Interaction between GC Box Binding Factors and Smad Proteins Modulates Cell Lineage-specific α2(I) Collagen Gene Transcription*

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Type I collagen is produced predominantly in mesenchymal cells, but molecular mechanisms responsible for cell type-specific expression are virtually unknown. During fibrogenic process in the liver, activated hepatic stellate cells (HSC) are the main producers of type I collagen, whereas parenchymal hepatocytes produce little, if any, of this protein. We have previously reported that Sp1 and an interacting unknown factor(s) bind to the −313 to −255 sequence of the α2(I) collagen gene (COL1A2) and play essential roles for basal and TGF-β-stimulated transcription in skin fibroblasts and HSC. Recently, Smad3 has been shown to bind to this region, and its interaction with Sp1 has been implicated in TGF-β-elicted COL1A2 stimulation. The present study demonstrates predominant binding of Sp3 rather than Sp1 to this regulatory element in parenchymal hepatocytes. In these cells, this region did not exhibit strong enhancer activity or mediate the effect of TGF-β. Transfection of HSC with an Sp3 expression plasmid abolished the COL1A2 response to TGF-β, whereas overexpression of Sp1 in hepatocytes increased basal COL1A2 transcription and conferred TGF-β responsiveness. Functional and physical interactions between Sp1 and Smad3, but not between Sp3 and Smad3, were demonstrated using the bacterial GAL4 system and immunoprecipitation-Western blot analyses. These results indicate that cell lineage-specific interactions between GC box binding factors and Smad protein(s) may account, at least in part, for differential COL1A2 transcription and TGF-β responsiveness in HSC and parenchymal hepatocytes.

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1 The abbreviations used are: COL1A1 and COL1A2, genes coding for the α1 and α2 chains of type I collagen, respectively; CAT, chloramphenicol acetyltransferase; CEBP, CCAAT/enhancer-binding protein(s); CMV, cytomegalovirus; FBS, fetal bovine serum; HSC, hepatic stellate cells; TbRE, TGF-β-responsive element; TGF-β, transforming growth factor-β; HA, hemagglutinin.
same substrata for a strict comparison in the experiments shown in Fig. 2. COS-7 cells were obtained from the American Type Culture Collection and were cultured in Dulbecco’s modified Eagle’s medium with 10% FBS.

**Chimeric Constructs—**Plasmids containing different lengths of COL1A2 upstream sequence linked to either a bacterial chloramphenicol acetyltransferase (CAT) gene or a firefly luciferase gene have been previously described (8, 9, 19) and are schematically shown in Fig. 1. A promoterless CAT construct, pBLCAT3 (20), was used as a negative control. An SV40 early promoter region was cloned into pBLCAT3 vector (designated p5VCAT3) and used as a positive control. Expression plasmids used were pCMV-Sp1 (21) kindly provided by Dr. G. Elder, CMV-Sp3 (22) from Dr. J. Gorovitz, and pCMV-Smad3 (23) from Dr. R. Derynck. In these plasmids, either Sp1, Sp3, or Smad3 is expressed under the control of cytomegalovirus (CMV) promoter. An empty CMV-driven expression vector, pcDNA3 (Invitrogen Corp., Carlsbad, CA), was used as a negative control. Bacterial GAL4 fusion protein expression plasmids, pMSP1, pMDSNp1, and pMSP5, which encode the active form Sp1, transactivation domain-deleted Sp1, and active form Sp3, respectively, were generously provided by Dr. Y. Sowa, as well as a control pm plasmid and the pG5-luc reporter construct (24).

**Cell Transfection Assays—**Preparation of plasmid DNA for cell transfection was previously described (8). CFSC-2G cells were transfected using the calcium phosphate coprecipitation technique (8) followed by a 15% glycerol shock for 90 s. FuGENE 6 transfection reagent (Roche Diagnostics Corp., Indianapolis, IN) was used for transfection of primary culture of rat hepatocytes and HSC. In some experiments, transfected cells were placed in medium containing 0.1% FBS and treated with 2 ng/ml of TGF-β1 (Collaborative Biomedical Products, Bedford, MA). Cells were harvested 48 h after transfection and subjected to either CAT or luciferase assays. Enzyme activity of the COL1A2/CAT chimeric constructs was normalized against that of co-transfected pSVXp1, in which the SV40 early promoter region was cloned into a promoterless luciferase gene construct, pXP1 (25). Transcriptional activity of the COL1A2/luciferase chimeric constructs and pG5-luc reporter construct was normalized against that of co-transfected pRLCMV (Promega, Madison, WI), in which a Renilla luciferase gene was driven by a CMV promoter. To avoid competition between the CMV promoter region of the expression plasmids and that of pRLCMV vector, the latter DNA was added to the plasmid mixture at 1:1,000 molar ratio against the former. CAT assays and the dual luciferase assays were carried as previously reported (26) and according to the manufacturer’s protocol (Promega), respectively.

**Preparation of Nuclear Extracts and Gel Mobility Shift Assays—**Nuclear extracts were prepared from culture cells as previously reported (27). After incubating nuclear extracts with an end-labeled probe, gel mobility shift assays were carried out as previously described (27). Binding conditions, as well as the sequences of the Box 5A (−330 to −297) and Box 3A (−313 to −286) oligonucleotides used as either probes or unlabeled competitors, have been previously described (9). Identical amounts of nuclear proteins from each cell source were added to the binding reactions. For antibody interference assays, antibodies against CCAAT/enhancer-binding proteins (C/EBPα, C/EBPβ, and C/EBPγ), NF1, or Sp1-related factors (Sp1, Sp2, Sp3, and Sp4) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

**Immunoprecipitation and Western Blot Analysis—**Immunoprecipitation of nuclear proteins was performed as previously described (11) using antibodies against Sp1 and Sp3. To analyze interactions between Smad3 and Sp1/Sp3, COS-7 cells were transfected with a Myc-tagged Smad3 expression plasmid (23) together with an expression vector encoding either Sp1 or Sp3, in the presence or absence of HA-tagged constitutive active TGF-β type I receptor (ALK5TD) (23). Forty-eight hours later, whole cell lysates were subjected to immunoprecipitation with anti-Myc antibodies (Santa Cruz Biotechnology, Inc.), followed by immunoblotting with either anti-Sp1 or anti-Sp3 antibodies. Some cell lysates from the transfected cells were directly immunoblotted with anti-Myc, anti-Sp1, anti-Sp3, or anti-HA antibodies (Roche Diagnostic Co.) to confirm expression of Myc-tagged Smad3, Sp1, Sp3, and HA-tagged ALK5TD, respectively, in the cells.

**Statistical Analysis—**Values were expressed as mean ± S.D. Either Student’s t test or the Mann-Whitney U test was used to evaluate the statistical differences between groups: a p value of less than 0.05 was considered significant.

**RESULTS**

COL1A2 Transcription and Response to TGF-β in HSC and Parenchymal Hepatocytes—We first compared COL1A2 tran-
Key findings:

- Cultures of activated HSC and primary culture of rat hepatocytes showed significantly different transcriptional activity of different lengths of the COL1A2 upstream sequence linked to a CAT reporter gene after transfection into CFSC-2G cells and primary culture of rat hepatocytes.

- Activity of each construct was normalized against the pSVXP1 vector.

- The values are mean ± S.D. obtained from four independent tests and shown below.

- A representative CAT assay film is shown in the upper part of the figure. Each lane of spots corresponds to the histogram shown below. Activity of each construct was normalized against the co-transfected SV40 early promoter-driven luciferase construct, pSV-XP1. The values are mean ± S.D. obtained from five independent tests and expressed relative to the activity in untreated CFSC-2G cells transfected with the −378COL1A2-CAT construct. An SV40 early promoter-driven CAT plasmid, pSV-βCAT (P), was used as a positive control.

- The asterisk signifies that the values are significantly different between the groups.

- There were no significant differences in the CAT enzyme activity among the −378, −313, and −183COL1A2-CAT transfectants (Fig. 2A). Deletion of the −378 to −183 sequence did not affect COL1A2 transcription in hepatocytes: there were no significant differences in the CAT enzyme activity among the −378, −313, and −183COL1A2-CAT transfectants (Fig. 2A). This was apparently different from the results obtained with CFSC-2G cells showing that transcriptional activity of the −378 and −313COL1A2-CAT constructs was significantly higher than that of the −183COL1A2-CAT construct (Fig. 2A).

- Administration of 2 ng/ml of TGF-β into the culture medium resulted in a significant increase in transcriptional activity of the −378 and −313COL1A2-CAT constructs in CFSC-2G cells, but not in primary culture of hepatocytes (Fig. 2A). Consistent with these results, a parallel experiment of Northern blot hybridization did not show any detectable amount of COL1A2 mRNA in either untreated or TGF-β-treated hepatocytes (data not shown). Transcription of the −183COL1A2-CAT construct did not show TGF-β responsiveness when transfected into either CFSC-2G cells or primary culture of hepatocytes (Fig. 2A).

- Similar results were obtained when comparing COL1A2 transcription between early passed HSC, instead of an immortalized HSC clone CFSC-2G, and primary culture of hepatocytes (Fig. 2B).

- Characterization of Nuclear Factors Bound to the COL1A2 Upstream Sequence in HSC and Parenchymal Hepatocytes—Because the above functional assays revealed a major difference in the promoter activity and TGF-β responsiveness of the −378 to −183 segment between HSC and primary culture of hepatocytes, we next examined the binding of transcription factors present in the nuclei of these cells to the COL1A2 upstream sequence. Nuclear extracts prepared from primary culture of hepatocytes and CFSC-2G cells exhibited similar gel shift patterns when using the Box 5A oligonucleotide as a probe, although the intensities and relative ratios of the retarded bands were somewhat different from each other (Fig. 3A). By contrast, gel mobility shift assays using the Box 3A probe indicated that nuclear proteins prepared from primary culture of hepatocytes and CFSC-2G cells demonstrated slightly different migrating patterns. The faster migrating complexes obtained with both cell types showed different mobilities from each other (Fig. 3A).

- Antibody interference assays revealed that anti-C/EBPβ antibodies effectively diminished the intensity of the Box 5A-bound complexes, particularly the fast migrating bands, in both primary culture of hepatocytes and CFSC-2G cells (Fig. 3B), as previously reported with NIH3T3 fibroblasts (28). None of the anti-C/EBPα, anti-C/EBPγ, and anti-NF1 antibodies interfered with the complex obtained with either hepatocytes or CFSC-2G cells (Fig. 3B). When using the Box 3A oligonucleotide as a probe, anti-Sp1 antibodies completely diminished the slowly migrating complex obtained with CFSC-2G nuclear extracts (Fig. 3B, arrowhead). By contrast, anti-Sp3 antibodies showed a relatively modest effect on both slowly and faster migrating complexes. On the other hand, in the case of primary culture of hepatocytes, anti-Sp3 antibodies completely diminished the intensity of the fast migrating Box 3A-bound complex (Fig. 3B, arrowhead), whereas anti-Sp1 antibodies had a modest effect on both slowly and faster migrating complexes. Neither anti-Sp2 nor anti-Sp4 antibodies interfered with the complex formation with Box 3A probe (Fig. 3B).

- To analyze semi-quantitatively the amounts of Sp1 and Sp3 proteins present in HSC and parenchymal hepatocytes, nuclear proteins prepared from both cell types were subjected to Western blot analyses using anti-Sp1 or anti-Sp3 antibodies. The results indicated that a larger amount of Sp1 protein was detected in nuclear extracts prepared from freshly isolated HSC than in those from hepatocytes (Fig. 3C). Conversely, more Sp3 protein was present in nuclei from parenchymal hepatocytes than those from HSC (Fig. 3C). Likewise, nuclear extracts prepared from CFSC-2G cells contained a larger amount of Sp1 and less Sp3 protein as compared with hepatocyte nuclei (data not shown).

Effects of Overexpression of Sp1 or Sp3 on COL1A2 Transcription in Activated HSC and Parenchymal Hepatocytes—The gel mobility shift assays and Western blot analyses of nuclear proteins indicated a difference in the relative amounts of Box 3A-bound Sp1 and Sp3 between CFSC-2G cells and primary culture of hepatocytes. Thus, we next examined the
antibodies. The stellate cells (HSC) were immunoblotted with anti-Sp1 or anti-Sp3 and nuclear proteins prepared from freshly isolated hepatocytes (HX) respectively affected by the addition of anti-Sp1 or anti-Sp3 antibodies. C, to the amount present in hepatocytes (in the case of Sp1) or relative to analyses of five independent tests, and the values are expressed relative to

FIG. 3. Gel mobility shift assays and Western blot analyses of the Box 5A- and Box 3A-bound nuclear factors. A, nuclear extracts prepared from primary culture of hepatocytes (HX) and CFSC-2G cells (2G) were incubated with Box 5A or Box 3A probes in the absence (−) or presence (+) of 100-fold molar excess of unlabeled homologous oligonucleotide competitor (comp.). Arrowheads indicate the specific bands, whereas NS shows nonspecific binding that was not affected by addition of molar excess of unlabeled competitors. B, nuclear proteins prepared from primary culture of hepatocytes and CFSC-2G cells were incubated without (−) or with antibodies (Ab) against CCAAT/enhancer-binding proteins (C/EBPα, C/EBPβ, and C/EBPγ) or NF1 prior to the binding reaction with the end-labeled Box 5A oligonucleotide (upper panel), or with antibodies against Sp1, Sp2, Sp3, or Sp4 before adding Box 3A probe (lower panel). The arrowheads indicate the complexes most effectively affected by the addition of anti-Sp1 or anti-Sp3 antibodies. C, nuclear proteins prepared from freshly isolated hepatocytes (HX) and stellate cells (HSC) were immunoblotted with anti-Sp1 or anti-Sp3 antibodies. The histograms summarize the results of densitometric analyses of five independent tests, and the values are expressed relative to the amount present in hepatocytes (in the case of Sp1) or relative to that in stellate cells (in the case of Sp3).

FIG. 4. Effects of overexpression of Sp1 or Sp3 protein on COL1A2 transcription. CFSC-2G cells and primary culture of hepatocytes were transfected with the −378COL1A2-LUC construct together with a control empty vector or expression plasmids encoding either Sp1 or Sp3, then untreated or treated with TGF-β. The values are mean ± S.D. obtained from five independent tests and expressed relative to the activity in untreated CFSC-2G cells co-transfected with the control expression vector. The asterisk signifies that the values are significantly different between the groups, NS, not significant.

The effects of overexpression of Sp1 or Sp3 on COL1A2 transcription in both cell types. Transfection of CFSC-2G cells with an Sp1 expression plasmid significantly increased basal transcription of the −378COL1A2-LUC construct (Fig. 4). TGF-β treatment further increased COL1A2 transcription in Sp1-transfected cells. Interestingly, overexpression of Sp1 in primary culture of hepatocytes not only increased basal COL1A2 transcription but also conferred TGF-β responsiveness to the cells (Fig. 4).

In contrast to these stimulatory effects of Sp1 overexpression, transfection of CFSC-2G cells with an Sp3 expression plasmid did not affect the basal transcription levels (Fig. 4). More importantly, overexpression of Sp3 in CFSC-2G cells completely abolished TGF-β-elicited COL1A2 stimulation (Fig. 4).

Functional Interaction between Sp1 and Smad3 in Stimulating COL1A2 Transcription—We next examined the effects of co-transfecting either Sp1 or Sp3 expression vector together with an expression plasmid encoding Smad3, an intracellular mediator of TGF-β signal transduction. Overexpression of Smad3 in CFSC-2G cells significantly increased transcription of the −378COL1A2-LUC construct (Fig. 5A). Whereas co-transfection with an Sp1 expression plasmid resulted in a further increase in −378COL1A2-LUC transcription, overexpression of Sp3 did not affect Smad3-stimulated COL1A2 transcription (Fig. 5A).

Because there are at least two more Sp1 binding sites downstream of the TbRE (Fig. 1; Refs. 29, 30), we also performed co-transfection experiments using the −378COL1A2-LUC construct containing an internal deletion of the −183 to −108 segment (−378COL1A2Δ108-LUC in Fig. 1). Basal transcriptional activity of this internally deleted construct was approximately one-fourth of that of the parental −378COL1A2-LUC construct (Fig. 5A), indicating that the two Sp1 binding sites present in the −183 to −108 segment are also important for basal COL1A2 transcription. However, co-transfection of Smad3 and Sp1 expression plasmids resulted in the same level (−3-fold) of transcriptional activation with both −378COL1A2-LUC and −378COL1A2Δ108-LUC constructs (Fig. 5A). It is therefore suggested that Smad3 might interact with the TbRE-bound Sp1, rather than with the downstream Sp1, to mediate TGF-β-elicited COL1A2 stimulation.
Functional interaction between Sp1 and Smad3, but not between Sp3 and Smad3, was further confirmed using the bacterial GAL4 system. Transfection of CFSC-2G cells with an expression plasmid encoding GAL4 DNA binding domain-fused Sp1 protein (pMSp1) increased transcription of the pG5-luc reporter in cis-acting Sp1 binding sites. In contrast, transfection with an expression plasmid encoding GAL4 DNA binding domain-fused Sp3 protein (pMSp3) did not increase transcription in cis-acting Sp3 binding sites. These results were also confirmed by co-transfection with Smad3 expression plasmids. Co-transfection with a Smad3 expression plasmid stimulated transcription of the pG5-luc reporter construct containing cis-acting Sp1 binding sites, but not Sp3 binding sites. These results suggest that Sp1 and Smad3 interact in cis-acting Sp1 binding sites, but not Sp3 binding sites.

**Physical Interaction between Sp1 and Smad3**—In the last set of experiments, we examined physical interactions between Sp1 and Smad3 and between Sp3 and Smad3 using immunoprecipitation followed by Western blot analysis. We first attempted to examine interactions between endogenous GC box binding factors and Smad3 using CFSC-2G cells, but failed to detect any co-immunoprecipitated proteins (data not shown). It was not clear whether this was because of a lack of interaction between Sp1/Sp3 and Smad3 or because of relatively small amounts of these proteins present in CFSC-2G cells. We therefore transfected COS-7 cells with a Myc-tagged Smad3 expression plasmid together with either Sp1 or Sp3 expression vector, in the presence or absence of HA-tagged constitutive active TGF-β type I receptor (ALK5TD). Direct immunoblotting of whole cell lysates with anti-Myc, anti-Sp1, or anti-Sp3 antibodies confirmed expression of Myc-tagged Smad3, Sp1, and Sp3, respectively, in transfected COS-7 cells (Fig. 6). In the absence of ALK5TD, anti-Sp1 antibodies hardly detected immunocomplexes, which had been first precipitated with anti-Myc antibodies recognizing Myc-tagged Smad3. By contrast, overexpression of ALK5TD markedly enhanced the physical interaction between Sp1 and Smad3 (Fig. 6), indicating that the interaction is TGF-β-dependent. On the other hand, immunoblotting using anti-Sp3 antibodies failed to detect Smad3-bound Sp3, either in the presence or absence of ALK5TD (Fig. 6).

**DISCUSSION**

In this study, we demonstrated that different molecular mechanisms control COLIA2 transcription in activated HSC and parenchymal hepatocytes. Our experimental data indicated the differential roles of Sp1 and Sp3 in COLIA2 regulation. They also suggested that, in parenchymal hepatocytes, predominant binding of Sp3 to the Box 3A sequence and a lack of interaction with Smad3 may account, at least in part, for relatively low levels of COLIA2 transcription and loss of TGF-β responsiveness. It should be noted that, although we confirmed more than 95% purity of hepatocytes, there still remained a small number of HSC contaminated in the culture (31), which may respond to TGF-β. Despite possible underestimate of the results because of this contamination of HSC, cell transfection assays clearly indicated that the COLIA2 promoter containing the TbRE did not show TGF-β responsiveness when transfected into primary culture of hepatocytes. Sp1 and Sp3 are closely related proteins with very similar structural features (32). They bind to the common GC-rich sequence named the GC box with the same specificity and affinity and regulate gene transcription (33). However, Sp3 often acts as a transcriptional repressor by competitively binding to the Sp1-bound GC box sequences (33) and/or by expressing internally initiated Sp3 proteins functioning as potent inhibitors of Sp1/Sp3-mediated transcription (34). It is now recognized that Sp3 can either activate or repress transcription of target genes depending on the cell type, the context of DNA binding sites, and the interactions with other nuclear factors (35).
It has been previously reported that both Sp1 and Sp3 stimulate COL1A2 transcription when transfected into Drosophila Schneider cells lacking endogenous Sp transcription factors (36). By contrast, it has been shown by others that co-transfection of an Sp3 expression plasmid inhibits Sp1-stimulated COL1A1 transcription in the same insect cells (37). However, neither of the studies examined the effects of Sp1 or Sp3 on TGF-β-elicited COL1A2 stimulation. The present study clearly demonstrated that, although Sp3 functioned as a weak trans-activator of transcription in the bacterial GAL4 system, it did not increase basal levels of COL1A2 transcription when transfected into CFSC-2G cells. More importantly, overexpression of Sp3 abolished TGF-β responsiveness in CFSC-2G cells. Different behaviors of Sp3 in regulating COL1A2 transcription in Drosophila Schneider cells and mammalian CFSC-2G cells might be attributed to the presence of interacting factor(s) in the latter cells (35). On the other hand, overexpression of Sp1 in primary culture of hepatocytes conferred TGF-β responsiveness with regard to COL1A2 transcription.

A family of proteins termed Smad have been identified (38) and found to play important roles in the intracellular signal transduction pathways of the TGF-β superfamily members (22). Some of them, Smad3 and Smad4, have been shown to bind to the so-called CAGACA sequence present in the promotors of several TGF-β-inducible genes including plasminogen activator inhibitor-1 (39), junB (40), p21WAF1/CIP1 (41), and Smad7 (42). Box B of the COL1A2 promoter also contains a CAGACA sequence (−265 to −260) (39, 40), and it has been shown that Smad3 binds to this sequence in vitro and stimulates COL1A2 transcription (12, 13).

We have recently revealed that an interaction between Sp1 and Smad3 is critical in mediating the stimulatory effect of TGF-β on COL1A2 transcription in NIH 3T3 fibroblasts (14). We demonstrated a functional interaction between Sp1 and Smad3/Smad4 using co-transfection experiments. In addition, it was found that, despite the presence of CAGACA sequence in Smad3/Smad4 using co-transfection experiments. It also revealed that similar interactions were not observed between Sp1 and Smad3.

A recent study has revealed that the glutamine-rich transactivation domain of Sp1 and the MH1 domain of Smad3 mediate the interaction between these two proteins bound to the p21WAF1/CIP1 gene promoter, which is stimulated by TGF-β (43). Consistent with these results, the present study showed that co-transfection of an expression plasmid encoding transactivation domain-deleted Sp1 together with a Smad3 expression vector did not increase transcription in the bacterial GAL4 system. It has been previously reported that the glutamine-rich transactivation domain of Sp1 cannot be replaced by the homologous sequence of Sp3 (33). Taken together, it is conceivable that a lack of interaction between Sp3 and Smad3 may account, at least in part, for the loss of TGF-β responsiveness of COL1A2 transcription in parenchymal hepatocytes.

It has been shown that Smad2, another TGF-β responsive Smad, is also capable of interacting with Sp1 (43, 44). Our recent study revealed that Smad2 did not bind to the T-box of the COL1A2 promoter (14). Nor did transfection of NIH 3T3 cells with a Smad2 expression plasmid stimulate basal COL1A2 transcription (14). On the other hand, under a certain experimental condition, overexpression of Smad2 in primary culture of skin fibroblasts markedly increased TGF-β responsiveness of COL1A2 transcription without affecting basal transcription level (45). Functional roles of Smad2 in TGF-β-stimulated COL1A2 transcription have not been fully understood, and experiments are in progress to clarify possible contribution of Smad2 to cell lineage-specific COL1A2 transcription.

In conclusion, the present study is the first to demonstrate, at the molecular level, differences in cell lineage-specific regulation of COL1A2 transcription in activated HSC and parenchymal hepatocytes. It illustrates that two members of the GC box binding transcription factor family participate in regulation of COL1A2 transcription through differential interaction with Smad3. These results lead to not only better understanding of regulatory mechanisms responsible for cell type-specific gene expression but also the development of novel therapeutic means for fibrotic diseases in various organs by suppressing pathologically activated collagen gene transcription.

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