Cell. Mol. Life Sci. 65 (2008) 3304 – 3311
1420-682X/08/203304-8
DOI 10.1007/s00018-008-8294-9

Cellular and Molecular Life Sciences

Research Article

Galectin-1 suppresses α2(I) collagen through Smad3 in renal epithelial cells

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Received 26 May 2008; received after revision 15 August 2008; accepted 19 August 2008
Online First 15 September 2008

Abstract. Transforming growth factor (TGF-β1) promotes renal fibrogenesis through activation of Smads. Galectin-1 is reported to prevent experimental glomerulonephritis. Here we investigated the fact that transfected galectin-1 significantly suppressed the transcription of α2(I) collagen (COL1A2) in TGF-β1-activated human renal epithelial cells. Conversely, galectin-1 silencing RNA reduced secretion of type I collagen by HKC cells. Galectin-1 significantly decreased activation of a TGF-β1-responsive reporter construct and of a minimal reporter construct that contains four repeats of the Smad binding element (SBE). Galectin-1 had no effect on phosphorylation of Smad3 at the linker region and C-terminus, whereas it decreased affinity of Smad3 to the SBE. Additionally, the inhibitory effect of galectin-1 disappeared using a mutated reporter construct, 376 m-LUC, in which a potential Smad recognition site within the promoter is mutated. Taken together, the results suggest that galectin-1 decreases Smad3-complex from binding to the SBE, down-regulating transcription of COL1A2 in TGF-β1-stimulated renal epithelial cells.

Keywords. Galectin-1, TGF-β1, Smad3, renal fibrosis, type I collagen, SBE.

Introduction

Transforming growth factor-β1 (TGF-β1) is one of the most important mediators for production of extracellular matrix (ECM) in kidney [1]. Upon binding of TGF-β1, the TGF-β receptor (TβR) leads to C-terminal phosphorylation of the R-Smad, Smad2 and Smad3, within five minutes in human mesangial cells [2] and renal tubular cells [3]. The phosphorylated R-Smads associate with the Co-Smad, Smad4, and then translocate to the nucleus. Smad can bind directly to specific DNA sequences called Smad binding elements (SBE) and regulate gene expression [4]. TGF-β1/Smad signaling stimulates expression of type I, -III, -IV collagen, laminin, fibronectin, and heparan sulfate proteoglycan in kidney cells [5–7]. Previously, we and colleagues found that the extracellular signal-regulated kinase (ERK) mitogen-activated protein (MAP) kinase enhances TGF-β1/Smad signaling for induction of α2(I) collagen expression (COL1A2) [8]. The phosphatidyl inositol 3-kinase (PI3K)/Akt pathway also participates in TGF-β1-induced collagen I expression [9]. These signaling molecules contribute to maximal activity of R-Smads in TGF-β1-activated renal cells.

Galectin-1 expression is detected in the kidney, specifically during embryogenesis [10], in podocytes

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in children with diffuse mesangial proliferation and focal segmental glomeruloscrelosis [11], and in cultured human tubular epithelial cells [12]. Peritoneal administration of recombinant galectin-1 ameliorates nephrotoxic serum nephritis in Wister Kyoto rats, suggesting that galectin-1 may play a protective role in renal fibrotic changes [13]. Intracellularly, galectin-1 is known as a binding partner of Ras [14], leading to stimulation of ERK pathways [15]. Galectin-1-Ras complex renders the activated molecule selective toward Raf-1, but not toward PI3K, which leads to antiproliferative effects resulting from the inhibition of ERK pathway [16]. Since galectin-1 is a bifunctional regulator [17], it is suggested that galectin-1 may have a pivotal role in renal fibrogenesis. However, little is known as to whether galectin-1 affects TGF-β/Smad signaling in renal cells. Here we show for the first time that galectin-1 decreases TGF-β/Smad signaling and inhibits COL1A2 expression in renal epithelial cells.

Materials and methods

Reagents and antibodies. Recombinant human TGF-β1 was obtained from R and D Systems (Minneapolis, MN, USA). For Galectin-1, a goat polyclonal antibody S-14 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used for immunoprecipitation and immunoblot. Anti-Smad1/2/3 (H-2) monoclonal antibody (mAb), anti-Smad4 (B-8), anti-c-Myc (9E10), and anti-Ets-2 (C-20) antibodies were also purchased from Santa Cruz Biotechnology. Anti-Flag M2 mouse anti-actin (A4700) mAb were obtained from Sigma (St. Louis, MO, USA); anti-phospho-Smad3 (Ser433/435) antibody from Cell Signaling technology (Beverly, MA, USA); rabbit anti-phosphoserine and rabbit anti-Smad3 antibodies from Zymed (San Francisco, CA, USA); goat anti-human type I collagen antibody from Southern Biotechnology Associates (Birmingham, AL, USA).

Cell culture. Immortalized human renal epithelial cell lines, HKC cells, were kindly gifted from L. C. Racusen and cultured as previously described [18].

Plasmid constructs. We isolated cDNA of human galectin-1 from cDNA library using 15 min TGF-β1-stimulated human mesangial cells, using the following primers containing sequences of restriction enzyme: 5'-ACAAGCTTCTGGACTCAGTCATG-3' and 5'-AAGGGCCCA-CATTTGATCTTGAAGT-3'. After digestion by HindIII and ApaI, the PCR products were subcloned into pcDNA3.1/myc-His A vector (Invitrogen). The inserted gene was checked again by DNA sequencing (Biotechnology Laboratory, Northwestern University, Chicago, IL, USA). The flag-tagged Smad3 construct was obtained from H. F. Lodish and X. Liu (Whitehead Institute, MA, USA). The p3TP-Lux and the Gal4-Smad3 constructs were kind gifts from J. Massague [19, 20]. The SBE-LUC reporter construct was kindly provided by B. Vogelstein [21]. The COL1A2-LUC construct containing the sequence 376 bp of α2(I) collagen promoter and 58 bp of the transcribed sequence fused to the luciferase reporter gene was described previously [2]. Dr. A. C. Poncelet generated a point mutant of COL1A2-LUC, 376 m-LUC, which contains mutated potential Smad recognition site (at –268/-260) [3]. The pFR-LUC plasmid was purchased from Stratagene (La Jolla, CA, USA).

Reporter assay. HKC cells were seeded on twelve-well plates at a density of 1 x 10^5/well. Twenty-four hours later, transfection was performed using FuGene6 (Roche, Indianapolis, IN, USA) with the indicated plasmid DNAs along with CMV-SPORT-β-galactosidase (Invitrogen) as a control for transfection efficiency. Five hours after the transfection, 1ng/ml of TGF-β1 was added and the cells were incubated for additional 20 hours. The cells were harvested with reporter lysis buffer (Promega, Madison, WI, USA) and luciferase activity was measured as described previously [8]. β-galactosidase activity was assayed (Promega) and used to standardize for transfection efficiency.

siRNA transfection. Chemically synthesized, double-stranded galectin-1 siRNA or control siRNA were purchased and transfected into the HKC cells following the protocol from Santa Cruz Biotechnology. HKC cells were seeded on six-well plates at a density of 5 x 10^5/well 20 hours before transfection. The final concentration of siRNAs was 10nM. Twenty hours later, the conditioned media was collected and subjected to western blot analysis with anti-human type I collagen antibodies.

SDS-PAGE and western blot. HKC cells were seeded on 60 mm dishes at a density of 1 x 10^6/dish. Four μg of Galectin-1/pcDNA3.1 or empty pcDNA3.1 were transfected using FuGene6 (2.5 μl/1 μg plasmid DNA) in serum-free media. Five hours after the transfection, the media was replaced with fresh 10% serum-containing media. On the following day, media was then replaced with fresh low-serum (0.5%) media followed by addition of TGF-β1 24 hours later. Equal volume of the conditioned media (40 μl), collected after 72-hour incubation, were resolved by SDS-PAGE, followed by immunobloting for type I collagen in a standard manner [20]. Total cell lysates (2.5 μg)

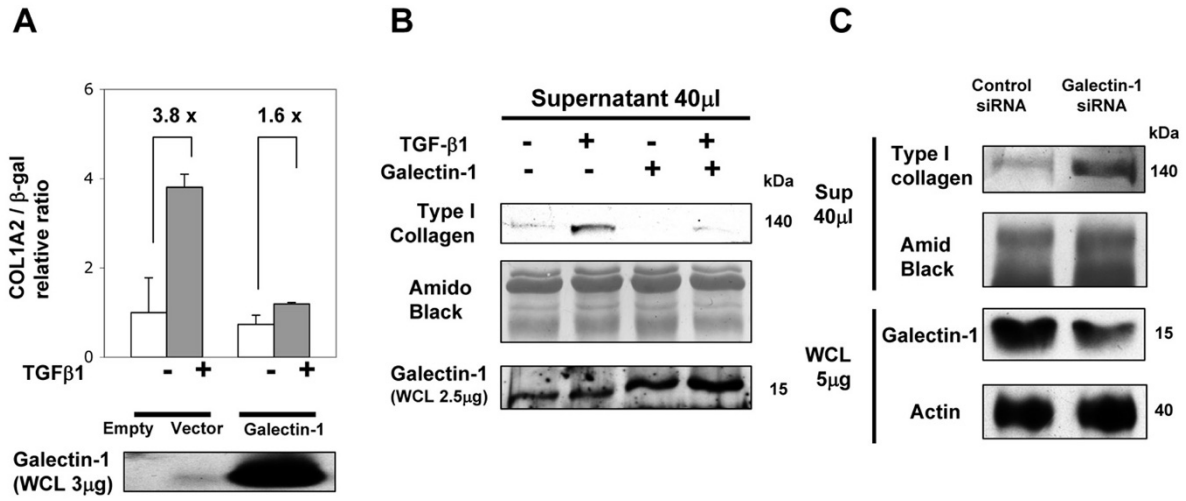

Figure 1. Effect of α2(I) collagen transcription and expression of galectin-1. (*A*) HKC cells were transfected with 0.25 μg COL1A2-LUC construct and 0.05 μg β-galactosidase construct together with either 0.25 μg of Galectin-1/pcDNA3.1 or empty pcDNA3.1. Relative ratios of TGF-β1–induced luciferase activity were corrected for transfection efficiency by dividing by β-galactosidase activity. Results are shown as mean ± S.D. of triplicate samples from a representative experiment. Similar results were obtained in three independent studies. A part of whole cell lysate (WCL) was resolved by SDS-PAGE and immunoblotted for galectin-1 to check the expression level. (*B*) Cells were transfected with 3μg of galectin-1/pcDNA3.1 or control construct. The cells were fed with regular media (10% serum) five hours after transfection, then replaced again with 0.5% serum-containing media next day. Twenty-four hours later, media was changed to 1.5 ml of new 0.5% serum-containing media with or without TGF-β1 for another 72 hours of culture. Levels of type I collagen in supernatants (40 μl) were examined by western blot analyses. The same membrane was re-blotted with amido black to confirm that same amount of proteins are containg in each samples. WCL was resolved and blotted with anti-galectin-1 antibody to detect levels of over-expressed galectin-1. (*C*) Chemically synthesized, double-stranded galectin-1 siRNA (final concentration; 10 μM) or control siRNA were transfected into HKC cells. Type I collagen secreted by TGF-β1 treatment was evaluated by Western blot analysis. The same membrane was re-blotted with amido black to confirm that amount of proteins. WCL was resolved and blotted to check the protein levels of galectin-1. The same membrane was re-blotted for anti-actin to check equivalency of protein loaded.

prepared as described below were analyzed to check the expression levels of transfected galectin-1. For immunoprecipitation, HKC cells (2 x 10^6 cells) were transfected with 1.5 μg of Flag-N-Smad3/pEXL along with 4.5 μg of Myc-C-Galectin-1/pcDNA3.1 or control pcDNA3.1. After TGF-β1-treatment, equal amounts of protein (0.5 mg) were immunoprecipitated overnight at 4°C with 1 μg of anti-Flag mAb, followed by absorption with 20 μl of protein G-sepharose (Zymed) for 90 min at 4°C as described previously [23], followed by SDS-PAGE and immunobloting with appropriate antibodies. For re-blotting, membranes were stripped with a stripping buffer (100 mM 2-mercaptoethanol; 2% SDS; 62.5 mM Tris-Hcl, pH 6.7) at 55°C for 30 min. Total cell lysates (10 μg) were resolved by SDS-PAGE and immunoblotted with anti-c-Myc mAb to check the expression levels of galectin-1.

DNA affinity precipitation assay (DAPA). HKC cells were transfected with either Galectin-1/pcDNA3.1 or control pcDNA3.1. After treating with TGF-β1 (1 ng/ml, 30 min), nuclear extracts were prepared and an equal amount of protein (50 μg) was incubated with 4 μg biotin-labeled double stranded probes (Sigma Genosys, Woodlands, TX, USA) containing the SBE sequence for three hours at 4°C as previously described [22]. Samples were then incubated with 20 μl of streptavidin beads in 50% slurry (Pierce, Rockford, IL, USA) for 90 min, and then washed four times with cold RIPA buffer containing protease and phosphatase inhibitors [24]. After eluting with SDS-loading buffer, the samples were subjected to immunoblotting analysis. Nuclear extracts (15 μg) were also analyzed to detect expression levels of the transfected protein in the nucleus.

Statistical analyses. The data are presented as mean ± S.D. Statistical differences between the experimental and control groups were determined by analysis of variance, and a value of $p < 0.05$ by subsequent Student's *t* test was considered significant.

Results

Galectin-1 reduces transcription and expression levels of type I collagen in TGF-β1-stimulated renal epithelial cells. First, we examined whether galectin-1 has a negative role in collagen production in renal cells. Over-expression of galectin-1 significantly inhibited TGF-β1-induced transcriptional activity of COL1A2-

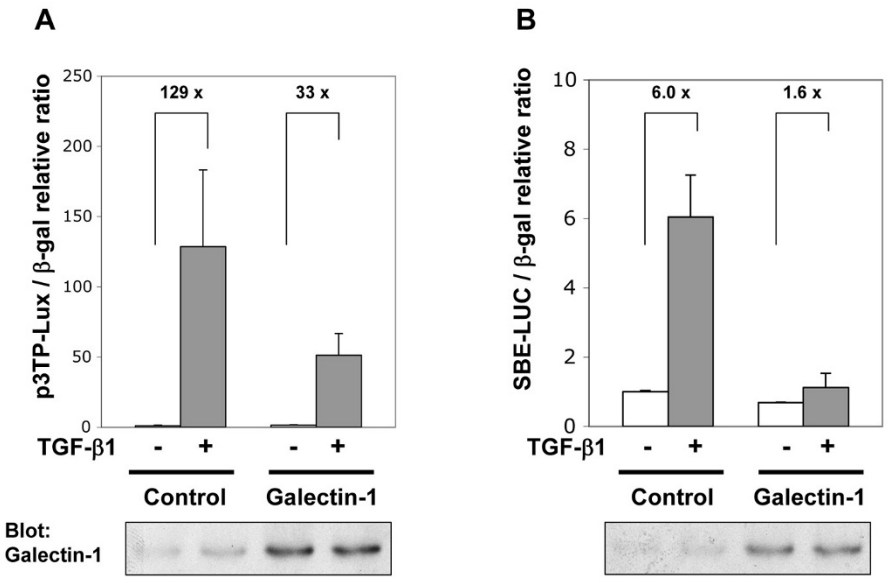

Figure 2. Decrease of TGF-β1-mediated and Smad3/4-mediated reporter activity by galectin-1. (*A*) Cells were transfected with 0.25 μg Galectin-1/pcDNA3.1 or control pcDNA3.1 along with 0.05 μg β-galactosidase construct and 0.25 μg p3TP-Lux construct. Luciferase activity was measured as mentioned above. We repeated the experiments three times and representative data are shown as mean ± S.D. from triplicate samples. (*B*) Transfection was performed under the same conditions above, except that the SBE-LUC construct was used instead of p3TP-Lux. In both experiments, 2 mg of WCL was resolved by SDS-PAGE and immunoblotted for galectin-1 to check the expression levels of galectin-1.

LUC in HKC cells (Fig. 1A). Secreted type I collagen was decreased in conditioned media from galectin-1-transfected cells compared with the control (Fig. 1B). The whole cell lysate was resolved and blotted with anti-galectin-1 antibodies, which showed no clear difference between those with- and without TGF-β1 stimulation (Fig. 1B, bottom panel). Next, we examined the role of endogenous galectin-1 using siRNA to galactin-1. Endogenous galectin-1 expression was decreased by approximately 50% (Fig. 1C, third panel). The secreted levels of type I collagen were doubled by suppression of galectin-1 expression by siRNA (Fig. 1C, upper panel). Equivalence of loaded protein was shown for supernatant and whole cell lysate (Fig. 1C, second and lower panel). These data suggested that galectin-1 suppresses TGF-β1-induced type I collagen production in HKC cells.

Galectin-1 decreases TGF-β1- and Smad3-mediated transcriptional activity. Next, we investigated whether galectin-1 affects TGF-β1/Smad signaling in renal HKC cells, employing two reporter constructs that are responsive to TGF-β1; p3TP-Lux that contains part of PAI-1 promoter and AP1 binding repeats [20], and SBE-LUC that has four repeats of SBE [21]. Over-expression of galectin-1 decreased fold induction of TGF-β1-mediated reporter activity in both reporters, by 60% for p3TP-Lux (Fig. 2A) and by 80% for SBE-LUC (Fig. 2B). Bottom panels of the histograms shows levels of galectin-1 expression with or without transfection. These results suggest that galectin-1 exerts an inhibitory effect on TGF-β1/Smad signaling in HKC cells.

Galectin-1 reduces direct binding of Smad3 to the SBE motif. The level of phosphorylated flag-tagged Smad3 was measured with or without over-expression of galectin-1. No effect of galectin-1 over-expression was observed in the levels of Smad3 phosphorylation either at the receptor-specific C-terminal residues (Fig. 3A, top panel) or total phosphoserine (Fig. 3A, second panel). There was no significant difference in association of Smad4 to Flag-Smad3 between cells expressing either galectin-1 or empty vector (Fig. 3A, third panel). The same membrane was re-blotted with anti-Smad1/2/3 mAb and equivalent amount of Smad3 immunoprecipitation was verified (Fig. 3A, forth panel). Galectin-1 over-expression significantly increased levels of Myc-tagged galectin-1 in cell lysates (Fig. 3A, lower panel). These results suggest that galectin-1 over-expression does not affect Smad3 phosphorylation or assembly with Smad4 in TGF-β1-treated renal epithelial cells.

We next performed DAPA to test the possibility that galectin-1 inhibits TGF-β1/Smad signals by interfering with Smad binding to the SBE sequence. Galectin-1 over-expression reduced Smad3 binding to the SBE motif compared to control (Fig. 3B, top panel). In contrast, Smad4 binding to SBE probes was not affected by galectin-1 expression (Fig. 3B, second panel). Galectin-1 had no effect on nuclear translocation of Smad3 and Smad4 (Fig. 3B, third and forth panel). Interestingly, TGF-β1 increased nuclear localization of galectin-1 compared to non-stimulated HKC cells (Fig. 3B, fifth panel). Immunoblot for Ets-2 and p300 shows that equal amounts of nuclear protein were loaded in each condition (Fig. 3B, two bottom panels). DAPA assay also showed decreased

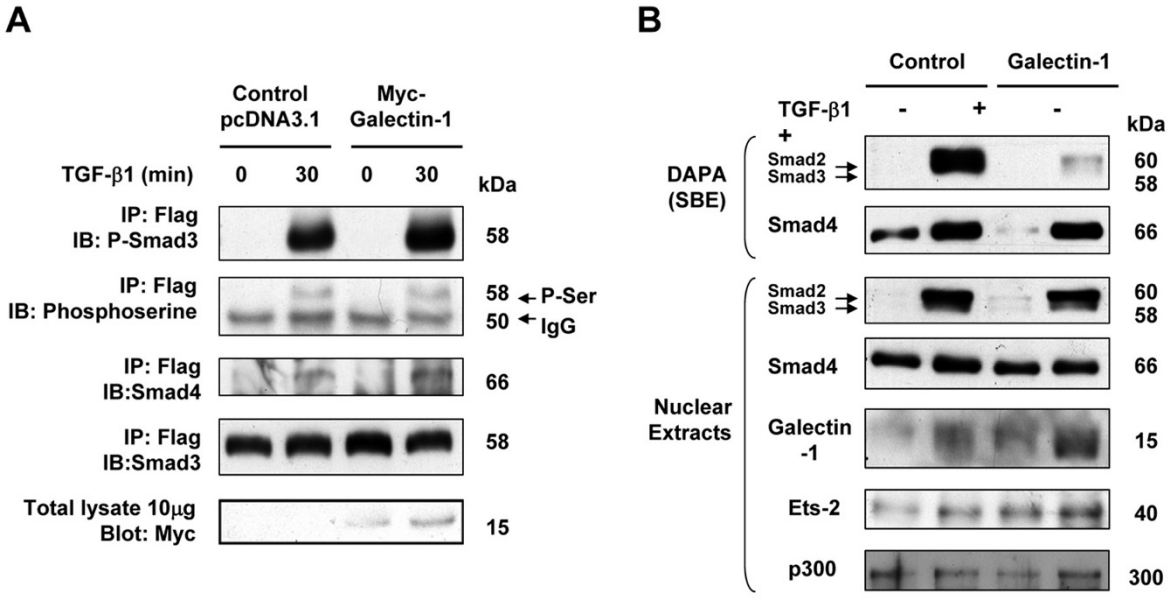

Figure 3. Decrease of direct Smad3-binding to SBE by galectin-1. (*A*) HKC cells were transfected with 4.5 μg Galectin-1/pcDNA3.1 or the empty pcDNA3.1 along with 1.5 μg Flag-N-Smad3 construct. After TGF-β1 stimulation, 0.5 mg of WCL was immunoprecipitated with 1 μg of anti-Flag mAb. Protein complexes were resolved and immunoblotted with anti-phosphoserine antibodies, anti-phospho-Smad3 antibodies, or anti-Smad4 mAb. The same membrane was stripped and re-blotted with anti-Smad3 mAb to check levels of immunoprecipitated Flag-Smad3 proteins. WCL (10 μg) was analyzed to check the levels of myc-tagged galectin-1 with anti-Myc mAb. (*B*) HKC cells were transfected with either Galectin-1/pcDNA3.1 or empty pcDNA3.1. After 48 h of transfection, they were stimulated with TGF-β1. Nuclear extracts (50 μg) were incubated with biotinylated SBE probe and precipitated with 20 μl of 50% streptavidin-agarose overnight. After washing and eluting, proteins bound to the probes were resolved and immunoblotted with anti-Smad1/2/3 and anti-Smad4 antibodies. Nuclear extracts (15 μg) were also analyzed by western blot using anti-Smad1/2/3, anti-Smad4, anti-Galectin-1, anti-Ets-2, or anti-p300 antibodies. Representative immunoblots are shown.

Smad2 detection in the complex (Fig. 3B, top panel). These data suggested that galectin-1 inhibits TGF-β1-stimulated collagen production by an effect on direct binding of Smad3 to a specific DNA motif, but not affecting Smad translocation into the nucleus.

SBE motif is critical for the inhibitory effect of Galectin-1. We and others previously reported that Smad binding to the SBE motif in the COL1A2 promoter is a critical event in TGF-β1-stimulated COL1A2 transcription [1]. Therefore, we speculated the inhibitory effect of galectin-1 on TGF-β1-stimulated collagen expression in HKC cells to result mainly through inhibition of Smad binding to the SBE motif by galectin-1. To confirm this hypothesis, we employed another promoter constructs, Gal4-Smad3 and pFR-LUC. The Gal4-Smad3 construct contains full-length Smad3 fused to Gal4 DNA binding domain. Once Smad3 is activated, the Gal4 DNA binding domain interacts with Gal4 binding site that is fused to the luciferase promoter in the pFR-LUC construct. The assay using these constructs is completely independent of direct binding of Smad3 to SBE motifs but indicates transactivation integrating Smad3, such as complex formation of Smad3 with transcriptional factors or other cell signal molecules to bind DNA outside SBE motif. In contrast to the SBE-LUC reporter, over-expression of galectin-1 increased Gal4-Smad3 reporter activity induced by TGF-β1 in HKC cells (Fig. 4A). The results suggest that over-expressed galectin-1 does not interfere, but rather facilitates Smad3 forming a transcriptionally active complex in the presence of TGF-β1. To test whether this Smad3 transcriptional complex acts on DNA motif(s) other than SBE region within -376 bp of the COL1A2 promoter region, we employed reporter assay using the 376 m-LUC in which SBE motif was mutated. Galectin-1 over-expression inhibited COL1A2 promoter activation by TGF-β1 as expected (Fig. 4B, left most pair). Mutation of the SBE motif also abolished the response to TGF-β1 to the same extent as galectin-1 over-expression (Fig. 4B, second pair from the right). Co-transfection of galectin-1 did not show any additional effect on 376 m-LUC activity in regard to fold induction (Fig. 4B, right-most pair). These results imply that increased Smad3 transcriptional complex activity by galectin-1 expression (as shown in Fig. 4A) did not exert any additional modulation on 376COL1A2 transcription other than that at the SBE motif. To observe the difference more clearly, we repeated the experiment in the presence of co-transfected Smad3 along with galectin-1 and

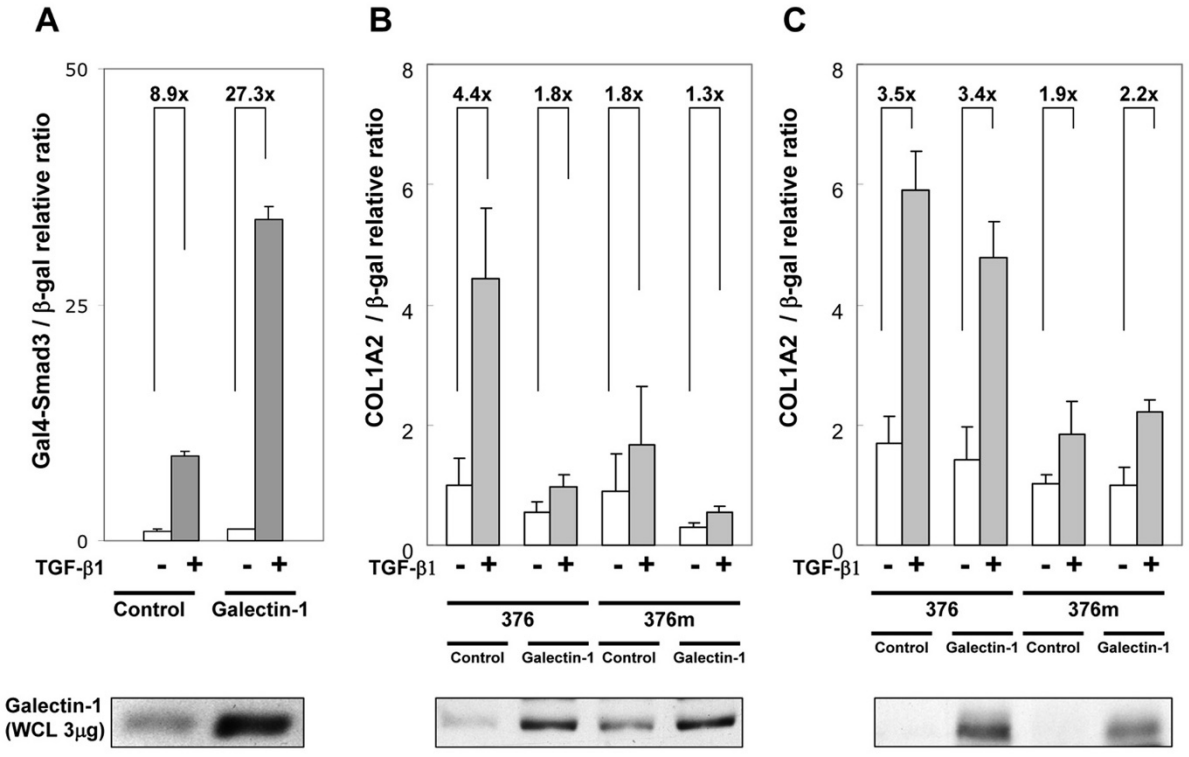

Figure 4. The SBE motif on COL1A2 promoter is critical for the inhibitory effect of Galectin-1. (*A*) HKC cells were transfected with 0.25 μg pFR-LUC, 0.25 μg Gal4-Smad3, and 0.05 μg β-galactosidase constructs. Cells were also cotransfected with an expression vector for galectin-1 (0.25 μg) or the empty vector. Reporter activity was measured and results are shown as mentioned in previous figures. (*B*) HKC cells were cotransfected with the indicated reporter construct, 0.25 μg of COL1A2-LUC, 0.05 μg of β-galactosidase construct along with 0.25 μg of Galectin-1/pcDNA3.1 or the empty vector. Reporter activity was measured and results are shown with same way as above. (*C*) HKC cells were cotransfected with the indicated reporter construct, 0.25 μg of COL1A2-LUC, 0.25 μg of Flag-tagged Smad3, 0.05 μg of β-galactosidase constructs along with 0.25 μg of Galectin-1/pcDNA3.1 or the empty vector. Reporter activity was measured and results are shown in the same way as above. Bottom panels of the histograms shows levels of galectin-1 expression with- or without transfection in WCL. Representative immunoblots are shown.

376 m-LUC promoter constructs (Fig. 4C). The inhibitory effect of galectin-1 over-expression is compensated by over-expression of Smad3, suggesting that abundant Smad3 overcomes inhibition of Smad3 binding to the SBE motif in COL1A2 promoter by galectin-1. These results suggest that inhibitory effect of galectin-1 is solely mediated through blockade of Smad3 binding to the SBE motif in TGF-β1-stimulated renal epithelial cells.

Discussion

Although Smad acts as a major signaling molecule in TGF-β1-stimulated renal cells, a variety of signaling molecules are reported to regulate TGF-β1/Smad signaling. Our major interest is to understand how Smads and other signaling pathways cross-talk in modulation of TGF-β1-mediated renal fibrogenesis. Peritoneal administration of recombinant galectin-1 ameliorates nephrotoxic serum nephritis in Wister Kyoto rats, suggesting that galectin-1 may play a protective role in renal fibrotic changes [13]. It has been suggested that ERK MAP kinase is one of the mediators for intracellular signaling for galectin-1. ERK2 [25] and transcriptional factors, NFAT and AP-1 [26] are activated by galectin-1 in T cell. Proliferation of hepatic stellate cells is mediated by galectin-1 through ERK1/2 [27]. These studies suggested that galectin-1 may enhance ERK MAP kinase pathway in renal cells, and possibly plays an important role in TGF-β1/Smad in renal fibrogenesis.

Our major finding in the study is that galectin-1 blocks Smad3-complex binding to the SBE motif, likely by sequestering Smad3 from transcriptional complex within the collagen promoter. DAPA shows decreasing levels of Smad2 binding to SBE motif as well as Smad3. The SBE motif (GTCTAGAC motif) is specific for Smad3 and Smad4, but not for Smad2 that lacks DNA binding sequence [21]. It is likely that Smad2 found in the complex with SBE motif derived from those that associated with Smad3, and that a decrease in Smad2 amounts bound to SBE reflects decreased binding of Smad3 complexes containing

Smad2. GST-fused Smad4 directly binds to the SBE motif in the *in vitro* experiments [28], suggesting that association between Smad4 and SBE motif may be independent from complex formation of Smad3 and Smad4 in this system. The experiment using point-mutated COL1A2 reporter construct, 376 m-LUC, indicates that inhibitory effect of galectin-1 is mediated mainly through SBE sequence of COL1A2 promoter region. As previously reported, co-transfection of wild-type Smad3 with galectin-1 suggests that the motif is essential for TGF-β-induced COL1A2 production and for negative effect of galectin-1 in HKC cells [29].

Recently, several putative binding sites for Smad3 and Smad4 complex in entire galectin-1 gene were identified by computer assisted gene sequence analysis [30]. In the present study, we showed that galectin-1 modulates TGF-β1/Smad pathway via interfering Smad binding to the specific sequence of a target gene. In the nucleus, galectin-1 is known as a redundant factor involved in pre-mRNA splicing [31]. Therefore, it is possible that observed functional interaction between the Smad and galectin-1 could also involve physical interaction in the nucleus. On the other hand, galectin-1 also abundantly exists in the cytoplasm. Even though the complex is formed in the nucleus or cytoplasm, characterization of this potential physical interaction warrants further study.

Decreasing levels of galectin-1 in TGF-β1-treated HKC cells cause up-regulation of secreted type I collagen. Furthermore, TGF-β1-stimulation slightly increases the amount of galectin-1 localizing in the nucleus. These support a hypothesis that the inhibitory effect is not mainly mediated by extracellular, secreted galectin-1, but rather by that secreted intracellularly. Nuclear galectin-1 has a crucial role in pre-mRNA splicing [32]. Intracellular localization of galectin-1 is consistent with several interacting proteins, such as membrane-associated Ras [14]. However, altered distribution of intracellular galectin-1 by various stimulations is left for a future study because galectin-1 abundantly exists within the cytoplasm and the nucleus.

In conclusion, our results provide evidence for a novel mechanism of galectin-1 inhibiting type I collagen transcription in TGF-β1-stimulated renal tubular epithelial cells. The inhibitory function of galectin-1 is thought mainly to occur through the prevention of activated Smad3-complex binding the unique DNA sequence in COL1A2 promoter. Our study revealed a new function of galectin-1 as a regulator of TGF-β1/Smad signaling in renal fibrogenesis. The new information warrants this protein being considered as a potentially promising therapeutic target.

Acknowledgements. This work was supported in part by grant DK49362 from the National Institute of Diabetes (H. W. Schnaper). We thank Dr. H. W. Schnaper and the members of the Pediatric Nephrology laboratory at Northwestern University for helpful suggestions.

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