Cooperation between GATA4 and TGF-β signaling regulates intestinal epithelial gene expression

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Belaguli NS, Zhang M, Rigi M, Aftab M, Berger DH. Cooperation between GATA4 and TGF-β signaling regulates intestinal epithelial gene expression. Am J Physiol Gastrointest Liver Physiol 292: G1520–G1533, 2007. First published February 8, 2007; doi:10.1152/ajpgi.00236.2006.—Members of the transforming growth factor-β (TGF-β) family have been shown to play an important role in the regulation of gut epithelial gene expression. We have used the intestinal alkaline phosphatase (IAP) and intestinal fatty acid binding protein (IFABP) promoters to dissect the mechanisms by which TGF-β1 signaling regulates gut epithelial gene expression. TGF-β signaling alone was not sufficient for activation of IAP and IFABP promoters. However, TGF-β signaling cooperated with the gut epithelial transcription factor GATA4 to synergistically activate IAP and IFABP promoters. Coexpression of GATA4 along with the TGF-β1 signal transducing downstream effectors such as Smad2, 3, and 4 resulted in synergistic activation of both IAP and IFABP promoters. This synergistic activation was reduced by simultaneous expression of dominant-negative Smad4. —40 and −89 IAP binding sites in the IFABP promoter were required for the synergistic activation by Smad2, 3, and 4 and GATA4, GATA4 and Smad2, 3, and 4 physically associated with each other and this interaction was mediated through the MH2 domain of Smad2, 3, and 4 and the second zinc finger and the COOH-terminal basic domain of GATA4. The COOH-terminal activation domain and the Smad-interacting second zinc finger domain of GATA4 were required for the synergistic activation of the IFABP promoter. Naturally occurring oncogenic mutations within the GATA4-interacting MH2 domain of Smad2 reduced the coactivation of IFABP promoter by Smad2 and GATA4. Our results suggest that the TGF-β signaling regulates gut epithelial gene expression by targeting GATA4.

GATA4; transforming growth factor-β; Smads; intestinal fatty acid binding protein; intestinal alkaline phosphatase

The mammalian intestine is one of the organs with rapid and perpetual epithelial cell turnover. In the small intestine, stem cell and stem daughter cell proliferation gives rise to a progeny of cells that subsequently undergo differentiation into distinct cell types such as absorptive enterocytes and secretory enteroendocrine, goblet, and Paneth cells (19, 78). The acquisition of the differentiated phenotype is associated with repression of genes that drive cell proliferation and activation of differentiation marker genes characteristic of a particular differentiated cell type. A variety of growth factors including members of the TGF-β family are known to regulate gut epithelial gene expression.

Members of TGF-β family have been implicated in maintaining gut homeostasis through their role in cellular proliferation, differentiation, migration, tissue repair, and apoptosis. TGF-β family members are expressed in the adult intestinal epithelium and epithelial cell lines. In the intestines, TGF-β1, activin A, and activin βA are expressed in a gradient along the crypt-villus axis with a maximal expression in the differentiated cells of the villus tip in the jejunum and above the crypt zone in the colon (6, 7, 50, 74). Both type I and type II TGF-β receptors and activin IB and IIA receptors are expressed in intestinal epithelial cells and gut epithelial cell lines (66, 71, 74, 81, 83). Smad4, an obligate downstream signal transducer common to TGF-β family members, is strongly expressed in the crypt region of developing mouse embryos (51) and in differentiated cells of human colon (44).

Several studies have suggested that TGF-β1 and activins play an important role in differentiation of gut epithelial cell lines. TGF-β1 induces differentiation of IEC-6 intestinal epithelial cells and antagonizes the proliferative effect of insulin-like growth factor-I (43, 48). Differentiation of Caco2 cells induced by the addition of sodium butyrate or postconfluence is associated with upregulation of TGF-β1 and activin βA, respectively (69, 74). Neutralization of TGF-β activity by addition of specific antibodies interferes with sodium butyrate induced differentiation of Caco2 cells, suggesting an essential autoregulatory role for TGF-β1 in gut epithelial cell differentiation (69). Similarly, differentiation of HT29 cells induced by hexamethylene-bis-acetamide is associated with increased production and secretion of TGF-β1. Hexamethylene-bis-acetamide induced HT29 cell differentiation is inhibited by TGF-β1 neutralizing antibodies (34). Addition of TGF-β1 is sufficient for induction of differentiation of T84 intestinal epithelial cells cultured in three-dimensional collagen gels and further organization of these differentiated cells into luminal structures resembling crypts, providing definitive evidence for the role of TGF-β in intestinal differentiation (35).

The mechanisms by which TGF-β family members and their downstream signal transducing Smad proteins regulate gut epithelial-specific gene expression are not known. Considering the ubiquitous expression of Smads, binding of Smads to potential Smad binding elements within the regulatory regions of gut-expressed genes will not account for the ability of TGF-β/Smads to drive gut epithelium-restricted gene expression. Gut epithelium-restricted gene expression by TGF-β may be achieved by combinatorial interaction of Smads with gut tissue-enriched transcription factors, as demonstrated for several other tissues (29, 46, 54, 61, 62).

GATA4, a member of the GATA zinc finger protein family, has been shown to play an important role in early endoderm development.
development (5, 58, 63, 75). GATA4 and the related GATA6 are sufficient to induce early endoderm formation in pluripotential embryonal carcinoma cells (32). Additionally, ablation of either GATA4 or GATA6 severely impairs endodermal differentiation (47, 57, 58, 60, 63, 75). GATA binding sites occur within the regulatory regions of several gut epithelium-restricted genes such as sucrase isomaltase, lactase-phlorizin hydrolase, liver- and intestine-type fatty acid binding proteins, sodium-hydrogen exchanger isoform 3, and trefoil factors 1 and 2. Furthermore, GATA4 activates these gut epithelium-restricted genes in both gut epithelial and nonepithelial cells (2, 13, 20, 22, 27, 31, 33, 39, 40, 45, 64, 73, 79, 80). Within the adult murine small intestine GATA4 is expressed in a gradient along the crypt-villus axis with strongest expression in differentiated enterocytes (13, 22, 28). This pattern of expression is similar to that of TGF-β and its type I receptor. The overlapping expression pattern of GATA4 and TGF-β suggests that TGF-β signaling may target GATA4 to activate gut epithelial gene expression. It has been shown previously that the interaction between TGF-β signaling and GATA family members is involved in conferring cell-type specificity on gene expression. T cell-specific expression of IL-5 was mediated by interaction between the T cell-expressed GATA factor, GATA3, and the TGF-β signal-transducing Smad3 (11). Similarly, expression of the cardiomyogenic factor, Nkx2-5, is regulated by physical and functional interaction between GATA4 and Smad3 and Smad4 (14). More recently, Anttonen et al. (4) showed that TGF-β upregulated GATA4 expression in granulosa cells and GATA4 activated the inhibin-α gene expression through interaction with Smad3. Here we show that GATA4 mediates the activation of intestinal alkaline phosphatase (IAP) and intestinal fatty acid binding protein (IFABP) promoters by TGF-β and Smad signaling by physically and functionally interacting with Smad2/3/4. We demonstrate that the second zinc finger of GATA4, the region that mediates the physical interaction of GATA4 with Smad2/3/4, and the COOH-terminal-activation domain of GATA4 are essential for coactivation of IAP and IFABP promoters. Additionally, we also show that the oncopgenic mutations within the GATA4 interacting MH2 domain of Smad2 compromise the ability of Smad2 to coactivate IAP and IFABP promoters, suggesting that the interaction between TGF-β/Smad2 signaling and GATA4 may be essential for maintenance of gut homeostasis.

**MATERIALS AND METHODS**

**Plasmids.** IAP promoter-luciferase reporter containing 2,550 bp of human IAP promoter cloned upstream of pGL3 basic reporter has been described earlier (41). IFABP promoter-luciferase reporter was constructed by inserting PCR-amplified −306 to +24 fragment of murine IFABP between MluI and XhoI sites of the pGL3 basic vector. HA epitope-tagged GATA4 was generated by inserting PCR amplified rat GATA4 between XhoI and BamHI sites of cytomegalovirus promoter-enhancer-based pCGN vector. GATA42ZF2, GATA4ΔC, GATA4ΔN, and GATA4 ZF1+2 deletion mutants of GATA4 used for transfection experiments and GATA5 and GATA6 vectors used for in vitro translation have been described earlier (9). GATA4 N1, NT3, CT3, CT4, and ZF-B deletion mutants of GATA4 used for in vitro translation were kindly provided by Dr. Schwartz (14). FLAG epitope-tagged Smad2/3/4 expression vectors have been described earlier (14). pCMV5-based dominant-negative Smad4 expression vector with truncation of the COOH terminus (amino acids 1–514) has been reported earlier (49). Expression vectors for Smad2 NL (aa 1–251), Smad2 C (aa 241–467), Smad3 N (aa 1–147), Smad3LC (aa 145–425), and Smad3C (aa 199–425) were generated by cloning of PCR-amplified human Smad2/3 fragments into FLAG epitope-tagged pCMV5 vector. Constitutively active (T204D) and wild-type TGF-β type 1 receptor expression vectors were kindly provided by Dr. Attisano (38).

**Mutagenesis.** Mutations at the −40, −89, and −181 GATA sites of the IFABP promoter were created by PCR-based mutagenesis. The top strand sequence for wild-type and GATA site-mutated oligos is shown below. Mutated sequences are in lower case. −40 wild type: TTGTTCGAAGATGAAATAGA-3; −40 mutant: 5′-TTGTTCGAAttcAGAAGAAATAGA-3; −89 wild type: 5′-AGGTAGTTAATCTCTGACTT-3; −89 mutant: 5′-AGGTAGTTGtaaTTCCT-GAACC-3; −181 wild type: 5′-TGGATGTAAAAGTTAATTAT-3; −181 mutant 5′-TGGATGTAAAAGTTAATTAT-3. GATA binding sites at −89 and −181 were on the opposite strands. Inability of the mutated GATA sites to bind GATA4 from RIE and IEC-6 cell nuclear extracts was confirmed by EMSA. P454H and D450E point mutations in pCMV5 FLAG epitope-tagged Smad2 were generated by QuickChange II XL mutagenesis system (Stratagene). The sense strand of the mutagenic oligonucleotides is shown with the mutated nucleotides in lower case. p454H: 5′-GAACCTCTACT-GATGGGAGGGCCGGAAGGCG-3′; D450E: 5′-GACCTCATTGAATGATggaaAGATTTTAAT-3′. p454H and deletion mutants of GATA4 used for in vitro translation system (Promega). Full-length Smad2 and 3 and GATA4 were generated by QuickChange II XL mutagenesis system (Stratagene). The sense strand of the mutagenic oligonucleotides is shown with the mutated nucleotides in lower case. 89 mutant: 5′-AGGGTTAGGGTTgaaTCCT-3′; 89 wild type: 5′-AGGGTTAGGGTTAATCGGTAACC-3′. The ability of the transfected siRNA to downregulate endogenous Smad2/3/4 was on the opposite strands. Total amount of transfected DNA was adjusted to 1 μg by using pCGN empty vector. Cells were harvested 36 h posttransfection, and the luciferase values were normalized to total protein. For experiments in which TGF-β was used, transfected CV1 cells were serum starved overnight in 0.5% FBS-containing medium and stimulated with 5 ng/ml of porcine TGF-β1 (R and D Systems) or vehicle for 24 h. For small-interfering RNA (siRNA) transfections, chemically synthesized negative control siRNA 1, Smad2 siRNA, Smad3 siRNA (Ambion), and Smad4 siRNA pool (Dharmacon) were transfected at 100 nM final concentration along with IFABP luciferase reporter. The top strand sequence for the most effective Smad2 siRNA, siRNA-2, was 5′-GGUCUCAUCAAUUAAAGCAT-3′. The previously validated Smad3 siRNA sequence was 5′-GGACGAGAGGUCGCGUGAA-Utt-3′. The ability of the transfected siRNA to downregulate endogenous Smad2/3/4 was confirmed by Western blotting with Smad2 and Smad3 antibodies (Zymed) and Smad4 antibody (Santa Cruz Biotechnology). All transfection experiments were performed in duplicate or triplicate using Lipofectamine 2000 (Invitrogen) and repeated at least three times.

**Cell culture and transfections.** Subconfluent IEC11 colon carcinoma cells were transfected with 0.2 μg of luciferase reporter and 0.4 μg of expression vectors by use of Lipofectamine 2000 (Invitrogen). Total amount of transfected DNA was adjusted to 1 μg by using pCGN empty vector. Cells were harvested 36 h posttransfection, and the luciferase values were normalized to total protein. For experiments in which TGF-β was used, transfected CV1 cells were serum starved overnight in 0.5% FBS-containing medium and stimulated with 5 ng/ml of porcine TGF-β1 (R and D Systems) or vehicle for 24 h. For small-interfering RNA (siRNA) transfections, chemically synthesized negative control siRNA 1, Smad2 siRNA, Smad3 siRNA (Ambion), and Smad4 siRNA pool (Dharmacon) were transfected at 100 nM final concentration along with IFABP luciferase reporter. The top strand sequence for the most effective Smad2 siRNA, siRNA-2, was 5′-GGUCUCAUCAAUUAAAGCAT-3′. The previously validated Smad3 siRNA sequence was 5′-GGACGAGAGGUCGCGUGAA-Utt-3′. The ability of the transfected siRNA to downregulate endogenous Smad2/3/4 was confirmed by Western blotting with Smad2 and Smad3 antibodies (Zymed) and Smad4 antibody (Santa Cruz Biotechnology). All transfection experiments were performed in duplicate or triplicate using Lipofectamine 2000 (Invitrogen) and repeated at least three times.

**In vitro translation and GST pull-down assays.** Deletion mutants of GATA4, Smad2 and Smad3 were hot translated in vitro in the presence of [35S]methionine by using a coupled in vitro transcription-translation system (Promega). Full-length Smad2 and 3 and GATA4 fused in frame to glutathione S-transferase (GST) were expressed in bacteria and purified on glutathione Sepharose columns. Approximately 10 μg of purified proteins were immobilized on glutathione Sepharose beads and incubated with 5 μl of hot translated wild-type and deletion mutants of GATA4, Smad2, and Smad3 in binding buffer (20 mM Tris · HCl pH 7.5, 100 mM NaCl and 0.5% Nonidet P-40) for 4 h at 4°C with constant rocking. Beads were washed five times in binding buffer. Bound proteins were eluted by boiling in SDS sample buffer, resolved on SDS-10% denaturing gels, and analyzed by autoradiography.
Immunoprecipitation and Western blotting. HCT116 cells were transfected in six-well plates with 1 μg each of HA epitope-tagged GATA4 or FLAG epitope-tagged Smad2/3/4 expression plasmids either alone or in combination. Cells were lysed for 36 h posttransfection in lysis buffer (20 mM Tris·HCl pH 7.5, 100 mM NaCl, and 0.5% Nonidet P-40). Equal amounts of protein lysates were immunoprecipitated for 4 h at 4°C with constant rocking with use of EZview Red anti-HA or FLAG affinity beads (Sigma). Beads were washed extensively and the bound proteins were eluted by boiling in SDS sample buffer and resolved on SDS-10% denaturing gels. Resolved proteins were electroblotted on to polyvinylidene difluoride membranes and analyzed by Western blotting with rabbit HA and FLAG antibodies (Santa Cruz Biotechnology).

Immunohistochemistry. Jejunal segments from 6-mo-old C57BL/6 mice were fixed overnight in 4% paraformaldehyde and embedded in paraffin. Five-micrometer-thick sections were boiled in citrate buffer for antigen retrieval. Following inactivation of endogenous peroxidases with H2O2 treatment and blocking, sections were treated with no antibody or 1:100 diluted rabbit GATA4 or rabbit Smad4 antibodies (Santa Cruz Biotechnology) overnight in a humidified chamber. Sections were washed extensively in PBS. Specifically bound antibodies were visualized with a Vectastain Elite ABC kit (Vector Laboratories) and nickel-enhanced 3,3’-diaminobenzidine substrate. Sections were counterstained with eosin.

RESULTS

TGF-β signaling augments GATA4 activation of the IAP and IFABP promoters. TGF-β is an important cytokine involved in maintaining gut homeostasis through its role in cellular proliferation, differentiation, migration, tissue repair, and apoptosis. TGF-β1 and members of the TGF-β superfamily such as activin A and activin βA are expressed in a gradient along the crypt-villus axis with a maximal expression in the differentiated cells of the villus tip in the jejunum and above the crypt zone in the colon (6, 7, 50, 74). TGF-β1 has been shown to induce differentiation in gut epithelial cell lines such as IEC-6 and Caco2. Since the expression pattern of TGF-β1 overlaps with that of GATA4, we hypothesized that TGF-β1 regulates intestinal epithelial gene expression by targeting GATA4. Therefore we analyzed whether TGF-β signaling regulates intestinal marker genes requires GATA4. Since the majority of intestinal cell lines express GATA4, we used heterologous GATA4-deficient monkey kidney epithelioid CV1 cells. CV1 cells have been shown to be receptive to TGF-β signaling and constitute various GATA4 target genes (9, 10, 15, 70). CV1 cells were transfected with 2.5-kb IAP and −306 IFABP promoter-luciferase reporter plasmids along with the pCGN empty vector or the GATA4 expression vector, serum starved overnight, and then treated with 5 ng/ml TGF-β or control vehicle in low-serum media for 24 h. TGF-β treatment alone did not elicit strong IAP or IFABP promoter activities in the absence of transfected GATA4. Whereas GATA4 alone activated both IAP and IFABP promoters strongly, TGF-β treatment enhanced this activity further. These data suggest that TGF-β induced activation of IAP and IFABP promoter activity requires GATA4 (Fig. 1A).

Fig. 1. TGF-β signaling and GATA4 cooperate to activate intestinal alkaline phosphatase (IAP) and intestinal fatty acid binding protein (IFABP) promoters. A: subconfluent CV1 cells were transfected with IAP, IFABP, or pGL3 basic luciferase reporter vectors along with the GATA4 expression vector or the pCGN empty control vector. At 24 h after transfection, cells were serum starved overnight and stimulated with 5 ng/ml porcine TGF-β (T) or vehicle (V) for an additional 24 h. B: subconfluent HCT116 cells were transfected with IFABP or pGL3 basic luciferase reporter vectors along with expression vectors for GATA4 or wild-type TBRI or constitutively active TBRI or the pCGN empty control vector. Luciferase activity was measured 36 h posttransfection and normalized to total protein. Results from 3 experiments performed in duplicate are represented as means ± SD. P < 0.05 for GATA4 transfected and TGF-β-treated cells compared with GATA4 transfected and vehicle-treated cells for A. For B, P < 0.001 for activated TBRI+GATA4 transfection compared with wild-type TBRI+GATA4 transfection.

Signaling by TGF-β is initiated and transduced through a heteromeric complex of type I and type II transmembrane serine/threonine kinases. Following ligand binding, type I receptor becomes activated and phosphorylated by the constitutively active type II receptor kinase and propagates signals to the downstream Smad proteins. We examined whether activation of TGF-β signaling through overexpression of wild-type and constitutively active type I receptor leads to enhanced activation of IFABP promoter by GATA4. HCT116 cells, which express low levels of GATA4, and wild-type TGF-β signal-transducing Smad2/3/4 factors were cotransfected with the −306 IFABP promoter and GATA4 along with either the wild-type or active type I receptor. Transfection of wild-type and constitutively active type I receptors alone did not activate the IFABP promoter. However, in the presence of GATA4 both wild-type and active type I receptors robustly activated the IFABP promoter, suggesting that TGF-β signaling targets GATA4 to activate intestinal differentiation marker gene expression (Fig. 1B). The active receptor was more potent than the wild-type receptor for activation of IFABP promoter.
Promoterless control pGL3 basic vector was not activated by coexpression of GATA4 and type I receptors.

Smad2, 3, and 4 are required for IFABP promoter activity. TGF-β signaling initiated by type I TGF-β receptor following ligand binding leads to phosphorylation and activation of pathway-specific Smads such as Smad2 and 3, which then associate with Smad4 and translocate to the cell nucleus to modulate gene expression. To explore whether TGF-β signaling transduced through the Smad pathway is involved in the activation of IFABP, we assessed the effect of downregulation of Smad2, Smad3, and Smad4 on the activity of IFABP promoter. HCT116 cells that express wild-type Smad2, Smad3, and Smad4 were transfected with −306 IFABP promoter along with Smad2 siRNA, Smad3 siRNA, Smad4 siRNA, or negative control siRNA. Whereas Smad3 siRNA completely knocked down Smad3 protein expression, Smad4 and Smad2 proteins were downregulated by ~50% by Smad4 siRNA pool and one of the Smad2 siRNAs (no. 2), respectively (Fig. 2A). Compared with the control siRNA-transfected cells, Smad2, Smad3, and Smad4 siRNA-transfected cells showed decreased IFABP promoter activity, suggesting that both the receptor-activated Smads, Smad2 and Smad3, and the common Smad, Smad4, are required for IFABP promoter activity (Fig. 2B).

GATA4 and Smads coactivate IAP and IFABP promoters. The ability of TGF-β signaling to augment GATA4-mediated activation of IAP and IFABP promoters and the requirement for Smad2/3/4 for the activation of the IFABP promoter suggested that TGF-β signal-transducing factors such as Smad2/3/4 may be involved in enhancing the GATA4 activation. Therefore we hypothesized that GATA4 and Smad2/3/4 cooperatively activate IAP and IFABP promoters. Consistent with our hypothesis, cotransfection of GATA4 with Smad2 or Smad3 or Smad4 into HCT116 colon cancer cells synergistically activated both IAP and IFABP promoters (Fig. 3). Additionally, the lactase phlorizin hydrolase promoter was also synergistically activated by GATA4 and Smad2/3/4 cotransfections (data not shown). Although cotransfection of GATA4 with Smad3 resulted in strongest activation of IFABP promoter, Smad2/3/4 were equivalent in synergistic activation of IAP promoter when cotransfected with GATA4. Transfection of CV1 cells also demonstrated coactivation of IAP and IFABP promoters by GATA4 and Smad2/3/4 (data not shown). Smad2/3/4 by themselves activated the IAP promoter weakly. The promoterless control vector, pGL3 basic, was not activated by cotransfection of GATA4 and Smad2/3/4, suggesting that IFABP promoter elements were mediating this synergistic activation.

Smad4-dependent TGF-β signaling is mediated by heterooligomerization of activated Smad2 and Smad3 with Smad4 and their translocation into the nucleus. Since Smad2 or Smad3...
alone synergistically activated GATA4-dependent activation of IAP and IFABP promoters, we examined whether this coactivation was Smad4 dependent by coexpressing dominant-negative Smad4. Dominant-negative Smad4 has been shown previously to interfere with Smad4-dependent activation of TGF-β target genes (49). Coexpression of dominant-negative Smad4 along with combination of GATA4 and Smad2/3/4 resulted in a decrease in the IFABP promoter activity, suggesting that the coactivation of IFABP promoter activity by GATA4 and Smad2/3/4 is Smad4 dependent (Fig. 3C).

GATA4 binds to multiple sites in the IFABP promoter. The −306 IFABP promoter reporter we have used in our assays contains potential GATA binding sites located at −40, −89, and −181 and one overlapping the TATA box. Before examining the role of these potential GATA sites in GATA4 and Smad2/3/4 mediated coactivation of IFABP promoter, we examined whether these sites are authentic GATA4 binding sites by gel mobility shift assays. As a positive control, a consensus GATA binding site from the rat brain-derived natriuretic peptide (rBNP) promoter was used. In addition to IFABP promoter GATA sites, one of the nine potential GATA sites from the IAP promoter, the −725 GATA site, was also examined. The control rBNP and the wild-type GATA probes from IAP and IFABP promoters formed GATA complexes with RIE nuclear extracts (Fig. 4A). We used supershifting GATA4 and GATA6 antibodies to determine the identity of GATA complexes. Although both GATA4 and GATA6 bound to all of the wild-type GATA probes analyzed, there were relative differences in the binding of GATA4 and GATA6 to individual probes with strong GATA6 binding to the control rBNP and −89 IFABP GATA sites (Fig. 4A, lanes 2–4 and 22–24). GATA5 antibody was not used in the supershift analysis since GATA5 is not expressed in RIE cells (data not shown). GATA site-mutated −89 and −181 IFABP probes did not bind GATA factors whereas a non-GATA4/6 binding activity, as determined by lack of reactivity to supershifting GATA4 and GATA6 antibodies, was detected over the GATA site-mutated −40 GATA site probe (Fig. 4A, lanes 17–19). In addition to the GATA sites, the −306 IFABP promoter contains two potential Smad binding CAGA box sequences located at −178 and −196. However, by gel shift assays using these potential Smad binding site probes and extracts from RIE, IEC-6, and HCT116 cells, we could not detect Smad binding to these sites (data not shown).

−40 and −89 GATA sites are required for coactivation of IFABP promoter by GATA4 and Smad2/3/4. Since GATA4 binds to multiple sites within the IFABP promoter, we examined which of the GATA binding sites mediated the coactivation of IFABP promoter by GATA4 and Smad2/3/4. Whereas mutation of −40 GATA site abolished the activation of the mutant promoter by GATA4 and coactivation by GATA4 and Smad2/3/4, mutation of the −89 site significantly reduced (P < 0.001) but did not abolish coactivation (Fig. 4B). Mutation of the −181 GATA site did not affect the activation of this mutated promoter by GATA4. Although coactivation of this mutated promoter by GATA4 and Smad2/3 was reduced, the reduction was not significant. Together, these results indicate that the coactivation of IFABP promoter by GATA4 and Smad2/3/4 is mediated by multiple GATA binding sites.

GATA4 physically interacts with Smad2/3/4. Since Smad2/3/4 and GATA4 functionally interacted with each other to
synergistically activate the IAP and IFABP promoters, we examined whether GATA4 could physically interact with Smad2/3/4 by coimmunoprecipitation experiments. HA epitope-tagged GATA4 was transfected with or without FLAG epitope-tagged Smad2 or Smad3 or Smad4 into HCT116 cells. Precleared cell lysates were immunoprecipitated with HA epitope-specific antibody and the immunoprecipitates were analyzed by Western blotting with FLAG antibody. As shown in Fig. 5A, FLAG-tagged Smad2/3/4 were present in the immunoprecipitate of HA-GATA4, demonstrating that GATA4 and Smad2/3/4 physically interact in vivo. We performed converse experiments in which the cell lysates were immunoprecipitated with FLAG antibody and the immunoprecipitates were analyzed by Western blotting with HA antibody. HA-tagged GATA4 was present in the FLAG immunoprecipitates of FLAG-Smad2/3/4, confirming that GATA4 and Smad2/3/4 associate in vivo (Fig. 5A). By in vitro GST pull-down assays, we found that Smad2/3/4 interacted directly with other gut tissue expressed GATA factors such as GATA5 and GATA6 (Fig. 5B, data not shown for Smad4).

To evaluate whether GATA4 and Smad4, which are required for the coactivation of IFABP promoter, are coexpressed in the gut epithelial cells, we performed GATA4 immunohistochemistry in the colon and jejunum of wild-type and Smad4−/− mice. As shown in Fig. 5C, we found a significantly lower number of GATA4-positive cells in the colon and jejunum of Smad4−/− mice, demonstrating that GATA4 and Smad4 are coexpressed in vivo. These findings are consistent with our previous studies demonstrating that GATA4 and Smad4 are coexpressed in the gut epithelial cells and that they physically interact in vivo. Furthermore, these findings suggest that GATA4 and Smad4 are important for the coactivation of IFABP promoter in the gut epithelial cells.
intestine epithelium and within the same subcellular compartment(s) to permit the physical association, we examined successive jejunal sections from adult mouse by immunohistochemistry with antibodies to GATA4 and Smad2/3/4 alone or in combinations were used for in vivo communoprecipitation experiments. Equal amounts of lysates were preadsorbed with protein A + G agarose beads and immunoprecipitated with either HA or FLAG immunosafinity beads. Following extensive washing, affinity beads were boiled in SDS sample buffer and eluted proteins were analyzed by Western blotting with rabbit HA or FLAG antibodies. B: in vitro glutathione S-transferase (GST) pull-down experiments were performed by incubating in vitro translated [35S]methionine-labeled GATA4, GATA5, GATA6, or luciferase proteins with bacterially expressed purified GST or GSTSmad3 proteins immobilized on glutathione Sepharose beads. Following extensive washing, proteins bound to GST or GSTSmad3 were eluted, resolved under denaturing conditions, and analyzed by autoradiography. C–E: expression of GATA4 (C) and Smad4 (D) in adult murine jejunum was examined by immunohistochemistry. Staining was visualized by nickel-enhanced diaminobenzidine staining. Sections were counterstained with eosin. E did not receive any primary antibody. Images are shown at a magnification of \( \times 100 \). Magnification for villus tips shown in inset is \( \times 400 \). Arrowhead indicates nuclear staining for GATA4 and Smad4.

Fig. 5. GATA4 and Smad2/3/4 physically associate in vivo and in vitro. A: cell lysates prepared from HCT116 cells transfected with expression vectors for HA epitope-tagged GATA4 and FLAG epitope-tagged Smad2/3/4 alone or in combinations were used for in vivo communoprecipitation experiments. Equal amounts of lysates were preadsorbed with protein A + G agarose beads and immunoprecipitated with either HA or FLAG immunosafinity beads. Following extensive washing, affinity beads were boiled in SDS sample buffer and eluted proteins were analyzed by Western blotting with rabbit HA or FLAG antibodies.

B: in vitro glutathione S-transferase (GST) pull-down experiments were performed by incubating in vitro translated [35S]methionine-labeled GATA4, GATA5, GATA6, or luciferase proteins with bacterially expressed purified GST or GSTSmad3 proteins immobilized on glutathione Sepharose beads. Following extensive washing, proteins bound to GST or GSTSmad3 were eluted, resolved under denaturing conditions, and analyzed by autoradiography.

C–E: expression of GATA4 (C) and Smad4 (D) in adult murine jejunum was examined by immunohistochemistry. Staining was visualized by nickel-enhanced diaminobenzidine staining. Sections were counterstained with eosin. E did not receive any primary antibody. Images are shown at a magnification of \( \times 100 \). Magnification for villus tips shown in inset is \( \times 400 \). Arrowhead indicates nuclear staining for GATA4 and Smad4.

COOH-terminal zinc finger and the basic domain of GATA4 mediate physical association with Smad2. To map the domains of GATA4 that mediate the physical association with Smads, purified Smad2 protein fused in frame with GST was immobilized on glutathione-agarose beads and tested for its ability to interact with radiolabeled in vitro translated wild-type and deletion mutants of GATA4. GST-fused Smad2 bound to both wild-type GATA4 and a mutant of GATA4 lacking the COOH-terminal activation domain (G4NT1) (Fig. 6, A and B). A mutant GATA4 protein containing the NH2-terminal activation domains and the first zinc finger (G4NT3) failed to bind Smad2, suggesting that these domains were dispensable for
interaction with Smad2. A mutant protein containing the second zinc finger, the immediate COOH-terminal basic domain, and the COOH-terminal activation domain (G4CT3) interacted with Smad2, suggesting that the domain mediating the interaction of GATA4 with Smad2 is located within this region. Mutant proteins containing both zinc fingers but lacking the basic domain (G4 ZF-B) or containing the basic domain and the COOH-terminal activation domain (G4CT4) bound Smad2 poorly but detectably, suggesting that both the second zinc finger and the immediately COOH-terminal basic domain are required for efficient binding of GATA4 to Smad2 (Fig. 6, A and B).

MH2 domain of Smad2/3 mediates physical association with GATA4. The ability of GATA4 to interact with all three Smads indicates that highly conserved domains among Smad2, 3, and 4, such as MH1 and/or MH2 domains, may be involved in the physical interaction. To delineate the domains of Smad2/3 and GATA4 that mediate the physical interaction between these two classes of factors we used GST protein pull-down assays. GATA4 protein fused inframe to GST was expressed in bacteria and purified over the glutathione affinity beads. Purified GST or GST-GATA4 was immobilized on affinity beads and incubated with [35S]methionine labeled, in vitro translated wild-type or deletion mutants of Smad2/3 proteins and analyzed for the ability of GATA4 to interact with the radiolabeled Smad proteins. As shown in Fig. 6D, wild-type Smad2 interacted with GATA4. A mutant protein containing the MH1 domain and the linker domain but lacking the MH2 domain failed to interact with GATA4, suggesting that the MH2 domain mediates the interaction between Smad2 and GATA4. An isolated MH2 domain interacted with GATA4, confirming that the MH2 domain of Smad2 mediates the in vitro interaction between Smad2 and GATA4 (Fig. 6D). Since Smad3 also physically interacted with GATA4 and coactivated the IFABP

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Fig. 6. Physical association between GATA4 and Smad2/3 is mediated by the second zinc finger and the COOH-terminal basic region of GATA4 and the MH2 domain of Smad2/3. GST pull-down experiments were performed by incubating in vitro translated [35S]methionine-labeled wild-type and deletion mutants of GATA4 and bacterially expressed, purified GST or GSTSmad2 proteins immobilized on glutathione Sepharose beads. A: wild-type and deletion mutants of GATA4 are diagrammatically shown and their binding to GST-fused Smad2. B: representative autoradiogram of the pull-down experiment. C: representative autoradiogram of the in vitro synthesized [35S]methionine labeled wild-type and deletion mutants of GATA4. D: GST pull-down experiments were performed using in vitro synthesized [35S]methionine labeled wild-type and deletion mutants of Smad2 and Smad3 and bacterially expressed, purified GST or GSTGATA4 proteins immobilized on glutathione Sepharose beads. Wild-type and deletion mutants of Smad2 and Smad3 are diagrammatically shown and their binding to GST or GST fused GATA4 is shown. E: cell lysates prepared from HCT116 cells transfected with expression vectors for HA epitope-tagged GATA4 and FLAG epitope-tagged Smad3, Smad3N (MH1 domain) and Smad3C (MH2 domain) alone or in combinations were used for in vivo coimmunoprecipitation experiments. Equal amounts of lysates were preadsorbed with protein A + G agarose beads and immunoprecipitated with HA immobilization beads. The immunoprecipitates (IP) and the inputs were analyzed by Western blotting with rabbit FLAG antibody. The blot was stripped and reprobed with rabbit HA antibody (bottom). Asterisks indicate the signals from incomplete stripping of the FLAGSmad3 signal, which masked HA GATA4 signal in lane 3.

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promoter, we examined which domains of Smad3 mediated its interaction with GATA4. While wild-type Smad3 associated with GATA4, deletion of the MH2 and linker domains abolished this interaction. Similar to Smad2, an isolated MH2 domain from Smad3 bound GATA4 demonstrating that MH2 domains of Smad2/3 mediate their association with GATA4 (Fig. 6D).

To examine whether the MH2 domain is sufficient for interaction with GATA4 in vivo, we performed coimmunoprecipitation experiments. FLAG epitope-tagged full-length Smad3 or MH1 and MH2 domains from Smad3 were cotransfected with HA epitope-tagged GATA4. Cell lysates were immunoprecipitated with HA antibody, and the immunoprecipitates were analyzed by FLAG antibody. The full-length Smad3 and the MH2 domain of Smad3 communoprecipitated with GATA4, suggesting that the MH2 domain is sufficient for in vivo interaction between Smad3 and GATA4 (Fig. 6E). The MH1 domain of Smad3 failed to associate in vivo with GATA4, confirming that the interaction between GATA4 and Smad3 is mediated by MH2 domain. There was no evidence for interaction between Smad3N and GATA4 when the gel shown in Fig. 6E was overexposed, suggesting that the failure of MH1 domain to interact with GATA4 is not related to the lower expression of the MH1 domain fragment compared with that of MH2 domain. Similar to the MH2 domain of Smad3, the MH2 domain of Smad2 was found to be sufficient for interaction with GATA4 in vivo (data not shown).

Domains of GATA4 were required for coactivation of IFABP promoter. GATA4 contains two zinc finger motifs in addition to two NH2-terminal-independent activation domains and a conformation-dependent activation domain in the COOH terminus. We examined the domains of GATA4 required for the coactivation of IFABP promoter. Deletion of the second zinc finger (GATA4ΔZF2) abolished GATA4-mediated activation of the IFABP promoter as well as the GATA4-Smad2/3/4-mediated coactivation of the IFABP promoter, suggesting that the DNA binding domain of GATA4 is essential for coactivation (Fig. 7A). Additionally, it also suggests that the interaction between GATA4 and Smad2/3/4 is essential for coactivation since this deletion affects the domain of GATA4 required to mediate its interaction with Smad2/3/4. Similarly, deletion of both the NH2- and COOH-terminal-activation domains in the GATA4ZF1+2 construct (aa 198–332) eliminated the ability of the mutant GATA4 to activate and coactivate the IFABP promoter, demonstrating the requirement for GATA4 activation domains. Interestingly, deletion of the COOH-terminal activation domain (GATA4ΔC aa 1–332) abolished coactivation without affecting the ability of the mutant GATA4 to activate the IFABP promoter, indicating that whereas the NH2-terminal activation domains are sufficient for the activation of the IFABP promoter, coactivation requires the COOH-terminal activation domain. Accordingly, a mutant GATA4 containing the zinc fingers and the COOH-terminal activation domain (GATA4ΔN aa199–443) was impaired for activation of the IFABP promoter and a moderate decrease in coactivation (Fig. 7A). Together, these results suggest that the activation domains of GATA4 required for activation and coactivation of IFABP promoter are distinct. Although the NH2-terminal activation domain is sufficient for activation, coactivation requires the COOH-terminal activation domain.

Mutation of Smad2 MH2 domain affects IFABP promoter coactivation. Several studies have shown that Smad2 is frequently mutated in colorectal cancers. The majority of these mutations occur within the MH2 domain. Mutations in Smad2 result in disruption of TGF-β regulation of target genes. Since the physical association between GATA4 and Smad2 mediated by the MH2 domain of Smad2 appears to be important for coactivation of IFABP and IAP promoters, we examined whether missense mutations within the MH2 domain affect the ability of Smad2 to cooperate with GATA4 in IFABP promoter coactivation. A mutation that substitutes proline 445 to histidine (P445H) significantly impaired the ability of the mutant Smad2 to coactivate the IFABP promoter (Fig. 7B). A second Smad2 mutant, D450E, was also significantly impaired for coactivation, suggesting that the oncogenic mutations in Smad2 affect coactivation functions of Smad2.
GUT EPITHELIAL GENE REGULATION BY GATA4 AND TGF-β

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DISCUSSION

The TGF-β family of growth factors plays an essential role in gut formation starting from the early embryonic stages of endoderm formation through the successive stages of endoderm differentiation, formation of the primitive gut tube, and regional specialization of the gut tube (42, 77). Subsequently, in the adult TGF-β is involved in maintaining gut homeostasis through its role in cellular proliferation, differentiation, migration, tissue repair, and apoptosis. Although several studies have suggested the involvement of TGF-β in gut epithelial differentiation, such studies are limited and the underlying mechanisms have not been addressed (34, 35, 69, 74). Our work demonstrates that TGF-β signaling regulates the expression of intestinal differentiation markers such as IFABP and IAP. Although TGF-β signaling by itself was not sufficient to elicit the expression of IAP and IFABP promoter activities, the TGF-β signaling factors, Smad2/3/4, were required for the expression of IFABP promoter in gut epithelial cells. This finding suggests that TGF-β signaling may be necessary for gut epithelial differentiation. Considering the relatively high frequency of the less complex Smad binding elements in the genome, it is likely that members of the TGF-β signaling pathway interact with gut epithelium enriched factors to execute the gut epithelial gene program. Accordingly, reconstitution of GATA4 in GATA4 deficient nonenteric epithelial CV1 cells strongly enhances the activity of both the IAP and IFABP promoters. Additionally, activating the TGF-β signaling pathway in gut epithelial cells by cotransfecting constitutively active type I receptor along with GATA4 strongly enhanced the GATA4-mediated activation of IFABP promoter. In a recent study Anttonen et al. (4) showed that reconstitution of GATA4 in GATA4-deficient Cos-7 cells, which are derived from the CV1 cells that we have used in our experiments, was essential for the upregulation of the TGF-β target gene, inhibin-α. Together these results demonstrate that the TGF-β signaling pathway converges on the gut epithelium enriched GATA factor, GATA4. The cooperativity between TGF-β signaling and gut tissue-enriched GATA factors, GATA4/5/6, appears to be evolutionarily conserved. GATA4/5/6 mediates the TGF-β-regulated elaboration of early endoderm and subsequent gut morphogenesis in Xenopus (1).

The convergence of the TGF-β pathway on GATA4 in modulating gut epithelial promoter activities involved Smads, the downstream effectors of the TGF-β signaling pathway. Both GATA4 and the common Smad, Smad4, were expressed in jejunal epithelial cells with maximal expression in terminally differentiated cells toward the villus tip, highlighting the relevance of TGF-β pathway convergence on GATA4 to differentiation of enteric epithelial cells. IFABP promoter activity was affected by knockdown of individual Smads. Although Smad3 knockdown was nearly 100%, there was only 50% downregulation in IFABP promoter activity, suggesting that Smad2 and Smad4 may be sufficient for partial activation of the promoter. Furthermore, a partial knockdown of Smad4 was more effective in suppressing IFABP promoter activity, indicating an important role for Smad4 in IFABP promoter activity. The role for Smad4 in GATA4-dependent synergistic activation of IFABP promoter by Smad2/3/4 was demonstrated by simultaneous expression of dominant-negative Smad4. GATA4-dependent synergistic activation of IFABP promoter by Smad2, Smad3, and Smad4 was reduced by dominant-negative Smad4, suggesting that the coactivation by GATA4 and individual Smads is Smad4 dependent.

GATA4-dependent coactivation of IAP promoter by Smad2, Smad3, and Smad4 was comparable for all three Smads. However, the combination of Smad3 and GATA4 was the most potent in activating the IFABP promoter, suggesting that GATA4 cooperates preferentially with Smad3 to activate this promoter. Differences in the activation of these two promoters by GATA4 and Smad2/3/4 combination could be related to the differences in 1) the type of GATA factors and their binding affinities to GATA elements in these promoters, 2) differences in the factors that bind to flanking sequence that surround the core GATA site, or 3) the relative strengths of binding of GATA cofactors such as Smads. In support of the first possibility, our gel mobility shift assays indicate considerably more GATA4 binding and less GATA6 binding to the −40 and −181 IFABP GATA sites compared with the −725 IAP GATA site. The −89 GATA site appears to bind more GATA6 compared with the −40 and −181 GATA sites. Colon cancer cells such as Caco2, HT29, and the cells that we have used for our transfection experiments, HCT116, express low levels of GATA4, abundant levels of GATA6, but no GATA5 (33). Although GATA4, GATA5, and GATA6 are known to bind to similar sequences, they differ in their ability to activate their target genes including IFABP and the related liver-type fatty acid binding protein, Fabpl (22, 33). Therefore, preferential binding of GATA4 vs. GATA6 to IAP and IFABP GATA sites may result in differential activation of these promoters. Under in vivo conditions in which endodermal GATA family members are coexpressed in enteric epithelial cells and interact physically and functionally (12, 22, 23), such preferential occupation of GATA sites by intestinal GATA factors could result in differential regulation of GATA target genes. In support of the second possibility, a multitude of factors such as Sp1, Sp3, KLF4, HNF4, thyroid hormone receptor, Cdx1 and Cdx2, and Kruppel type zinc finger protein, ZBP-89, have been reported to bind to IAP promoter and regulate IAP promoter activity (3, 37, 41, 52, 53, 65). Several of these factors bind in close proximity to the nine potential GATA sites present in the IAP promoter. Studies from the evolutionarily conserved zebrafish IFABP promoter, related intestinal epithelial cell-expressed, GATA4-regulated Fabpl promoter (8), and potential transcription factor binding site prediction suggest that the same factors, such as GATA factors, C/EBP and Cdx1/2, and many distinct factors, may be involved in regulating IAP and IFABP promoters (21, 36, 76). However, considerable differences in terms of trans-factors that bind to IAP and IFABP promoters and the context of their binding sites relative to the core GATA sites may have contributed to differences in the activity of IAP and IFABP promoters. Besides, the 2.5-kb IAP promoter contains several CAGA box sequences that may serve as authentic Smad binding sites in contrast to the IFABP promoter sequences that resembled Smad sites but did not bind Smads. In support of the third possibility, there appear to be differences in relative binding of GATA4 to Smads with the strongest binding to Smad4. Compared with Smad2, in vitro binding of GATA4 to Smad3 was stronger. It is possible that differences in binding strengths of Smads to GATA4 may be related to the differences in the coactivation of IAP and IFABP promoters by GATA4 and Smad2/3/4 combination. Alter-
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nately, differences in the promoter regulatory regions of these two promoters and the _trans_-factors that bind to these sites as discussed above may be involved in recruiting Smads independent of GATA4. For example, IAP promoter activity is dependent on promoter proximal Sp1 sites (41). Such Sp1 sites are not readily discernible in the −306 IFABP promoter. Sp1 has been shown to recruit Smad2/3/4 and mediate activation of TGF-β target genes such as p15 (ink4B), plasminogen activator inhibitor 1, collagen alpha 2(I) and amyloid beta precursor protein (18, 24, 30, 68).

Preferential interaction between GATA and Smad family members on T-cell, cardiac myocyte and granulose cell expressed promoters has been reported previously (4, 11, 14). GATA3 cooperates with Smad3 but not Smad1, 2, or 4 to activate the T-cell expressed IL-5 promoter (11). Similarly, GATA4 preferentially cooperates with Smad3 and Smad4 and weakly with Smad1, Smad2, and Smad5 in activating Smad-responsive promoters in p19 embryonal carcinoma cells (14). In granulosa cells, preferential interaction between GATA4 and Smad3 was involved in cell type-specific expression of TGF-β target gene inhibin-α (4). Thus preferential interaction between specific members of the GATA and Smad families appears to be the basis by which TGF-β signaling discriminates among distinct target genes in different cell types. Further specificity may be achieved by cell type- or promoter-specific utilization of distinct functional domains of GATA4. Coactivation of the cardiac myocyte-specific Nkx2.5 enhancer in p19 mouse embryonal carcinoma cells requires both the NH2- and COOH-terminal activation domains of GATA4. However, coactivation of the gut epithelial-specific IFABP promoter requires the conformation-dependent COOH-terminal activation domain but not the independent NH2-terminal activation domains of GATA4.

The physical association among GATA and Smad family members appears to be a prerequisite for their cooperativity at target gene promoters. Whereas GATA3-interacting Smad, Smad3, coactivated the IL-5 promoter in T-cells, non-GATA3-interacting Smads involved in either the TGF-β pathway (Smad2) or in the BMP pathway (Smad1) or in both pathways (Smad4) failed to do so (11). Smad2, which interacted weakly with GATA4, was a weak coactivator of the cardiac myocyte-restricted Nkx2.5 enhancer, whereas strong GATA4-interacting Smads, Smad3 and Smad4, were the strongest coactivators of this enhancer (14).

In addition to the ability of specific interaction of different GATA and Smad family members to discriminate among different target genes, distinct domains of GATAs and Smads are utilized to achieve activation of target genes in specific cell types. Interaction between the T-cell-enriched GATA3 and Smad3 was mediated by the MH1 domain of Smad3 and a region of the GATA3 activation domain just NH2-terminal to the first zinc finger domain (11). Interestingly, our results and those of Brown et al. (14) and Anttonen (4) show that gastrointestinal tissue-enriched GATA4 interacted with Smad3 through the second zinc finger and the immediate COOH-terminal basic region. GATA factors share a high degree of sequence and structural similarity over the zinc finger regions, and the second zinc finger region is the most frequently utilized in interactions with various other transcription factors (9, 14, 15, 25, 57, 59, 70). Our results differ substantially from those of Brown et al. (14) and Blokzijl et al. (11) in that the MH2 domain rather than the MH1 domains of Smad2 and Smad3 were the GATA-interacting domains in both in vivo coimmunoprecipitations and in vitro GST pull-down assays. Although the MH2 domain of Smad3 was sufficient for strong binding to GATA4 in vivo, adjoining linker region appears to contribute to binding in vitro. Both MH1 and MH2 domains of Smads are known to mediate interactions with various transcription factors including several zinc finger proteins. The discrepancy between our results and the previously published studies could be related to the technique used to map the GATA-interacting domain of Smad3. Brown et al. and Blokzijl et al. used GST-fused MH1 and/or MH2 domains of Smad3 to determine their interactivity with in vitro translated GATA. We have used isolated MH1 and MH2 domains of both Smad3 and the structurally related Smad2, translated in rabbit reticulocyte lysates to determine their interactivity with GST-fused GATA4. Such isolated MH1 and MH2 domains from Smad3 and Smad2 devoid of large fusion tags such as GST have been shown to fold appropriately to permit DNA binding and interaction with cofactors by cocry stallization and structure analysis studies (72, 82). We have used the HCT116 gut epithelial cells rather than the more commonly used heterologous HEK293 or Cos-1 cells to validate our in vitro GST pull-down results by in vivo coimmunoprecipitation assays. Therefore it is also possible that the utilization of distinct interacting domains may be a function of the cell type in which the assays are performed.

The physical interaction of various transcription factors such as Sp1, EKLF, etc., with GATA and Smad family members has often been shown to result in recruitment of these factors to GATA- and Smad-responsive gene regulatory elements (29, 55, 56, 67). The synergy between GATA3 and Smad3 on the T-cell-expressed IL-5 promoter was shown to be related to the ability of GATA3 to recruit Smad3 to IL-5 promoter. Likewise, on the cardiac myocyte-expressed Nkx2.5 enhancer GATA4 was recruited via Smad binding elements. The mutual ability of the tissue enriched GATA factors and the ubiquitously expressed Smads to recruit each other to DNA regulatory elements permits modulation of cell type-specific genes responsive to external signals. The ability of IFABP promoter that bound GATA4/6 but not Smads to support coactivation suggested that the coactivation was GATA site dependent and mediated by recruitment of Smads to the promoter by GATA4. Accordingly, mutation of the −40 GATA site or the −89 GATA site individually severely affected coactivation of mutated IFABP promoters by GATA4 and Smad2/3/4 combination. In further support of Smad recruitment to the IFABP promoter by GATA4, DNA binding defective second zinc finger deleted GATA4 was also impaired for coactivation of the IFABP promoter. Considering the highly restricted expression of gut epithelial regulatory factor, GATA4, and the various GATA4-responsive differentiation markers such as IAP, lactase phlorizin hydrolase, and sucrase isomaltase, it is likely that Smads are also recruited via the GATA elements in these genes.

In the highly dynamic environment of the adult intestines, several physiological processes such as cell proliferation, differentiation, cell migration, and apoptosis occur in harmony, resulting in rapid turnover of the entire epithelium. GATA factors are known to play important roles in the regulation of such physiological processes in several different cell types. Targeting of a multifunctional gut tissue enriched GATA4...
regulatory factor by TGF-β signaling may therefore underlie the diverse role of TGF-β in gut homeostasis. Perturbation in homeostatic mechanisms caused by inactivating mutations in TGF-β signaling pathway such as mutational inactivation of type II receptor, Smad4, and to a lesser extent Smad2 is associated with malignancies in gut epithelium (16, 17, 26, 29). Impaired ability of GATA4 to cooperate with mutated Smad2 may also affect other potential activities of GATA4 required for gut homeostasis maintenance in addition to the demonstrated role of GATA4 in gut epithelial differentiation.

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