 10 ng/ml. Cells were also treated with PMA/ionomycin. Normalized results are expressed relative to control as the average ± SD of triplicate determinations.

(D) Smad3 binding to DNA is not required for synergistic cooperation with GATA-3. Wild-type or K81R mutant Smad3 were transfected in HepG2 cells along with the *IL-5* reporter construct in the presence or absence of GATA-3 and TGF-β (10 ng/ml). Cells were also treated with PMA/ionomycin. The K81R Smad3 mutant is not able to bind DNA but can still cooperate with GATA-3. Normalized results are expressed relative to control as the average ± SD of triplicate determinations.

(E) Luciferase activity in lysates of HepG2 cells transfected with a synthetic multimerized GATA reporter construct in the presence or absence of Smad3, GATA-3, and TGF-β (10 ng/ml). In this case, the cells were not treated with PMA/ionomycin. Normalized results are expressed relative to control as the average ± SD of triplicate determinations.

common partner of all receptor-regulated Smads) have intrinsic DNA binding activities, the large diversity of genes regulated by TGF-β proteins can not be solely explained bases on the presence of Smad binding elements in the promoters of target genes. Smads have been found to require other sequence-specific factors to bind efficiently to the promoters of certain responsive genes [9–11]. Although a number of general transcription factors, coactivators, and corepressors have been found to associate with Smad proteins, these are expressed in most cell types and are therefore insufficient to explain the manifold tissue-specific target gene selection observed in response to TGF-β family proteins.

In the immune system, TGF-β proteins have complex and cell context-dependent effects on cell proliferation, survival, and differentiation [12, 13]. In particular, these proteins play a crucial instructive role in the specification of distinct profiles of cytokine production by effector cells. The importance of Smad proteins in the regulation of immune function by TGF-β is underscored by the profound deficits in T cell activation, mucosal immunity, and cytokine production observed in mice lacking Smad3 [14, 15]. This Smad protein is particularly abundant in cells of immune origin and is therefore likely to be one of the main mediators of TGF-β responses in these cells. The specific transcription factors that partner with Smad3 to allow regulation of gene expression by TGF-β in immune cells are, however, unknown.

In the work reported here, we have investigated the possibility that GATA-3 integrates TGF-β signaling at target promoters via interaction with Smad proteins. Our results indicate that GATA-3 interacts physically and functionally with Smad3, allowing TGF-β regulation of GATA-3 target genes.

Results

Synergistic Cooperation of GATA-3 and Smad3 in the Activation of the *IL-5* Promoter

Prompted by parallels between the activities of GATA-3 and some of the effects of TGF-β proteins, we decided to investigate the possibility that GATA-3 may cooperate with Smads to mediate TGF-β responses in GATA target genes.

We first investigated whether GATA-3-dependent gene transcription can be regulated by TGF-β signaling. To this purpose, we utilized a DNA fragment containing 1.2 kb from the *IL-5* promoter coupled to a luciferase reporter gene. This construct contains GATA-3 DNA binding sites and can be transactivated by GATA-3 in response to T cell activation or in cells stimulated with PMA and ionomycin (used as a surrogate of antigen receptor stimulation, also see the Experimental Proce-

dures) [16]. Neither TGF-β nor BMP4 were able to stimulate transcription from the *IL-5* promoter in naïve HepG2 cells, an epithelial cell line that is highly responsive to several members of the TGF-β superfamily (Figure 1A). Treatment with PMA and ionomycin or transfection of GATA-3 had no effect on the *IL-5* promoter on their own, nor did they allow responsiveness to TGF-β ligands (Figure 1A). However, GATA-3 was able to stimulate transcription from the *IL-5* promoter in HepG2 cells treated with PMA and ionomycin, in agreement with previous reports (Figure 1A). Under these conditions, TGF-β, but not BMP4, augmented the activity of this promoter (Figure 1A). The effects of TGF-β were dose dependent, with a plateau between 5 and 10 ng/ml (Figure 1B). Together, these data indicated that the regulatory effects of TGF-β on the *IL-5* promoter were dependent on the presence of GATA-3.

We evaluated the role of individual Smad proteins on the activation of the *IL-5* promoter by GATA-3 and TGF-β. In the absence of GATA-3, overexpression of Smad proteins had no effect on the activity of the *IL-5* promoter in HepG2 cells, even after stimulation with TGF-β ligands (Figure 1B). However, transfection of Smad3 increased the activity of the *IL-5* promoter in a dose-dependent manner in cells that also received GATA-3 (Figure 1C, solid bars). Smad2 had only a modest effect, while Smad1 and Smad4 were inactive (Figure 1B). In the presence of GATA-3, small amounts of Smad3 dramatically increased the responsiveness of the *IL-5* promoter to TGF-β (Figure 1C, stippled bars), indicating a synergistic cooperation of these two factors to regulate ligand-dependent gene transcription. Smad3 contains DNA binding activity with low affinity toward 5′-TCTGAGAC-3′, termed the Smad binding element (SBE) [17, 18]. The SBE is absent from the 1.2-kb *IL-5* promoter fragment, suggesting that Smad3 binding to DNA is not required for transactivation of this promoter in the presence of GATA-3. To examine this possibility directly, we made use of a point mutant in the conserved β hairpin of the MAD homology 1 (MH1) domain of Smad3, which is the DNA binding domain of Smads [19]. As predicted from the crystal structure, mutation of Lys-81 into Arg (K81R) completely abolishes binding of Smad3 to the SBE [20]. The K81R Smad3 mutant still retained the ability to potentiate transcription from the *IL-5* promoter in the presence of GATA-3 and TGF-β (Figure 1D), indicating that Smad3 may not need to bind DNA directly in order to cooperate with GATA-3 to regulate gene transcription. The transcriptional activity of a construct containing a concatemerized GATA binding site in front of a minimal promoter was also stimulated by GATA-3 in HepG2 cells (Figure 1E). GATA-3 allowed TGF-β to upregulate the activity of this synthetic promoter in cells that also received additional Smad3 (Figure 1E). Importantly, Smad3 was unable to bind to the GATA oligonucleotide used to generate this promoter (see below). TGF-β had no effect in the absence of GATA-3 (Figure 1E), even in the presence of additional Smad3, suggesting that GATA-3 may have the ability to confer TGF-β responsiveness to a variety of promoters containing GATA binding sites.

We also examined the influence of cellular context on the responsiveness of the *IL-5* promoter to TGF-β

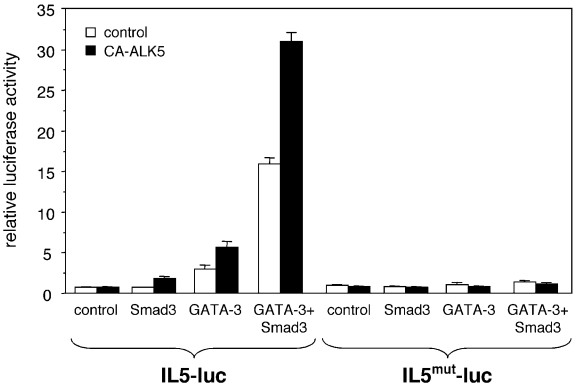

Figure 2. Mutation of the GATA-3 Binding Site in the *IL-5* Promoter Abolishes TGF-β Responsiveness

Luciferase activity in lysates of Jurkat cells transfected with a wild-type (IL5-luc) *IL-5* promoter construct or a mutant construct lacking the GATA-3 binding element (IL5^{mut}-luc). Promoter activity was tested in the presence or absence of exogenous GATA-3 and Smad3 (the latter at 150 ng plasmid DNA). Cells were also treated with PMA/ionomycin. TGF-β signaling was activated in Jurkat cells by the introduction of a constitutively activated TGF-β receptor (CA-ALK5, solid bars). Normalized results are expressed relative to control as the average ± SD of triplicate determinations.

signaling by testing the activity of the *IL-5* promoter construct in Jurkat cells, a lymphoid T cell line expressing moderate levels of endogenous GATA-3. Due to the low levels of TGF-β receptors expressed in these cells, a constitutively active form of the type I TGF-β receptor TβRI (CA-ALK5) was used to activate the TGF-β-signaling pathway. CA-ALK5 was able to increase *IL-5* promoter activity in Jurkat cells that also received moderate levels of Smad3 (Figure 2, left side). Exogenous GATA-3 elevated the activity of the *IL-5* promoter, and this could be further increased by CA-ALK5 (Figure 2, left side). Thus, GATA-3 and Smad3 cooperated synergistically to transactivate this promoter in Jurkat cells and to confer responsiveness to the constitutively activated TGF-β receptor (Figure 2, left side). These results indicated that, in the presence of GATA-3, activation of the TGF-β-signaling pathway can also elevate the activity of the 1.2-kb *IL-5* promoter fragment in cells of lymphoid origin. Finally, to further test the role of GATA-3 in conferring responsiveness to TGF-β signaling to the *IL-5* promoter, we examined the activity of a mutated *IL-5* promoter construct in which the GATA binding element had been replaced by an irrelevant sequence. As predicted by our previous results, this promoter construct was no longer responsive to activation of the TGF-β-signaling pathway (Figure 2, right side).

TGF-β-Dependent Formation of Transcription Factor Complexes on GATA-Specific DNA Binding Sites

The results presented above suggested that GATA-3 may be able to recruit Smad3 to DNA binding sites specific for GATA proteins, thereby conferring TGF-β responsiveness to target promoters. We therefore investigated protein/DNA interactions using an oligonucleotide probe containing a consensus binding site for GATA

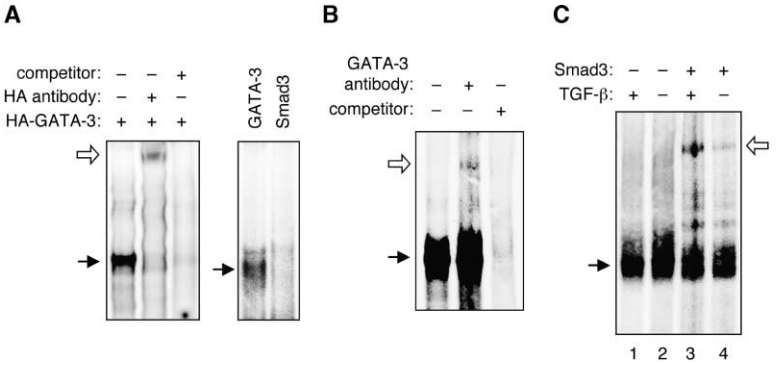

Figure 3. Interaction of the Smad3/GATA-3 Complex with GATA DNA Binding Sites and Regulation by TGF-β

(A) Specificity of the GATA oligonucleotide probe. Nuclear extracts of COS cells transfected with HA-tagged GATA-3 or myc-Smad3 constructs as indicated were used in electrophoretic mobility shift assays with a DNA probe based on a consensus binding site for GATA family factors. Excess unlabeled oligonucleotide was used as specificity control (competitor). Supershift by an anti-HA antibody demonstrated the presence of GATA-3 in the complex (open arrow). Smad3 was unable to shift the GATA oligonucleotide. Specific protein/DNA complexes are indicated by arrows.

(B) Binding of endogenous GATA-3 from HT-2 cells to GATA DNA binding sites. Nuclear extracts of HT-2 cells were used in electrophoretic mobility shift assays with a GATA DNA probe. No PMA/ionomycin treatment was done for this experiment. A 100-fold excess of unlabeled oligonucleotide was used as specificity control (competitor). Supershift by an anti-GATA-3 antibody demonstrated the presence of GATA-3 in the complex (open arrow).

(C) Electrophoretic mobility shift assays with nuclear extracts from HT-2 cells expressing endogenous GATA-3. Extracts from control cells or cells transfected with a Smad3 expression construct were used as indicated. Prior to the preparation of the extracts, some cells were treated with 10 ng/ml TGF-β for 50 min as indicated. No PMA/ionomycin treatment was done for this experiment. GATA-3/DNA complexes are indicated with a solid arrow. The open arrow indicates supershifted complexes.

factors in electrophoretic mobility shift assays. Hemagglutinin (HA)-tagged GATA-3 produced in nuclear extracts of transiently transfected COS cells was able to specifically bind to the radiolabeled GATA oligonucleotide probe (Figure 3A). This complex could be displaced with cold competitor oligonucleotide and supershifted with anti-HA antibodies (Figure 3A), demonstrating its specificity. Smad3 did not bind to the GATA oligonucleotide probe (Figure 3A). We then examined nuclear extracts from the TGF-β-responsive T helper cell line HT-2. An endogenous GATA DNA binding activity similar to that observed in transfected COS cells was detected in these cells (Figure 3B). This could be displaced by cold competitor oligonucleotide and supershifted with anti-GATA-3 antibodies (Figure 3B). Overexpression of Smad3 in HT-2 cells resulted in the appearance of small amounts of a higher-molecular weight complex (open arrow in Figure 3C, lane 4). Formation of this complex was greatly stimulated following a short exposure (15 min) to TGF-β (Figure 3C, lane 3), indicating that TGF-β can induce the formation of high-order protein/DNA complexes on GATA-specific DNA sequences in immune cells. The fact that this complex could only be observed in cells after transfection of Smad3 suggests that it may contain this Smad protein in complex with GATA-3 bound to the DNA oligonucleotide probe. The lack of effect of the TGF-β treatment in the absence of overexpressed Smad3 may be due to insufficient endogenous levels of this Smad protein for detection in the shift assay.

The Interaction between GATA-3 and Smad3 Is Direct

The results described so far could be best rationalized by a direct interaction between Smad3 and GATA-3. We first investigated this in vitro using a GST-Smad3 fusion protein produced in bacteria and ^{35}S-labeled GATA-3 produced by in vitro translation. A GST fusion of full-length Smad3, but not GST alone, was able to pull down ^{35}S-labeled GATA-3 (Figure 4A). GST-Smad3, but not GST alone, was also able to pull down endogenous GATA-3 from lysates of Jurkat cells (Figure 4B). The interaction between Smad3 and GATA-3 was then studied in vivo in mammalian cells. A myc-tagged Smad3 and a HA-tagged GATA-3 were transiently transfected into COS cells. Overexpression of Smad3 in COS cells results in ligand-independent accumulation of this protein in the nucleus (unpublished data). Immunoprecipitation with anti-HA antibodies allowed the recovery of myc-tagged Smad3 only from nuclear extracts of cells that received the HA-GATA-3 construct (Figure 4C), demonstrating the formation of a complex between Smad3 and GATA-3 in vivo. In agreement with the specificity observed in the transactivation of the *IL-5* promoter in HepG2 cells, GATA-3 associated preferentially with Smad3 over other Smad proteins after overexpression in COS cells (Figure 4C). Only very low levels of Smad1 could be recovered following GATA-3 immunoprecipitation, while Smad2 and Smad4 were undetectable in GATA-3 immunoprecipitates (Figure 4C). Under the same conditions, we could neither recover Smad4 in GATA-3 immunoprecipitates prepared from cells that also overexpressed Smad3 (Figure 4C, right panel), suggesting that Smad4 does not form part of the Smad3/GATA-3 complex. In agreement with this observation, overexpression of Smad4 did not affect the ability of CA-ALK5 and Smad3 to transactivate the *IL-5* promoter in Jurkat cells (Figure 4D), although it had dramatic effects on the activity of a synthetic promoter carrying multimerized SBE sites (Figure 4D).

To identify which of the domains of Smad3 mediated its interaction with GATA-3, we tested the ability of different GST fusion constructs to pull down in vitro-translated GATA-3. A GST fusion of the C-terminal MH2 domain of Smad3, with or without the linker region, was unable to interact with GATA-3 (Figure 4A), suggesting that the GATA-3 binding site in Smad3 may be in the N-terminal MH1 domain. Indeed, a GST fusion of the MH1 domain of Smad3 was able to pull down ^{35}S-labeled

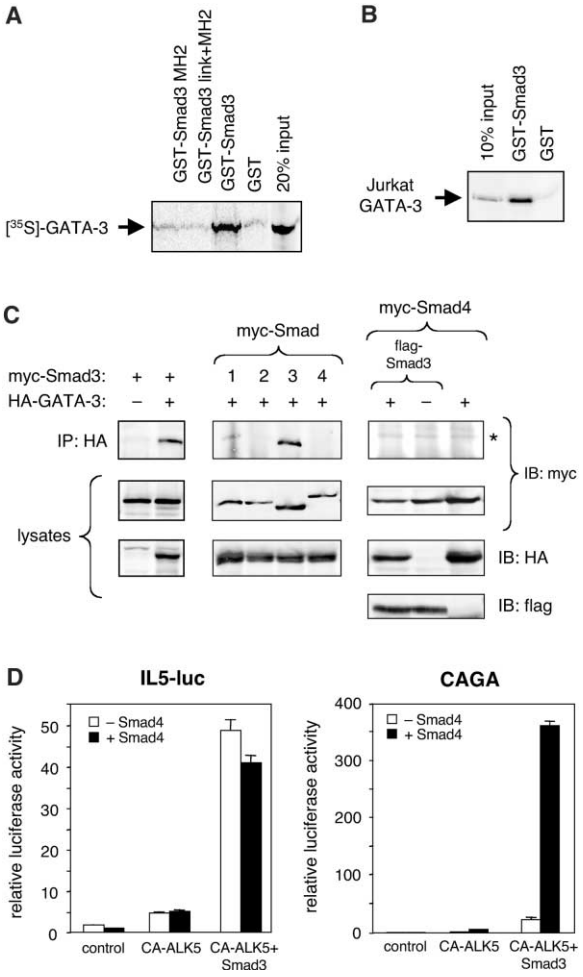

Figure 4. Physical Interaction of Smad3 with GATA-3 In Vitro and In Vivo

(A) Full-length ^{35}S-labeled GATA-3 produced by in vitro translation was used in coprecipitation assays together with equal amounts of the indicated GST-Smad fusion proteins. As control, 20% of the ^{35}S-labeled GATA-3 used for the precipitations was run in each gel (20% input).

(B) Coprecipitation assay of endogenous GATA-3 from lysates of Jurkat cells and GST-Smad fusion proteins. This experiment shows that GST-Smad3 exogenously added to a lysate of Jurkat cells can associate with endogenously expressed GATA-3. The cells had been pretreated with PMA/ionomycin, but similar results were obtained with extracts from untreated cells. Ten percent of the cell lysate was run as control (10% input).

(C) Coimmunoprecipitation of Smad3 and GATA-3 in nuclear lysates of transiently transfected COS fibroblasts. The anti-HA antibody directed against HA-tagged GATA-3 brings down myc-epitope-tagged Smad3 only in cells that received the HA-tagged GATA-3 construct (upper left panel). Using a similar coimmunoprecipitation assay, but with different Smad proteins, the upper middle panel shows that GATA-3 associates preferentially with Smad3 over other Smads after overexpression in COS cells. The upper right panel shows that Smad4 does not associate with the Smad3/GATA-3 complex in cells transfected with all three components. A weak Smad4 band of equal intensity can be seen in all three lanes (asterisk). The panels below show Western blots of 20% of the lysates used as input and reflects the different amounts of Smad and GATA-3 proteins expressed in the lysates. No PMA/ionomycin treatment was done for these experiments.

(D) Activity of the *IL-5* (left) and CAGA (right) promoters in Jurkat cells in the presence (solid bars) or absence (empty bars) of Smad4. Jurkat cells received exogenous GATA-3 as well as CA-ALK5 and/or Smad3 as indicated. Cells transfected with the *IL-5*, but not the CAGA, reporter construct were also treated with PMA/ionomycin. Normalized results are expressed relative to control as the average ± SD of triplicate determinations.

GATA-3 as efficiently as the full-length protein (Figure 5B). In order to identify the region in GATA-3 involved in Smad3 binding, we tested several deletion constructs produced as ^{35}S-labeled in vitro-translated products (Figure 5A). A GATA-3 construct spanning the first N-terminal 146 residues did not interact with GST-Smad3 (Figure 5B). However, extension of this construct to residue 215 allowed binding to a GST fusion protein containing the MH1 domain of Smad3 (Figure 5B). This construct did not interact with GST alone or a GST fusion of the MH2 domain of Smad3 (Figure 5B). A construct of the C-terminal region of GATA-3, encompassing the two zinc finger domains (Zn1 and Zn2 in Figure 5A) was also pulled down by a GST-Smad3 fusion protein (Figure 5B). Analysis of different GATA-3 deletion constructs in nuclear lysates of transfected COS cells confirmed the specific interaction of the first 215 N-terminal residues of GATA-3 with Smad3 (Figure 5C). The shorter construct (1–146) did not bind to Smad3 in COS cells (data not shown). In contrast to the in vitro analysis, however, the zinc finger region of GATA-3 did not bind to Smad3 in transfected cells (Figure 5C). This discrepancy could be due to interference or masking of the GATA-3 zinc finger region by nuclear DNA or other GATA-interacting proteins or simply that, in vivo, this region is not a bona fide Smad3 binding site. Together, these data indicate the presence of a domain in the central portion of the GATA-3 molecule, between residues 146 and 215, that interacts with the Smad3 MH1 domain.

TGF-β Stimulates the Formation of an Endogenous Smad3/GATA-3 Complex in the Nucleus of HT-2 Cells

We investigated Smad3/GATA-3 interactions in a physiological context, taking advantage of the endogenous expression of these two components in the T helper cell line HT-2. In untreated cells, immunoprecipitation with anti-GATA-3 or control antibodies did not bring down Smad3 in nuclear lysates of HT-2 cells (Figure 6). However, after treatment with TGF-β for 50 min, endogenous Smad3 could be detected in GATA-3 immunoprecipitates of HT-2 nuclear lysates (Figure 6). Control antibodies failed to immunoprecipitate Smad3 in any condition (Figure 6). These results demonstrate a specific and ligand-dependent interaction between endogenous GATA-3 and Smad3 in T helper cells.

TGF-β Regulation of an Endogenous GATA-3 Target Gene in HT-2 Cells Depends upon the Activities of Smad3 and GATA-3

In the last set of experiments, we examined whether TGF-β was able to regulate expression of endogenous GATA-3 target genes in HT-2 cells. Production of several cytokines by Th2 helper cells, including IL-4, IL-5, and IL-10, is controlled by GATA-3 [7]. HT-2 cells were found to secrete IL-10 to the culture medium (Figure 7A). IL-4 and IL-5 could not be detected in HT-2 cell supernatants

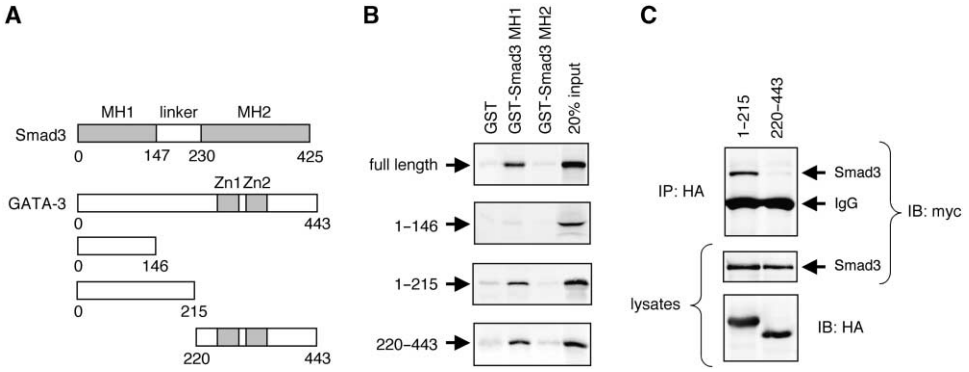

Figure 5. Identification of Protein Domains in Smad3 and GATA-3 Mediating Their Interaction

(A) Domain organization of Smad3 and GATA-3. Deletion constructs of GATA-3 used in in vitro coprecipitation assays are indicated. MH1 and MH2 denote Mad homology domains 1 and 2, respectively. Zn1 and Zn2 denote the two zinc fingers in GATA-3.

(B) The indicated GATA-3 deletions were ^{35}S-labeled by in vitro translation and used in coprecipitation assays together with equal amounts of the indicated GST-Smad fusion proteins. As control, 20% of each ^{35}S-labeled GATA-3 protein construct used for the precipitations was run in each gel (20% input).

(C) Coimmunoprecipitation of Smad3 and GATA-3 deletion constructs in nuclear lysates of transiently transfected COS fibroblasts. See the legend to Figure 4C for details. No PMA/ionomycin treatment was done for these experiments.

(data not shown). IL-10 production could be further stimulated by treatment of HT-2 cells with PMA and ionomycin (Figure 7A). In the presence of PMA and ionomycin, TGF-β was able to stimulate IL-10 production in supernatants of HT-2 cells maintained in serum-free medium for 16 hr (Figure 7A). This effect was observed at very low doses of TGF-β (i.e., 0.5–50 pg/ml) with a peak at 1 pg/ml. At higher doses (i.e., ≥ 500 pg/ml), TGF-β had no significant effect on IL-10 production by HT-2 cells (Figure 7A).

In order to determine whether the effect of TGF-β on IL-10 production by HT-2 cells was dependent on activation of Smad3, we overexpressed a dominant-negative form of Smad3 (D407E) [21] or the inhibitory

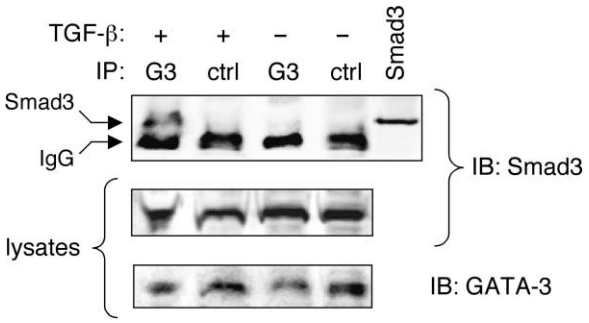

Figure 6. TGF-β Induces the Formation of an Endogenous Smad3/GATA-3 Complex in the Nucleus of the T Helper Cell Line HT-2

Nuclear extracts of HT-2 cells treated with TGF-β (50 min at 10 ng/ml), as indicated, were immunoprecipitated with anti-GATA-3 (G3) or control (ctrl) antibodies and probed with anti-Smad3 antibodies. Smad3 and IgG bands are indicated. In the experiment shown, the cells had also been treated with PMA/ionomycin. Note that Smad3 coimmunoprecipitates with GATA-3 only in cells treated with TGF-β. Smad3 produced in transfected COS cells was run in a parallel lane as a size marker. Reprobing of aliquots of the corresponding lysates (middle and bottom panels) indicates comparable amounts of GATA-3 and Smad3. Similar results were obtained in cells that had not been treated with PMA/ionomycin.

Smad7 protein [22] in HT-2 cells using recombinant adenoviruses. Treatment with TGF-β stimulated IL-10 production in uninfected cells and in cells infected with a control lacZ virus, but failed to increase IL-10 levels in the supernatant of cells infected with viruses expressing dominant-negative Smad3 or Smad7 (Figure 7B). These data suggest that regulation of IL-10 production by TGF-β in HT-2 cells is dependent on Smad3.

We then investigated whether GATA-3 activity was required for the regulatory effects of TGF-β on IL-10 production in HT-2 cells. To this purpose, we generated stable subclones of HT-2 cells overexpressing a dominant-negative GATA-3 isoform (KRR-GATA-3) [23]. This GATA-3 molecule carries a triple alanine substitution in the basic KRR triplet at positions 305–307 that interferes with GATA-3 acetylation, but not with DNA binding [24], and abolishes the ability of GATA-3 to transactivate target promoters in vitro [23] and in vivo [25]. The KRR mutation, however, did not interfere with the ability of GATA-3 to interact with Smad3 as assessed in an in vitro pull-down assay using GST-Smad3 and in vitro-translated KRR-GATA-3 (Figure 7C). We generated two independent clones of HT-2 cells expressing KRR-GATA-3 (KRR #19 and KRR #20) as well as a clone expressing wild-type GATA-3 (GATA3 #37). Expression was confirmed by immunoblotting with antibodies against an HA tag introduced in all the constructs and was comparable among the three lines (data not shown). Subclones and parental HT-2 cells had comparable growth rates and morphology. In the presence of PMA and ionomycin, parental HT-2 cells produced about 6 ng/ml of IL-10 in the conditioned medium, while the subclones GATA3 #37 and KRR #19 and #20 made 3 ng/ml and 1 ng/ml, respectively. Treatment with TGF-β stimulated IL-10 production in parental HT-2 cells and in cells overexpressing wild-type GATA-3 (Figure 7D). Cells overexpressing GATA-3 showed a TGF-β response comparable to that of parental cells, indicating that GATA-3 is not limiting in HT-2 cells for IL-10 regulation by TGF-β. In contrast, TGF-β had no effect on IL-10

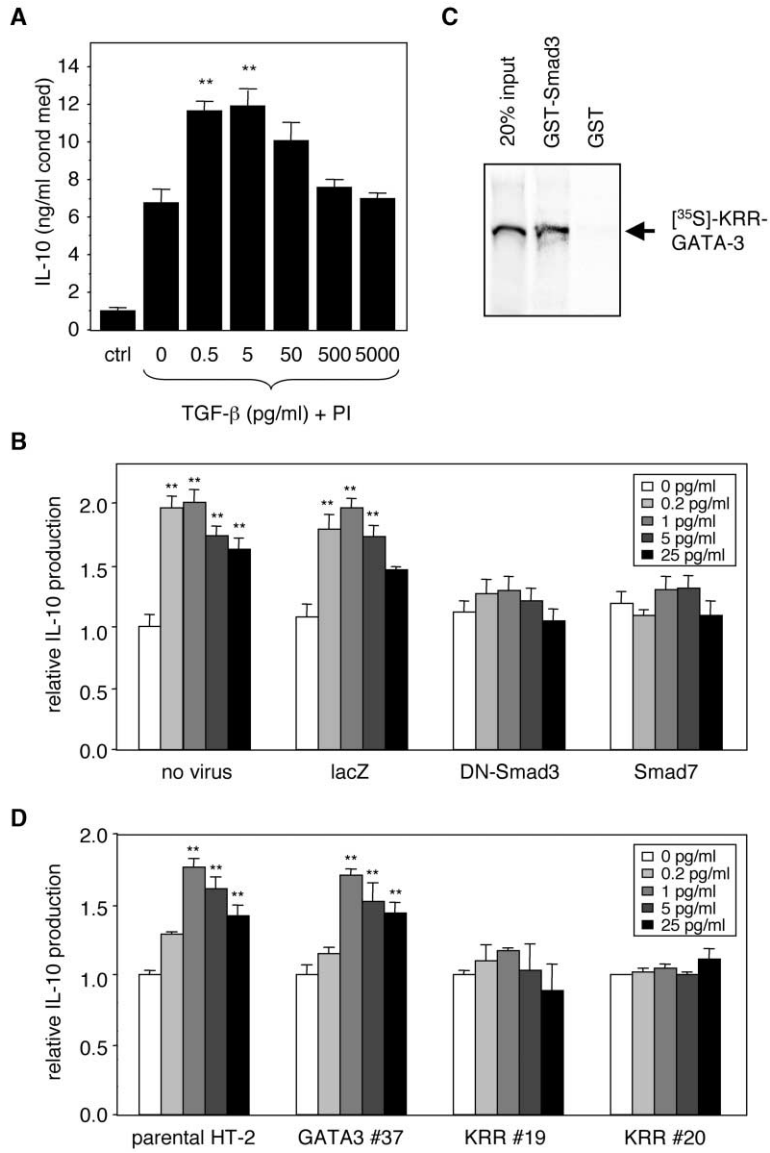

Figure 7. TGF-β Regulation of IL-10 Production in HT-2 Cells Is GATA-3 Dependent

(A) IL-10 production in HT-2 supernatants. HT-2 cells were incubated for 16 hr in serum-free medium in the presence or absence of PMA/ionomycin (PI) and TGF-β as indicated. A double asterisk indicates that $p < 0.01$.

(B) IL-10 production in supernatants of uninfected HT-2 cells (no virus) or cells infected with adenoviruses expressing β-gal (lacZ), dominant-negative Smad3 (DN-Smad3), or Smad7. Following a 2-day infection, cells were incubated for 16 hr in serum-free medium in the presence of PMA/ionomycin and TGF-β at the concentrations indicated. Results are denoted relative to IL-10 levels in uninfected HT-2 cells. A double asterisk indicates that $p < 0.01$.

(C) Interaction of KRR-GATA-3 with Smad3. ^{35}S-labeled KRR-GATA-3 produced by in vitro translation was used in coprecipitation assays together with GST-Smad3 or GST control fusion proteins. As control, 20% of the ^{35}S-labeled KRR-GATA-3 used for the precipitations was included (20% input).

(D) IL-10 production in supernatants of parental HT-2 cells and subclones overexpressing wild-type GATA-3 (GATA3 #37) or a dominant-negative GATA-3 (KRR #19 and KRR #20). Cells were incubated for 16 hr in serum-free medium in the presence of PMA/ionomycin and TGF-β at the concentrations indicated. Results are indicated relative to IL-10 levels in the absence of TGF-β produced by each cell line. A double asterisk indicates that $p < 0.01$.

production in the two subclones overexpressing the dominant-negative KRR-GATA-3 (Figure 7D). These results indicate that endogenous GATA-3 function is necessary for TGF-β to regulate the production of IL-10 in HT-2 cells.

Discussion

Despite recent significant advances in the identification of general transcription factors that collaborate with Smad proteins to regulate TGF-β-dependent gene transcription, a complete understanding of the mechanisms by which TGF-β proteins contribute to the establishment of distinct cell phenotypes will not be attained until the tissue-restricted factors that cooperate with Smads to control cell type-specific gene expression are identified. In the immune and nervous systems, in which TGF-β proteins play key roles in patterning and cell differentiation, the specific factors responsible for these effects are still uncharacterized. The many factors currently known to interact with Smads are simply not sufficient to explain the tissue-specific biology of TGF-β proteins. In the present work, we show that GATA-3, a master regulator of cell fate and differentiation in the immune and nervous systems, is a tissue-restricted partner of Smad proteins. We found that TGF-β induces a specific interaction between Smad3 and GATA-3 in T cells, thereby recruiting Smad3 to GATA DNA binding sites and allowing TGF-β regulation of GATA-3 target promoters.

Ligand binding to TGF-β receptors causes activation and nuclear translocation of Smad proteins. Receptor-specific Smads, such as Smad3, form heteromeric complexes with Smad4 to activate or repress genes in collaboration with cofactors. We found that Smad4 did not form part of the Smad3/GATA-3 nuclear complexes, nor did it affect the functional interaction of Smad3 and GATA-3 on the *IL-5* promoter, suggesting that Smad4

does not play a direct role in the regulation of GATA-3 target genes by TGF-β. Smad3 can bind DNA with low affinity, and recent studies have found a variable requirement of DNA binding for transactivation by this protein. Collaboration of Smad3 with c-Jun and transactivation of gene expression from AP-1 sites in the collagenase I gene promoter appears to require binding of Smad3 to DNA, although not in a DNA sequence-specific manner [26]. On the other hand, Smad3 can be recruited by the general transcription factor Sp1 to activate the $p21^{Waf1/Cip1}$ gene promoter independently of Smad3 binding to DNA [27]. Similar to this latter case, we also found that GATA-3 can recruit Smad3 to a GATA-specific DNA binding site, and that a Smad3 mutant deficient in DNA binding can still cooperate with GATA-3 to transactivate the *IL-5* promoter in a TGF-β-dependent manner.

Both MH1 and MH2 domains in Smad proteins have been implicated in protein-protein interactions with different cofactors [11], while, in Smad3, only the MH1 domain has the ability to interact with DNA [17, 18]. We found that Smad3 interacts with GATA-3 through its MH1 domain. This domain has been shown to mediate association of Smad3 with a number of general transcription factors, including c-Jun [28], ATF-2 [29], TFE3 [30], and Sp1 [27]. Cofactor interaction with MH1 versus MH2 domains does not appear to correlate with the requirement of Smad3 to bind DNA, as several examples have been found of protein-protein interactions involving the Smad3 MH1 domain that require [26, 30] or do not require [27] binding of this Smad to DNA. In the case of GATA-3, the Smad interaction domain appears to reside in the central portion of the molecule upstream of the two C-terminal zinc finger domains, which are the ones believed to mediate GATA-3 binding to DNA.

TGF-β family proteins have multiple and often perplexing effects on immune cell function, displaying both inhibitory and stimulatory activities. Many of the inhibitory effects of TGF-βs are due to their powerful antiproliferative activity, mediated in part through the induction of cell cycle inhibitors [27, 31–33]. TGF-β has also been shown to downregulate immune cell function independently of its antiproliferative effects through the regulation of specific genes required for lymphocyte differentiation and function [12, 13]. On the other hand, stimulatory activities of TGF-β in immune cells include the induction of extracellular matrix proteins, regulation of cytokine production, and antiapoptotic effects [12, 13]. In addition, TGF-β proteins are known to elicit different effects at different concentrations. Thus, for example, TGF-β has been shown to stimulate IL-2 production at very low concentrations (0.1–1 pg/ml) and to conversely inhibit IL-2 production at higher concentrations (1–10 ng/ml) in the same cell type [34], which is reminiscent of our own results on IL-10 production by HT-2 cells. TGF-β also has stimulatory [35–37] and inhibitory [38–40] effects on the differentiation and function of Th2 helper cells depending on the cellular context and the presence or absence of costimulatory signals. A big obstacle for the understanding of the paradoxical effects of TGF-β in immune cell differentiation and function has been the lack of specific molecular targets of the TGF-β-signaling pathway in immune cells.

Our finding of a physical and functional interaction between Smad3 and GATA-3 may allow us to rationalize some of the activities of TGF-β proteins in immune cells. As shown here and in other studies [41, 42], TGF-β can acutely upregulate cytokine production by effector cells, an effect likely to be mediated through the cooperation of Smad proteins with key transcription factors such as GATA-3. On the other hand, feedback regulatory loops activated by TGF-β signaling result in longer-term inhibitory effects on immune cell function. It has been shown, for example, that a 5-day treatment with TGF-β inhibits Th2 helper cell differentiation and cytokine production, interestingly enough, by preventing upregulation of GATA-3 expression during activation of naïve T helper cell precursors [39, 40]. This could represent an inhibitory feedback mechanism to limit the cooperation between GATA-3 and TGF-β signaling. Cellular context is also an important determinant of the activity of Smad proteins, as these are capable of interacting with both coactivators, such as p300/CBP [43–45], and corepressors, such as TGIF, c-Ski, and SnoN [46–48]. For example, a recent study showed that the interaction between Smad3 and the osteoblast transcription factor CBFA1 resulted in repression of the OSE2 promoter in mesenchymal cells, but in activation of the same promoter in epithelial cells [49]. In this regard, it should be noted that one study found that TGF-β inhibited IL-5 production by polyclonally activated human T cells [50]. Although we observed a synergistic cooperation between Smad3 and GATA-3 in the activation of the *IL-5* promoter in both epithelial and lymphoid cells, the construct that we used contained only 1.2 kb of upstream sequence, so we cannot rule out that Smad3 exerts a different effect on the endogenous, full-length *IL-5* promoter and that this may also be cell context dependent. Thus, although the Smad3/GATA-3 interaction can confer TGF-β responsiveness to specific target promoters, the functional outcome of this interaction, i.e., activation or repression, will likely depend on the presence or absence of additional cofactors, themselves subject to cell type-specific and temporal regulation.

In the T helper cell line HT-2, we found that TGF-β regulates IL-10 production in a Smad3- and GATA-3-dependent manner. Both TGF-β and IL-10 are often associated with downregulation of Th1-like immune responses and a shift toward Th2-like responses [35, 37]. TGF-β has been reported to increase IL-10 production in macrophages [41], and both cytokines contribute to the suppression of macrophage functions [35]. TGF-β and IL-10 have also been implicated in the induction of immune privilege in the anterior chamber of the eye as a result of downregulation of Th1 immune responses [42]. Thus, TGF-β contributes to the production of a cytokine, IL-10, that has a similar functional profile in immune cells, namely, suppression of monocyte and Th1 immune responses.

Our results also have implications for other systems in which GATA-3 and TGF-β signaling converge to control cell-specific differentiation events. For example, GATA-3 has been shown to be required for the specification of the neurotransmitter phenotype of sympathetic neurons [5]. Loss of GATA-3 leads to deficits in tyrosine hydroxylase (TH) and dopamine β-hydroxylase (DBH) expression, which results in reduced noradrenaline in

the sympathetic nervous system and embryonic lethality [5]. TGF-β proteins, in particular BMP-2, BMP-4, and BMP-7, direct noradrenergic differentiation of sympathetic neurons by inducing TH and DBH expression in collaboration with other transcription factors [51, 52]. Based on our present results, we hypothesize that GATA-3 may collaborate with Smad proteins to stimulate noradrenergic differentiation in sympathetic neuron precursors. In another recent example, adipocyte differentiation was shown to be regulated by GATA-3 through molecular control of the preadipocyte-adipocyte transition [53]. GATA-3 overexpression suppressed adipocyte differentiation, while GATA-3 loss resulted in an enhanced capacity to differentiate into adipocytes [53]. Interestingly, TGF-β is a well-known negative regulator of adipocyte differentiation [54, 55]. As with the GATA-3 experiments, interference with TGF-β receptor signaling enhanced adipocyte differentiation, while overexpression of Smad3 inhibited differentiation [55]. Thus, collaboration of these two pathways through the direct interaction of GATA-3 with Smad3 may also play a role, in this case inhibitory, in the regulation of adipocyte differentiation.

Conclusions

We present evidence that GATA-3, a tissue-restricted factor and "master" regulator of T helper cell development, can also integrate information from extracellular TGF-β signals by physically and functionally interacting with the signal-activated factor Smad3. The interaction between GATA-3 and Smad3 may allow TGF-β signaling to access the promoters of specific sets of genes involved in cell fate and differentiation in the immune and nervous systems. These results provide a molecular framework for the effects of TGF-β on the development and function of specific subsets of immune cells and possibly other cell types.

Experimental Procedures

Cell Lines and Plasmid Constructs

COS cells were grown in DMEM, HepG2 cells were grown in MEM, and Jurkat and HT-2 cells were grown in RPMI, all supplemented with 10% fetal bovine serum and antibiotics. HT-2 cells also received fresh IL-2 and β-mercaptoethanol every 2 days. The *IL-5* reporter construct contains 1.2 kb of upstream sequence of the *IL-5* gene (GenBank accession number D14461.1) followed by a luciferase reporter [16, 56] and was kindly provided by Naoko Arai. Mutagenesis of the GATA-3 binding site in this promoter construct was performed by replacing the 5′-TATCTGA-3′ heptamer in the conserved lymphokine element 2 (CLE2) motif [16, 56] with 5′-GCGACTC-3′ using the QuickChange Mutagenesis System from Stratagene. A synthetic GATA reporter construct was generated by introducing three tandem copies in the forward direction of an oligonucleotide containing two consensus binding sites for GATA factors (5′-CACT TGATAACAGAAAGTGATAACTCT-3′) upstream of the adenovirus major late promoter in the pGL3 plasmid from Promega. The CAGA reporter contains nine tandem copies of the SBE from the plasminogen activator inhibitor-1 (PAI-1) promoter and was described previously [17]. All Smad expression plasmids used the pCDNA3 backbone and have been described elsewhere [20]. GATA-3 expression plasmids were based in the pCDNA3 backbone. The mutant KRR-GATA-3 was kindly provided by A. Winoto. The constitutively activated TGF-β receptor (CA-ALK5) carries a mutation in the intracellular juxtamembrane domain (T204D) and has been described previously [57, 58].

Cell Transfection, Adenovirus Infection, Reporter Assays, and IL-10 ELISA

COS cells were transfected by the calcium phosphate method. HepG2 and Jurkat cells were transfected in complete medium with FuGene6 according to the manufacturer's instructions (Roche). After a 24-hr incubation, cell monolayers were washed with serum-free medium and incubated for an additional 16 hr in 0.1% serum-containing medium supplemented with TGF-β1 (R&D, 10 ng/ml, unless otherwise indicated) and PMA/ionomycin (50 ng/ml and 750 ng/ml, respectively) as indicated. Reporter assays were analyzed using the Dual-Luciferase Reporter Assay System kit from Promega. Firefly luciferase activity was normalized to the Renilla luciferase activity, and fold induction was calculated relative to the luciferase activity in control conditions. Luciferase expression was quantified on a 1450 Microbeta Jet luminescence counter (Wallac). All treatments and transfection conditions were analyzed in triplicate.

The treatment with PMA/ionomycin activates cAMP and Ca^{2+} pathways and mimics antigen stimulation of immune cells. Two assays utilized in the present study, namely, induction of the *IL-5*-Luc reporter construct and production of IL-10, require this treatment. Reporter assays based on multimerized GATA or CAGA sites, as well as the biochemical assays, do not require this treatment, and, in most cases, these were performed in the absence of PMA/ionomycin (exceptions are indicated in the figure legends). We found, however, that none of the biochemical assays were influenced by treatment with these drugs. The coimmunoprecipitation assay demonstrating interaction between endogenous Smad3 and GATA-3 in HT-2 cells shown in Figure 6 was performed in the presence of PMA/ionomycin, i.e., in the same cellular environment that allowed induction of IL-10 production by TGF-β. Of note, this interaction could also be observed in the absence of drug treatment.

HT-2 cells were transfected by electroporation. Stable populations of transfected cells were selected in G418 (Life Technologies). After amplification, clones of cells were obtained by limiting dilution and were analyzed for gene expression by Western blotting with anti-HA antibodies. Adenoviruses were kindly provided by Aristidis Moustakas, LICR, Uppsala, Sweden. HT-2 cells were infected in medium containing 3% serum at 100:1 multiplicity of infection for 48 hr prior to TGF-β stimulation. Infection was estimated to be greater than 70% by β-gal staining of cells infected with lacZ viruses. The IL-10 ELISA was performed using a kit from R&D. Cells were preincubated for 4–6 hr in IL-2/βME-supplemented serum-free medium, and were then plated in 96-well plates (2×10^5 cells in 200 μl) in HT-2 serum-free medium supplemented with PMA/ionomycin and TGF-β1 (R&D) as indicated. Conditioned media were harvested after an overnight incubation, and 50 μl was assayed following the manufacturer's instructions. All conditions were analyzed in quadruplicate.

Electrophoretic Mobility Shift Assays

Nuclear extracts were prepared from control and TGF-β-treated (50 min) HT-2 cells using high-salt extraction. Briefly, 2×10^8 cells from suspension cultures were collected by centrifugation and washed with phosphate buffer saline (PBS). The pellet was resuspended in 1 ml ice-cold homogenization buffer (25 mM Tris-HCl [pH 7.5], 0.32 M Sucrose, 30 mM NaF, 1 mM Na_3VO_4, 1× Complete protease inhibitor cocktail [Roche]) and was allowed to swell on ice for 20 min followed by 20 strokes of a Dounce all-glass homogenizer. The nuclei were collected by centrifugation, washed, and lysed in a high-salt buffer containing 420 mM NaCl, 10% glycerol, 20 mM Tris-HCl (pH 7.9), 1 mM DTT, 30 mM NaF, 1 mM Na_3VO_4, and 1× Complete protease inhibitor. After 30 strokes in a Dounce all-glass homogenizer, the nuclei fractions were incubated under agitation on ice for 40 min, then collected by centrifugation at 14,000 × g for 30 min, and the supernatant was aliquotted and stored at −80°C.

A total of 5 μg of nuclear extracts from HT-2 or COS cells were incubated in 20 μl binding buffer (10 mM Tris-HCl [pH 7.5], 50 mM NaCl, 1 mM $MgCl_2$, 4% glycerol, 0.5 mM DTT, 1 μg Poly [dI-dC]). After 30 min (45 min with antibody for supershift) on ice, complexes were separated on a 6% polyacrylamide gel and run in 0.5× TBE. Oligonucleotides containing two consensus binding sites for GATA factors were used: 5′-CACTTGATAACAGAAAGTGATAACTCT-3′ and its complementary strand. Oligonucleotides were end labeled with α-^{32}P-ATP using polynucleotide kinase (PNK).

GST Pull Downs, Immunoprecipitations, and Western Blotting

GST fusions were produced in *E. coli* and purified by chromatography on glutathione-conjugated agarose beads (Pharmacia). In vitro-translated products were produced using a kit from Promega. Antibody against GATA-3 was from Santa Cruz, anti-HA monoclonal was from Covance, anti-myc monoclonal antibody was obtained from the conditioned medium of the 9E1 hybridoma, and anti-Smad3 antibodies were previously described [59]. Nuclear extracts were obtained by high-salt extraction of nuclear pellets. Immunoprecipitations were done overnight at 4°C, followed by incubation with ProteinG Gamma-Bind beads (Pharmacia), repeated washing steps, and elution in SDS/PAGE sample buffer. Immunoprecipitates were fractionated by SDS-PAGE and blotted to PVDF membranes (Amersham). Blots were probed with the indicated antibodies, followed by alkaline phosphatase-conjugated anti-IgG and were developed with the ECF Western Detection System (Amersham). Gels with in vitro-translated products were fixed, dried, and exposed to phosphorscreens. All blots and gels were scanned in a Storm 840 phosphorimager/fluorimager and quantified with ImageQuant software (Molecular Dynamics).

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