Transcriptional Activation of Transforming Growth Factor β1 and Its Receptors by the Kruppel-like Factor Zf9/Core Promoter-binding Protein and Sp1

POTENTIAL MECHANISMS FOR AUTOCRINE FIBROGENESIS IN RESPONSE TO INJURY*

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We have explored the regulation of transforming growth factor β (TGF-β) activity in tissue repair by examining the interactions of Zf9/core promoter-binding protein, a Kruppel-like zinc finger transcription factor induced early in hepatic stellate cell (HSC) activation, with promoters for TGF-β1 and TGF-β2 receptors, types I and II. Nuclear extracts from culture-activated HSCs bound avidly by electrophoretic mobility shift assay to two tandem GC boxes within the TGF-β1 promoter but minimally to a single GC box; these results correlated with transactivation by Zf9 of TGF-β1 promoter-reporters. Zf9 transactivated the full-length TGF-β1 promoter in either primary HSCs, HSC-T6 cells (an SV40-immortalized rat HSC line), Hep G2 cells, or Drosophila Schneider (S2) cells. Recombinant Zf9-GST also bound to GC box sequences within the promoters for the types I and II TGF-β receptors. Both type I and type II TGF-β receptor promoters were also transactivated by Zf9 in mammalian cells but not in S2 cells. In contrast, Sp1 significantly transactivated both receptor promoters in S2 cells. These results suggest that (a) Zf9/core promoter-binding protein may enhance TGF-β activity through transactivation of both the TGF-β1 gene and its key signaling receptors, and (b) transactivation potential of Zf9 and Sp1 toward promoters for TGF-β1 and its receptors are not identical and depend on the cellular context.

Transforming growth factor β1 (TGF-β1) is a multifunctional cytokine that plays a key role in the response to injury in a wide variety of tissues (1). Increased TGF-β1 activity underlies the wound-healing response in kidney (2), lung (2), and vascular tissue (3, 4), among others.

The liver offers a particularly attractive paradigm in which to explore the role of TGF-β1 in tissue fibrosis. Cell-specific markers have been well characterized for in situ analysis, and methods are established for obtaining pure isolates of both parenchymal and nonparenchymal cell types. Such studies conclusively establish hepatic stellate cells as the primary source of extracellular matrix in liver fibrosis and a major cellular target of TGF-β1 (5, 6). Downstream effects of TGF-β1 in stellate cells include stimulation of matrix gene expression (7), up-regulation of plasminogen activator inhibitor (8) and receptors for platelet-derived growth factor (9), and suppression of collagenase synthesis (10). A role for TGF-β1 in vivo is further emphasized by the development of severe hepatic fibrosis in transgenic mice overexpressing the cytokine in liver cells (11).

Both in situ studies (12, 13) and isolated cell studies (14, 15) have also established hepatic stellate cells (previously called lipocytes, Ito, or fat-storing cells) as a major source of TGF-β1. Marked up-regulation of TGF-β1 gene expression and activation of latent cytokine are consistent features of both human (16) and experimental (14, 17, 18) liver injury.

Increased activity of TGF-β1 during hepatic stellate cell activation reflects not only up-regulation and activation of the cytokine but also enhanced responsiveness to TGF-β1 due to increased TGF-β1 receptor expression. We previously documented induction of TGF-β receptors types I, II, and III during stellate cell activation in vivo and in culture (19). Receptor induction in culture was paralleled by increased TGF-β1 binding and enhanced fibronectin gene expression in response to TGF-β1.

Increased fibrogenesis is only one of many phenotypic responses characterizing hepatic stellate cell activation. To define more broadly the nature of gene induction during this event, we used subtraction cloning to identify mRNAs up-regulated rapidly after acute injury (20). Interestingly, this approach identified the type II TGF-β receptor among the induced mRNAs. Another mRNA encoded a novel zinc finger partial cDNA; full-length cloning identified a Kruppel-like factor that we have termed Zf9 (21), which was also cloned from placenta, where it was termed CPBP (22). Zf9 contains three carboxyl-terminal C2H2 zinc fingers similar to other Kruppel-like factors and a serine-rich activation domain with homology in the most amino-terminal 47 amino acids to a new member of the Kruppel-like family, UKLF (23). Zf9 is expressed in all.

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The abbreviations used are: TGF-β, transforming growth factor β; HSC, hepatic stellate cell; EMSA, electrophoretic mobility shift assay; RI, type I receptor; RII, type II receptor; RII, type I receptor promoter; RIIP, type II receptor promoter; luc, luciferase; CPBP, core promoter-binding protein.
adult tissues and binds GC box promoter motifs, similar to binding by other Kruppel-like factors, including EKLF (24), BKLF (25), LKLF (26), and BTEB1 (27). Zf9 mRNA is rapidly induced during stellate cell activation in vivo and in culture, in association with nuclear localization of the protein. Most importantly, the factor transactivates a minimal collagen α(I) promoter, which contains several GC-rich Sp1 binding sites.

Our cloning and characterization of the promoters for both TGF-β1 (28, 29) and the types I (30) and II (31) TGF-β receptors has positioned us to explore the potential regulation of these genes. In particular, all three promoters contain GC-rich Sp1 binding sites. Such consensus sequences raise the possibility that one or more of these genes might be regulated by Zf9. In this study, we indeed demonstrate that Zf9 transactivates all three genes following transient transfection in mammalian cells, and we compare these interactions with those of Sp1, the prototype GC box-binding protein.

**EXPERIMENTAL PROCEDURES**

**Cells and Cell Lines**—Primary hepatic stellate cells were isolated from normal Sprague-Dawley rats by in situ perfusion and density gradient centrifugation as described previously (32). Cells were maintained on uncoated plastic in medium 199 with 20% serum (1:1, horse: calf). The immortalized rat hepatic stellate cells line HSC-T6 was generated by transfection of primary cells at day 15 after plating using LipofectAMINE containing an expression plasmid encoding the SV40 large T antigen. The resulting clone HSC-T6 retains all features of activated stellate cells, including expression of desmin, α smooth muscle actin, and glial acidic fibrillary protein, and it can esterify retinol into retinyl esters. The cells maintain a stable phenotype for at least 40 passages (more detailed characterization of this line will be reported elsewhere). These cells, as well as HepG2 cells, were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum (HyClone). Drosophila Schneider (S2) cells were maintained in S2 medium (Life Technologies, Inc.) with 10% fetal bovine serum.

**Cellular Fibronectin mRNA Quantitation—Expression of the EIIA isoform of cellular fibronectin mRNA by HSC-T6 cells was analyzed by RNase protection, exactly as described previously (19) in the absence or presence of recombinant TGF-β1 (R & D Laboratories). Fibronectin mRNA expression was normalized to that of S14 protein mRNA (33).

**Plasmids**—Expression plasmids containing rat Zf9 (pCIneo-Zf9) and human Sp1 (pCneo-Sp1) for mammalian and Drosophila cells (pFAC-Zf9; pFAC-Sp1) have been described previously (rat Zf9 GenBank TM accession number U73755) (21). Construction of the human TGF-β1 promoter-luciferase chimeric gene (phTG5luc) (30) and human TGF-β type II receptor promoter-luciferase chimeric genes (34) has been described previously. The human TGF-β type I promoter fragment (−867 to −67) was originally provided by D. R. H. Goldstein (35). Deletion constructs containing regions of the TGF-β1 promoter driving luciferase expression utilized previously described regions in chloramphenicol acetyltransferase (CAT) expression vectors (28, 36). These included phTG6, −323 to +11; phTG7, −175 to +11; and phTG7−4, −60 to +11. These promoter regions were subcloned into a luciferase reporter from their corresponding CAT vectors by digestion with HindIII, fill-in to create blunt ends, digestion with KpnI, and then ligation into the SmalKpnI sites of pGL2-basic (Promega).

The TGF-β receptor I reporter constructs pTβRIP-luc (−867 to −169) and pTβRIP-luc (−867 to −228) were constructed by digesting the RI promoter with either NcoI and KpnI or Smal and KpnI, respectively, and subcloning into KpnIBglII site of the pGL2-basic after BgII site was filled. Type I receptor constructs pTβIP-luc (−495 to −65), pTβRIP-luc (−425 to −65), and pTβRIP-luc (−283 to −65) were generated by digesting the RI promoter with BgIII, HindII, and PvuI, respectively, filling in the ends, digesting these fragments again with...
**Fig. 3. Transactivation of TGF-β1 promoter by Zf9 in different cell types.** A, relative transactivation in mammalian and nonmammalian (*Drosophila*) cells. Equal numbers (5 × 10⁵) of either primary rat stellate cells, Hep G2 cells, or *Drosophila* Schneider cells were cotransfected as described under "Experimental Procedures" with 1 μg of Zf9 (pCIneo-Zf9 for stellate and HepG2 cells and pPAC-Zf9 for *Drosophila* Schneider cells) and 1 μg of full-length TGF-β1 promoter reporter (pHTG5) and then assayed for normalized luciferase activity. Data are expressed as mean fold increase of triplicate determinations (± S.D.), compared with cells cotransfected with empty expression vector (pCIneo or pPAC) and TGF-β1 promoter reporter. B, concentration-dependent transactivation by Zf9 in *Drosophila* Schneider cells. 5 × 10⁵ cells were transfected with increasing amounts of pPAC-Zf9 and the TGF-β1 promoter reporter (pHTG5) and then harvested for luciferase activity assay (total DNA in the transfectant mix was maintained constant using empty pPAC vector DNA). Concentration-dependent transactivation is apparent.

XhoI, and subcloning into Smal/XhoI site of the pGL2-basic.

**Production of Recombinant GST-Fusion Proteins—** Recombinant full-length rat Zf9 fusion protein was expressed in JM109 bacteria and purified by affinity chromatography as described previously (21). Recombinant human Sp1 was purchased from Promega (catalog no. E3391).

**Electrophoretic Mobility Shift Assay (EMSA)—** Nuclear extracts from primary and immortalized rat stellate cells were prepared as reported previously (21). Purified Sp1 (Promega)-DNA or Zf9-GST fusion protein-DNA complexes were formed by incubating at room temperature for 20 min with 10,000 cpm of ³²P-labeled probe, 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5 mM MgCl₂, 1 mM dithiothreitol, 1 mM EDTA, 5% glycerol, and 300 μg/ml bovine serum albumin in 20 μl of binding mixture. Competition reactions were performed by adding an unlabeled double-stranded oligonucleotide to the reaction mixture. Reactions were electrophoresed on a 6% NOVEX precasted nondenaturing polyacrylamide gel at 100 V for 1 h in 0.025 M Tris borate-EDTA buffer. Gels were vacuum-dried and analyzed by autoradiography.

**Transient Transfection and Luciferase Assays—** For transient expression assays, cells were transfected with the indicated plasmids using Lipofectin (for HepG2 cells) or LipofectAMINE (for stellate cells) (Life Technologies, Inc.). Following transfection with either reagent, cells were incubated for 48 h. Luciferase activity was normalized to co-transfection assay, cells were transfected with the indicated plasmids using Lipofectin (for HepG2 cells) or LipofectAMINE (for stellate cells) (Life Technologies, Inc.). Following transfection with either reagent, cells were incubated for 48 h. Luciferase activity was normalized to co-transfection efficiency. *Drosophila* Schneider cells were transfected by the calcium phosphate coprecipitation method, using 10 μg of the appropriate reporter plasmid with 100 ng of either a control plasmid, pPAC; the Sp1 expression plasmid, pPAC-Sp1; or the Zf9 expression plasmid, pPAC-Zf9. Cells were harvested 48 h after addition of the DNA, and extracts were assayed for luciferase activity. All transfection were repeated at least three times.

**RESULTS**

**Hepatic Stellate Cells Respond to TGF-β1, and Their Nuclear Extracts Bind to the TGF-β1 Promoter—** We (19) and others (7, 15, 17) have previously documented that TGF-β1 increases matrix gene expression in primary stellate cells. This finding was confirmed in the immortalized HSC-T6 cells in order to establish the relevance of this line to studies of TGF-β regulation in hepatic fibrosis. As previously reported in activated primary stellate cells (19) a high level of basal cellular fibronectin mRNA was detected in HSC-T6 cells, which was increased an additional 42% in the presence of 1 ng/ml recombinant TGF-β1 (data not shown). We also examined the interaction of nuclear extracts from both primary and immortalized stellate cells with a GC-rich region located between −209 and −239 of the human TGF-β1 promoter (29). Nuclear extracts from both primary and HSC-T6 cells bound to a labeled oligonucleotide containing this sequence, and the interaction was abolished by competition with excess cold oligonucleotide (Fig. 1). Binding was more intense in the highly activated immortalized stellate cells than in the less activated primary cells, consistent with the observation that stellate cell activation is associated with increased TGF-β1 expression (17).

**Recombinant Zf9 Binds to GC-rich Regions in the TGF-β1 Promoter—** The TGF/β1 promoter contains three GC-rich regions, which were designated TGFβ1/1, TGFβ1/2, and TGFβ1/3 (Fig. 2A). Oligonucleotides TGFβ1/1 and TGFβ1/2 contain tandem Sp1 sites, whereas TGFβ1/3 contains only a single site. Oligonucleotides representing each of these motifs were used to assess interactions with recombinant Zf9-GST by EMSA. As shown in Fig. 2B, Zf9-GST bound in a specific manner to all three regions, but with much greater affinity to oligonucleotides TGFβ1/1 and TGFβ1/2 than TGFβ1/3. Addition of anti-Zf9 antiserum to nuclear extracts abolished protein-DNA interaction with TGFβ1/1 and TGFβ1/2 regions (not shown). This is similar to our previous result examining the interaction of Zf9-GST with an Sp1 consensus motif by EMSA (21).

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Zf9 Transactivates the TGF-β1 Promoter in Stellate Cells
Transient cotransfections were performed to determine whether Zf9 transactivated TGF-β₁ in stellate cells, as well as in other cellular contexts. As shown in Fig. 3, Zf9 transactivated the TGF-β₁ promoter in stellate cells and Hep G2 cells, although to a greater extent in Hep G2. Transactivation also was examined in Drosophila Schneider cells, which provide an important cellular context devoid of Sp1 and Zf9. Zf9 retained transactivating activity toward the TGF-β₁ promoter in Drosophila Schneider cells (Fig. 3A), which contrasts with its inability to transactivate collagen α(I) in this cell type (21). Transactivation by Zf9 in Drosophila Schneider cells was proportionate to the amount of transfected Zf9 expression plasmid (Fig. 3B).

TGF-β₁ promoter-luciferase constructs with progressive 5’ deletions were used to map the regions required for Zf9 responsiveness in stellate cells and Hep G2 (Fig. 4). Identical patterns of transactivation were observed between both cell types. Transactivation by Zf9 required at least the region from −175 to +11 in both cell types, whereas a reporter construct containing from −60 to +11 was unresponsive (Fig. 4). These functional data corresponded to the relative affinities of Zf9-GST for the three GC-rich regions (Fig. 2).

Transactivation of the TGF-β RI Gene by Zf9 Can Be Mediated through the Multiple GC Regions—We examined whether Zf9 might also contribute to up-regulation of the type I TGF-β receptor. Hep G2 cells were used in these and subsequent transfections because their transfection efficiency and relative transactivation were much higher than stellate cells, facilitating more reproducible functional mapping of the promoter. In part, this was due to a much higher basal transactivation of the reporter in stellate cells (not shown), similar to that seen for collagen α(I) (21). Transiently transfected Zf9 markedly transactivated a full-length TGF-β RI promoter-reporter in Hep G2. To identify the sequences responsible for regulation by Zf9, we tested a series of deletion constructs of the human TGF-β RI promoter fused to a luciferase reporter. Transfection of Zf9 cDNA induced the expression of the TGF-β RI-luciferase reporter genes containing 5’ sequences out to position −867 (pTgRIP-luc−867/−65) by 9-fold compared with transfection of the empty vector pCI-neo (Fig. 5). The induction was reduced when GC-rich sequences between −65 to −228 and sequences between −867 to −284 were deleted (Fig. 5). These regions contain multiple GC-rich sequences that may be target sequences for Zf9. Because transcription starts at −232, the results suggest that GC-rich sequences located at downstream of the transcription start site play an important role in the
TGF-β1 and TGF-β Receptor Transactivation by Zf9

Fig. 5. Functional mapping of the TGF-β receptor I promoter in response to Zf9. Left panel, structure of TGF-β1 promoter deletion constructs. Progressive deletants of the TGF-β RIP were cloned into the pGL2-basic luciferase expression construct as described under "Experimental Procedures." In addition, two constructs were tested that deleted the regions from −169 to −65 and from −228 to −65. Right panel, transactivation of the TGF-β RIP deletion constructs by Zf9 in Hep G2 cells. The expression constructs pCMVneo (1 μg) and pCMVneo-Zf9 (CMV-Zf9) (1 μg) were co-transfected with TGF-β1 promoter deletion constructs (described above) in Hep G2 cells. The relative luciferase activities for triplicate samples in two separate experiments are shown. Maximal transactivation occurred with the construct containing the entire region from −152 to −65. Deletion of the GC-rich region between −169 to −65 significantly reduced but did not eliminate transactivating capacity of Zf9. Progressive deletions reduced transactivation further, but some activity was retained with even a minimal promoter from −283 to −65.

Fig. 6. Interactions of recombinant Zf9 and Sp1 with the TGF-β receptor I promoter. Oligonucleotides incorporating the sequences between −360 and −349 and between −179 and −151 in the TGF-β RI promoter that contain GC-rich sequences were incubated with either affinity purified Zf9-GST or human Sp1 and analyzed by EMSA. Specific binding was present for both proteins, as shown by competition studies using wild type unlabeled excess competitor oligonucleotides. Sp1 also formed a specific complex with both sites. The effect of either Sp1 or Zf9 on transactivation of the TGF-β RI promoter was examined in Drosophila Schneider cells in order to determine whether Zf9 could activate in the absence of Sp1. The TGF-β RI-luciferase chimeric constructs were cotransfected with a Drosophila actin promoter-Sp1 or −Zf9 expression plasmid (Fig. 7). The activity of pTβRIIP-luc−867/−65 was induced 40-fold by Sp1, whereas the activities of constructs pTβRIIP-luc−867/−169 and pTβRIIP-luc−867/−228 were not induced. Moreover, constructs that deleted sequences between −283 and −867 were still responsive to Sp1, suggesting that the region between −65 and −169 is the target sequence for Sp1. In contrast to Sp1, Zf9 was unable to induce TGF-β RI gene expression in Drosophila Schneider cells (Fig. 7).

Transcriptional Activation of the TGF-β RI Promoter by Zf9—Zf9 transactivation of the type II TGF-β receptor was also examined (Fig. 8). Zf9 was a potent transcriptional activator of the TGF-β RI promoter-luciferase reporter in HepG2. As seen in Fig. 8, pTβRIIP-luc−1670/−36 construct was induced 7-fold by Zf9, and the construct pTβRIIP-luc−500/−36 was induced almost 10-fold. Transactivating activity of Zf9 was maintained with deletions to −219 to +36 (pTβRIIP-luc−219/+36) but dropped to the basal level when the deletion reached −70 (pTβRIIP-luc−70/+36). These results suggest that sequences between −219 and −70, including the multiple GC-rich sequences, are important for positively regulating TGF-β RI expression in the presence of high levels of the Zf9 protein.

EMSA was used to confirm the interactions between Zf9 or Sp1 and the TGF-β RI promoter (Fig. 9). To demonstrate that Zf9 and Sp1 can bind directly to the sequences between −219 and −70 in the TGF-β RI promoter, bacterially purified GST-Zf9 protein was used in EMSA using the three series of radio-labeled oligonucleotides and competitors, which represented sequences between −152 and −127, between −118 and −85, and between −39 and −10. Both GST-Zf9 and Sp1 bound to the −152/−127 and −118/−85 oligonucleotide probes with high affinity, whereas the −39/−10 oligonucleotide probe formed a
very low affinity complex with these proteins, consistent with the reduced transactivation of a reporter construct containing this region (Fig. 8).

Next, we examined the effect of either Sp1 or Zf9 on TGF-β RI promoter in Drosophila Schneider cells (Fig. 10). The activities of constructs of pTβRIIIP-luc−867/1, pTβRIIIP-luc−65 were induced 8- and 6-fold, respectively, by Sp1, whereas the activity of construct pTβRIIIP-luc−219/1 +36 was not induced. This finding suggests that one or more GC-rich sequences located between −219 and −70 in the TGF-β RI promoter are responsible for the Sp1-mediated transcription. Zf9 was unable to induce TGF-β RI gene expression in Drosophila Schneider cells, similar to its lack of effect on the type I receptor promoter in this cell type (Fig. 10).

**DISCUSSION**

Our results identify a potential role for the Kruppel-like factor Zf9/CPBP in the increases in TGF-β1 and its receptors which are characteristic of liver injury. A number of studies have identified TGF-β1 mRNA induction in liver injury (13, 14, 18) and during stellate cell activation in culture (17). The immediate-early induction of Zf9/CPBP prior to up-regulation of TGF-β1 and its receptors is consistent with the possible role of Zf9/CPBP in this response (20, 21).

Both nuclear extracts from activated stellate cells and recombinant Zf9-GST interact with GC-rich regions within the TGF-β1 promoter. The potential importance of GC-rich regions in this promoter has been emphasized in our earlier publication (28). Here, we have documented strong interaction of Zf9-GST with the tandem Sp1 binding sites between −228 to −65. This region contains GC-rich sequences, establishing it as essential for Sp1-mediated transactivation of the promoter (bottom left panel). In contrast, no specific transactivation of any of the constructs was observed for pPAC-Zf9 when compared with activity of the empty expression vector (bottom right panel).

**FIG. 7. Functional mapping of the TGF-β receptor I promoter in response to Sp1 and Zf9 in Drosophila Schneider cells.** Top panel, structure of TGF-β receptor I promoter deletion constructs. Progressive deletants of the TGF-β RIIP were cloned into the pGL2-basic luciferase expression construct as described in the legend to Fig. 5. Bottom panels, transactivation of the TGF-β receptor I promoter by Zf9 and Sp1 in Drosophila Schneider cells. The expression constructs pPAC (1 μg), pPAC-Sp1 or pPAC-Zf9 (1 μg) were co-transfected with TGF-β RIIP deletion constructs (described above) in Drosophila Schneider cells. The relative luciferase activity for triplicate samples in two separate experiments are shown. Marked transactivation by Sp1 was observed for the −867 to −65 promoter construct, which was completely lost with deletion of the regions from −228 to −65. This region contains GC-rich sequences, establishing it as essential for Sp1-mediated transactivation of the promoter (bottom left panel). In contrast, no specific transactivation of any of the constructs was observed for pPAC-Zf9 when compared with activity of the empty expression vector (bottom right panel).
and correlates with interaction between recombinant Zf9-GST and each of these regions. Similarly, transactivation of the TGF-β1 type II receptor promoter requires the presence of GC-rich regions from −152 to −127 and from −118 to −85. Zf9-GST did not bind to a third Sp1 site from −39 to −10, which correlated with a lack of transactivation of a reporter-luciferase construct containing only this region (Fig. 8). There was also no binding to GST alone.

In contrast to the TGF-β1 promoter, however, transactivation of TGF-β types I and II receptor genes by Zf9 occurred only in a mammalian cellular environment (Hep G2) and not in Drosophila. This finding underscores the unique requirements for each promoter in driving gene expression and raises the possibility that Sp1 is an essential component required for Zf9-mediated transactivation of the TGF-β types I and II receptors. This conclusion is based on several lines of evidence: 1) Sp1 is absent from Drosophila Schneider cells; 2) both this study and previous analyses by Ji et al. (37) and us (31) emphasize the importance of Sp1 binding sites for basal and stimulated expression of the type I receptor promoter; and 3) Zf9 and Sp1 can functionally synergize with one another in several promoter contexts (38). It also remains possible that Zf9 may interact with other members of the Kruppel-like family of transcriptional proteins, as has been demonstrated for EKLF and Sp1 (39).

Cooperation between Sp1 and Zf9 in the transcriptional activation of TGF-β1 during stellate cell activation is therefore possible. Not only is Zf9 induced (21), but there is also increased Sp1 protein and enhanced binding of stellate cell nuclear extracts during stellate cell activation (40). Whereas the cognate sequences recognized by Zf9 are similar to Sp1, the cellular factors required for transactivation by each protein are different, as evidenced by differing activities in Drosophila Schneider cells. In preliminary studies, we have confirmed direct interaction between these two proteins (data not shown). Interestingly, in this earlier study examining Sp1 binding in activated stellate cells (40), a significant amount of nuclear protein that bound to an Sp1 consensus sequence did not supershift with anti-Sp1 antibody. This suggests that other GC box-binding proteins may be present in activated stellate cells, including not only Zf9, but possibly BKLF (25), LKLF (26), UKLF (23), and BTEB1 (27), all of which are expressed in liver.

![Figure 8](http://www.jbc.org/)

**Fig. 8.** Transactivation of the TGF-β receptor II promoter by Zf9 in Hep G2. *Left panel,* structure of TGF-β receptor II promoter deletion constructs. Progressive deletants of the TGF-β RII P were cloned into the pGL2-basic luciferase expression construct as described previously (34). *Right panel,* transactivation of the TGF-β RII P promoter deletion constructs by Zf9 in Hep G2 cells. The expression constructs pCIneo (1 µg) or pCIneo-Zf9 (CMV-Zf9) (1 µg) were co-transfected with TGF-β RII P deletion constructs into Hep G2 cells. The relative luciferase activities for triplicate samples in two separate experiments are shown. Maximal transactivation was observed using a construct containing −500 to +36, which was completely lost by elimination of sequences between −219 to −70, a region that contains two GC-rich sequences, from −152 to −127 and from −118 to −85.

![Figure 9](http://www.jbc.org/)

**Fig. 9.** Interactions of recombinant Zf9 and Sp1 with the TGF-β receptor II promoter. Oligonucleotides incorporating the sequences between −152 and −127, between −118 and −85, and between −39 and −10 in the TGF-β RII P promoter that contain GC-rich sequences were incubated with either affinity purified Zf9-GST or human Sp1 and analyzed by EMSA. Specific binding to the two more 5′ regions is present for both proteins, as shown by competition studies using wild type unlabeled excess competitor oligonucleotides. Almost no binding was observed to the region between −39 and −10, corresponding to the lack of transactivation of the promoter construct containing only this region (Fig. 8). There was also no binding to GST alone.
These data provide a potentially important link in stellate cells between induction of a transcriptional activator, Zf9, and increased expression of TGF-β1, a key fibrogenic mediator. TGF-β1 induces a large number of matrix genes in stellate cells (17, 41–43), as in other tissues. The findings further suggest that Zf9 may enhance by the biologic effect of TGF-β1 (17, 41–43), as in other tissues. The findings further suggest that Zf9 may enhance by the biologic effect of TGF-β1, a key fibrogenic mediator.

In summary, our data suggest that Zf9/CPBP, a recently described Kruppel-like factor that is rapidly induced following liver injury (21), can transcriptionally activate TGF-β1, and its receptors. These findings suggest that Zf9/CPBP may contribute to TGF-β1-mediated autocrine fibrogenesis in stellate cells during liver injury.

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REFERENCES
1. Border, W. A., and Noble, N. A. (1994) N. Engl. J. Med. 331, 1286–1292
2. Border, W. A. (1994) Curr. Opin. Nephrol. Hypertens. 3, 54–58
3. Saltis, J., Agrotis, A., and Bobik, A. (1996) Clin. Exp. Pharmacol. Physiol. 23, 193–200
4. Schwartz, S. M., Reidy, M. A., and O’Brien, E. R. (1995) Thromb. Haemostasis 74, 541–551
5. Friedman, S. L. (1996) Prog. Liver Dis. 14, 101–130
6. Friedman, S. L. (1993) N. Engl. J. Med. 329, 1828–1835
7. Weiner, F. R., Giambonne, M. A., Caza, M. J., Shah, A., Annoni, G., Takahashi, S., Ephbal, M., and Zern, M. A. (1990) Hepatology 11, 111–117
8. Knittel, T., Pellmer, P., and Ramadori, G. (1996) Gastroenterology 111, 745–754
9. Pinzani, M., Gentilini, A., Caligiuri, A., De, F. R., Pellegrini, G., Milani, S., Marra, F., and Gentilini, P. (1995) Hepatology 21, 232–238
10. Okuno, M., Muraki, H., Imai, S., Muto, Y., Kawada, N., Sursuki, Y., and Kojima, S. (1997) Hepatology 26, 913–921
11. Cloughier, D. E., Comerford, S. A., and Hammer, R. E. (1997) J. Clin. Invest. 100, 2697–2713
12. Milani, S., Herbst, H., Schuppen, D., Stein, H., and Surrenti, C. (1991) Am. J. Pathol. 139, 1221–1229
13. Nakatsukasa, H., Nagy, P., Ervarts, R. P., Haia, C. C., Marsden, E., and Thorpestrinin, S. S. (1990) J. Clin. Invest. 85, 1833–1843
14. Bissell, D. M., Wang, S. S., Jarnagin, W. R., and Roll, F. J. (1995) J. Clin. Invest. 96, 447–455
15. Matsuoka, M., and Tsukamoto, H. (1990) Hepatology 11, 599–605
16. Castilla, A., Prieto, J., and Fausto, N. (1991) N. Engl. J. Med. 324, 933–940
17. Bachem, M. G., Meyer, D., Melchior, R., Sell, K. M., and Gressner, A. M. (1992) J. Clin. Invest. 89, 19–27
18. De Bleser, P. J., Niki, T., Rogiers, V., and Geerts, A. (1997) J. Hepatol. 26, 886–883
19. Friedman, S. L., Yamasaki, G., and Wong, L. (1994) J. Biol. Chem. 269, 10551–10558
20. Lalazar, A., Wong, L., Yamasaki, G., and Friedman, S. L. (1997) Gene 195, 233–243
21. Ratazzi, V., Lalazar, A., Wong, L., Dang, Q., Collins, C., Shaulian, E., Jensen,
S., and Friedman, S. L. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 9500–9505
22. Koritschoner, N. P., Bocco, J. L., Panzetta-Dutari, G. M., Dumur, C. I., Flury, A., and Patrito, L. C. (1997) J. Biol. Chem. 272, 9573–9580
23. Matsumoto, N., Lauf, F., Aldabe, B. W., Z., Ramírez, F., Yoshida, T., and Terada, M. (1998) J. Biol. Chem. 273, 28229–28237
24. Bieker, J. J. (1996) DNA Cell Biol. 15, 347–352
25. Crossley, M., Whitelaw, E., Perkins, M., Williams, G., Fujiwara, Y., and Orkin, S. H. (1996) Mol. Cell. Biol. 16, 1695–1705
26. Anderson, R. P., Kern, C. B., Crable, S. C., and Lingrel, J. B. (1995) Mol. Cell. Biol. 15, 5957–5965
27. Sogawa, K., Kikuchi, Y., Imataka, H., and Fujii-Kuriyama, Y. (1993) J. Biochem. (Tokyo) 114, 605–609
28. Kim, S. J., Glick, A., Sporn, M. B., and Roberts, A. B. (1989) J. Biol. Chem. 264, 402–408
29. Kim, S. J., Jeang, K. T., Glick, A. B., Sporn, M. B., and Roberts, A. B. (1989) J. Biol. Chem. 264, 7041–7045
30. Michelson, S., Alcami, J., Kim, S. J., Danielpour, D., Picard, L., Bessia, C., Paya, C., and Virelizier, J.-L. (1994) J. Virol. 68, 5730–5737
31. Bae, H. W., Geiser, A. G., Kim, D. H., Chung, M. T., Burmester, J. K., Sporn, M. B., Roberts, A. B., and Kim, S.-J. (1995) J. Biol. Chem. 270, 29460–29468
32. Friedman, S. (1993) in In Vitro Biological Systems (Tyson, C., and Frazier, J., eds) Vol. 1, pp. 292–310, Academic Press, San Diego, CA
33. Maher, J. J., and McGuire, R. F. (1996) J. Clin. Invest. 96, 1641–1648
34. Choi, S.-G., Yi, Y., Kim, Y.-S., Kato, M., Chang, J., Chung, H.-W., Hahn, K.-B., Yang, H.-K., Rhee, H. H., Bang, Y.-J., and Kim, S.-J. (1998) J. Biol. Chem. 273, 110–117
35. Bloom, B. B., Humphries, D. E., Kuang, P. P., Fine, A., and Goldstein, R. H. (1996) Biochim. Biophys. Acta 1312, 243–248
36. Kim, S. J., Park, K., Rudkin, B. B., Dey, B. R., Sporn, M. B., and Roberts, A. B. (1994) J. Biol. Chem. 269, 7373–7374
37. Ji, C., Casinghino, S., McCarthy, T. L., and Centrella, M. (1997) J. Biol. Chem. 272, 21260–21267
38. Ratziu, R., Kim, S. J., Kim, Y. S., Dang, S., Wong, L., and Friedman, S. L. (1997) Hepatology 26, 185A
39. Gregory, R. C., Taxman, D. J., Seshasayee, D., Renaimer, M. H., Bieker, J. J., and Wojchowski, D. M. (1996) Blood 87, 1793–1801
40. Rippe, R. A., Almounajed, G., and Brenzer, D. A. (1995) Hepatology 22, 241–251
41. Bedossa, P., and Paradis, V. (1995) J. Hepatol. 22, Suppl. 3, 37–42
42. Rosenbaum, J., Blazewiowski, S., Preaux, A. M., Mallat, A., Dhumeaux, D., and Mavric, P. (1995) Gastroenterology 108, 1986–1996
43. Knittel, T., Janneck, T., Muller, L., Fellner, P., and Ramadori, G. (1996) Hepatology 24, 352–360
44. Kretzschmar, M., and Massague, J. (1998) Curr. Opin. Genet. Dev. 8, 103–111
45. Heldin, C. H., Miyazono, K., and ten Dijke, P. (1997) Nature 380, 465–471
46. Reimann, T., Hempel, U., Krautwald, S., Axmann, A., Scheibe, R., Seidel, D., and Wenzel, K. W. (1997) FEBS Lett. 403, 51–60
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