Platelet-derived growth factor is a principal inductive factor modulating mannose 6-phosphate/insulin-like growth factor-II receptor gene expression via a distal E-box in activated hepatic stellate cells

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Hepatic stellate cells (HSCs) become activated during the early stages of hepatic injury associated with fibrogenesis. The mannose 6-phosphate/insulin-like growth factor-II receptor (M6P/IGFIIIR) plays an important role in early fibrogenesis by participating in the activation of latent transforming growth factor- β , a potent inducer of the matrix proteins in activated stellate cells that produce the hepatic nodule. Platelet-derived growth factor (PDGF), a potent HSC mitogen, is released early in hepatic injury and activates several signalling pathways in HSCs. In this study we examined the role of PDGF-BB in HSC regulation of M6P/IGFIIIR gene expression. Several promoter elements were found and characterized that modulate M6P/IGFIIIR expression in activated stellate cells. The presence of a distal CACGTG E-box at –2695 was required for M6P/IGFIIIR expression in transfected stellate cells. When the distal E-box was removed there was no significant M6P/IGFIIIR promoter activity. The distal E-box-binding protein responded specifically to PDGF-BB

with increased binding. This coincided with PDGF-BB up-regulation of M6P/IGFIIIR mRNA transcript levels. Downstream elements include two proximal (–2 to –48) CACGTG E-boxes that bind a different protein to the distal (–2695) E-box. The proximal E-boxes respond moderately to PDGF-BB. The promoter segment encompassing –144 to +109 is able to respond dramatically to serum but is refractory to PDGF-BB. However, a constitutively bound protein binding to the –611/–716 fragment appears to be a repressor that suppresses inductive changes in protein binding occurring downstream of –611. These results indicate that the M6P/IGFIIIR promoter responds primarily and specifically to PDGF-BB through a distal E-box element and possibly through two proximal E-box elements.

Key words: collagen, fibrosis, hepatic fibrogenesis, sinusoidal cell, TGF- β .

INTRODUCTION

During hepatic fibrogenesis the hepatic stellate cell (HSC) becomes activated, transforming from a vitamin-A-storing quiescent cell into a myofibroblast-like cell, devoid of vitamin A and overproducing type-I collagen as well as other matrix proteins that contribute to fibrosis [1]. This fibrogenic process is not limited to the liver, as parallel pathways exist in most solid organs (e.g. kidney, lung and heart) during pathologic fibrotic states [2–4]. Cytokine signalling also plays an integral role in fibrogenesis. Platelet-derived growth factor (PDGF) is released during hepatic injury [5]. During early stages of injury, HSCs express the PDGF β -receptor and respond specifically to PDGF-BB with altered gene expression, proliferation and chemotaxis, migrating to the site of injury [6]. Another major cytokine, transforming growth factor- β (TGF- β), is a powerful inducer of matrix gene expression in many cell types [7]. TGF- β stimulates HSCs under pathological conditions and is the most potent inducer of collagen $\alpha 1(I)$ expression, a major contributor to fibrosis [1]. TGF- β is secreted in a latent, biologically inactive, form and must be activated in order to bind to its own receptor [8]. The activation of TGF- β is substantially enhanced by the binding of latent TGF- β to the mannose 6-phosphate/insulin-like growth factor-II receptor (M6P/IGFIIIR) [9,10]. M6P/IGFIIIR is a dual-specificity receptor that binds both insulin-like growth factor-II as well as a variety of proteins bearing mannose

6-phosphate residues [11,12]. Administration of mannose 6-phosphate as well as anti-M6P/IGFIIIR antibodies to bovine aortic endothelial/bovine smooth-muscle co-cultures expressing M6P/IGFIIIR surface protein blocks the binding of TGF- β to M6P/IGFIIIR and specifically inhibits activation of TGF- β [9]. The latency peptide associated with the TGF- β dimer bears two N-linked mannose 6-phosphate residues, which bind latent TGF- β to membrane bound M6P/IGFIIIR. This binding, which concentrates latent TGF- β at the cell surface, allows the function of other molecules essential for TGF- β activation, which are not well understood *in vivo*, although plasmin is probably involved [9,13,14].

M6P/IGFIIIR expression is up-regulated early in the process of stellate activation. The M6P/IGFIIIR protein was found in rat stellate cells *in vivo* 48 h after administration of carbon tetrachloride [15], a potent inducer of hepatic fibrogenesis. We previously found an up-regulation of the M6P/IGFIIIR mRNA transcript *in vivo* 48 h after carbon tetrachloride administration and *in vitro* resulting from serum stimulation [16]. TGF- β mRNA levels in stellate cells increase dramatically during stellate-cell activation [17]. The parallel induction of M6P/IGFIIIR may serve to promote hepatic fibrogenesis by facilitating the TGF- β autocrine loop in the stellate cell through promotion of TGF- β activation. The co-regulation of M6P/IGFIIIR and TGF- β occurs not only in HSCs in early fibrogenesis [10,17,18], but also occurs in other cell types in which TGF- β participates in pathological

Abbreviations used: HSC, hepatic stellate cell; PDGF, platelet-derived growth factor; TGF- β , transforming growth factor- β ; M6P/IGFIIIR, mannose 6-phosphate/insulin-like growth factor-II receptor; JNK, c-Jun N-terminal kinase; CAT, chloramphenicol acetyltransferase; C/EBP, CCAAT-enhancer-binding protein; ERK, extracellular signal-related protein kinase; XRE, xenobiotic response element.

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states, including hepatocytes in rats exposed to phenobarbital [19], hepatocellular carcinoma [20] and mammary tumours [21].

We previously investigated the proximal region of the M6P/IGFIIR promoter and characterized two CACGTG E-boxes occurring between positions -2 and -48 [16]. E-boxes are transcription-factor-binding elements that conform to a CANNTG motif and bind proteins that carry a basic helix-loop-helix domain [22]. The specificity of E-box binding relies not only on the central two nucleotides of the CANNTG sequence, but also on the one or two nucleotides immediately flanking CANNTG [23]. The two M6P/IGFIIR proximal E-boxes both bound a novel 75-kDa repressor termed P75 that was down-regulated during stellate cell activation. Eliminating the E-boxes greatly increased the ability of the full promoter to drive chloramphenicol acetyltransferase (CAT) expression in transfected stellate cells [16]. Here we report another CACGTG E-box present at -2695 (the distal E-box) that binds a protein which induces M6P/IGFIIR gene expression. Binding is up-regulated as a result of both serum and PDGF-BB stimulation, and the promoter is unable to respond to either stimulus when this distal E-box is removed. A constitutively bound putative repressor was found between -611 and -716 of the promoter, which *in vitro* can only be overcome with the presence of the distal E-box. The distal E-box is an essential component to the up-regulation of M6P/IGFIIR *in vitro* and PDGF-BB appears to be the primary serum factor that up-regulates M6P/IGFIIR in HSCs.

EXPERIMENTAL PROCEDURES

Stellate cell isolation and culture

HSCs were isolated from Sprague-Dawley male rats by methods described previously [24,25]. The cells were used directly for RNA isolation or nuclear-extract preparation, or for culture on tissue-culture flasks precoated with type-I collagen and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 10% newborn calf serum (10/10 media) [26]. Cultures were passaged after reaching confluence; experiments were carried out with cells between passages 2 and 6. Cells quiesced in culture (serum-deprived) were prepared from serum-activated cultured cells placed into 0.4% serum. Cells were incubated in serum-deprived medium for 48 h before harvesting.

RNA isolation and ribonuclease protection assay

RNA was isolated using triagent (Sigma). Cells were homogenized in triagent (4 ml of triagent per 75 cm² of confluent cells), phase separation achieved with addition of 0.1 vol. of bromochloropropane (Molecular Research Center, Cincinnati, OH, U.S.A.) and centrifugation at 12000 g for 5 min. RNA was precipitated from the aqueous phase with addition of isopropanol at half the volume of the original triagent volume and quantified spectrophotometrically. RNA was isolated from cultured stellate cells activated in serum or quiesced in 0.4% serum for 48 h. Additionally, RNA was isolated from cultured stellate cells under various conditions within the second to fifth passages; cells were initially serum deprived for 48 h and then given one of three treatments: 15 ng/ml PDGF-BB (rat recombinant; R&D Systems) for 16 h; 25 pmol/ml cholate (Sigma) for 16 h; and UV irradiation for 24 s at 1 m from a 30 W UV bulb (cells were harvested 16 h after UV treatment). We have determined that 24 s of UV light is sufficient to activate c-Jun N-terminal kinase (JNK) [27]. A single-stranded RNA probe complementary to rat M6P/IGFIIR mRNA nucleotides 836-1247 was synthesized with [³²P]UTP using the MAXIscript kit (Ambion, Austin, TX,

U.S.A.). The template for RNA synthesis was generated by PCR with a T7 promoter linked to the downstream primer. Primers used were 5'-CAGGCTCGTTCTGACTTATG-3' and 5'-GGA-TCCTAATACACTCACTATAGGGAGGCTTCCTGCTAT-TTTGAC-3', with the T7 promoter underlined. The T7 promoter and 5' flanking nucleotides were described in the MAXIscript kit manual. The M6P/IGFIIR rat cDNA used for the PCR template was provided kindly by Professor Richard G. MacDonald [11]. PCR DNA was ethanol-precipitated, resuspended in water and used directly in RNA synthesis without further purification. The template for rat cyclophilin was obtained from Ambion and yields a 124-bp protected fragment. The synthesized RNA probes were gel purified in 5% acrylamide/8 M urea; RNA probes were eluted from the cut bands overnight in 0.5 M ammonium acetate/0.1% SDS/1 mM EDTA, at 37°C. To account for probable differences in mRNA abundance the cyclophilin probe was labelled at 800-fold less specificity than the M6P/IGFIIR probe by adding unlabelled UTP to the transcription reaction. Ribonuclease protection was carried out with the RPA II kit (Ambion). M6P/IGFIIR probe (38 000 c.p.m.) and cyclophilin probe (31 000 c.p.m.) were added to 5 µg of whole RNA, incubated for 16 h at 45 °C, treated with RNase (1:100 dilution of the RNase provided by the kit), and run on a 5% 19:1 native acrylamide gel. M6P/IGFIIR probe was hybridized to yeast RNA provided with the kit and treated with RNase as a negative control. The dried gel was exposed to a PhosphorImaging system (PhosphorImager SI, Molecular Dynamics, Sunnyvale, CA, U.S.A.); the radioactivity in each band was quantified by computer-aided densitometry of the PhosphorImage using IPLab Gel (Signal Analytics Co., Fairfax, VA, U.S.A.). The marker was DNA and so its migration relative to RNA was approximate.

Gel-shift assay

Nuclear extracts (5 µg/lane; method described in [28]) were incubated with [³²P]-labelled double-stranded oligonucleotides (Operon Technologies, San Francisco, CA, U.S.A.) and electrophoresed on a native 6% 80:1 polyacrylamide gel. The probes used for gel shifts are shown below in Figure 2 (top panel). Competition assays were performed with unlabelled probes at a 100 × concentration. The integrity of the extracts was determined by probing them with a double-stranded [³²P]-labelled Sp1 consensus sequence (5'-ATTCGATCGGGCGGGCGAGC-3') for a gel-shift assay, resulting in distinct Sp1 shifts from all extracts (results not shown).

Transfection studies

Stellate cells were transfected using the LipofectAMINE reagent (Life Technologies). CAT and luciferase raw data were adjusted for transfection efficiency determined by co-transfection of a β -galactosidase reporter. The level of expression was determined using chemiluminescence assay kits, a Tropix kit for β -galactosidase, and a Promega kit for luciferase. CAT assays were carried out as described previously [16]. The original M6P/IGFIIR promoter DNA was provided kindly by Dr. Peter Rotwein in a luciferase reporter plasmid [29]. This was used in constructions in Figure 4 (below). The full promoter was removed via *Hind*III and subcloned into a CAT vector, which was used in the Figure 1 constructions (see below). The full-length promoter includes sequence to -2824 of the M6P/IGFIIR 5'-flanking region plus 109 bp of the 5'-untranslated region. Deletion mutants of the promoter were constructed utilizing a unique *Sna*BI site at -2808 double-digested with a variety of downstream sites to

Figure 1 A distal 130-bp segment of the M6P/IGFIIIR promoter that contains an enhancer

CAT constructs containing the full promoter and serial deletions of the 5' promoter region were transfected into culture-activated stellate cells. On the left are illustrated the CAT constructs: -2824wt, contains full-length promoter; for -2730del, -2600del, -2490del, -1370del and -716del the promoter was truncated at the indicated nucleotide and the upstream segment removed. The right-hand side shows transfection results adjusted to efficiency of co-transfected β -galactosidase reporter and expressed per mg of cellular protein extract ($\pm$ S.D., $n = 6$).

Figure 2 The distal 130-bp segment contains an E-box which binds a protein that responds to serum

Top panel. Map of the M6P/IGFIIIR promoter indicating derivation of the probes. Shown are the E-box probe sequences, which contain the E-box plus the flanking seven nucleotides. Bottom panel. Gel shifts with nuclear extracts obtained from stellate cells cultured either with serum (A, activated) or without serum for 48 h (Q, quiescent), and probed with the indicated probes. Competition with unlabelled ('cold') probes was carried out in lanes 3, 6 and 9 with either the proximal E-box probe (p), or the distal E-box probe (d). Arrows indicate the shifted bands.

remove promoter segments and create truncated promoter-luciferase or promoter-CAT plasmids: *NheI*, *BstZ17I*, *PpuMI*, *BclI*, *BstEII*, *AflII*, *BsgI*, *BstXI* and *KpnI* were used to make the -2730del, -2600del, -2490del, -1370del, -716del, -611del, -523del, -292del and -144del plasmids, respectively.

RESULTS

Transfection and gel-shift assay

Transfections of M6P/IGFIIIR-promoter deletion mutants revealed the presence of an upstream enhancer residing between -2600 and -2730 that functions in culture-activated HSCs (Figure 1). Removing the distal 234 bp (to -2600) dramatically reduces the ability of the promoter to drive luciferase expression, whereas adding back the adjacent upstream 130 bp (to -2730) restores luciferase activity to the level seen with the full wild-type promoter. The sequence of this 130-bp enhancer segment was analysed for the presence of known consensus protein-binding elements. A CACGTG E-box was found at -2693 to -2698. This sequence was identical to that of two E-boxes found more proximally in the same promoter between -2 and -48 [16]. We had found previously that these two proximal E-boxes were both binding a novel 75-kDa repressor (P75) that was down-regulated during activation [16]. In contrast, the gel-shift assays herein show that the distal E-box bound a protein that increased binding in activated cells (Figure 2, bottom panel). This increase is coincident with up-regulation of M6P/IGFIIIR-transcript abundance in cultured HSCs [16], suggesting that it functions as an activator of transcription. Both the full 130-bp fragment and

Figure 3 PDGF-BB up-regulates M6P/IGFIIIR mRNA transcript abundance

Ribonuclease protection assay performed on total RNA from cultured stellate cells grown in serum-deprived media for 48 h (lane 1), in 20% serum (culture activated, lane 2) or in 48-h serum-deprived media and then treated with PDGF-BB (15 ng/ml for 16 h; lane 3), UV irradiation (24 s, 30 W bulb at 1 m; lane 4) or cholate (25 μ M, 16 h; lane 5). Total RNA was probed with M6P/IGFIIIR cRNA (412-bp protected fragment) and cyclophilin cRNA (124-bp protected fragment). Positive and negative controls: probed culture-activated RNA without RNase (lane 6) and probed yeast RNA with RNase (lane 7). The ladder is double-stranded DNA and therefore its migration relative to RNA is approximate. Radioactivity of the bands was quantified via densitometry of a PhosphorImage. The levels of the hybridized M6P/IGFIIIR were adjusted to cyclophilin levels, revealing a 2.4-fold increase of M6P/IGFIIIR in PDGF-treated cells but negligible differences with UV- or cholate-treated cells.

Figure 4 The distal 334 bp of the M6P/IGFIIIR promoter is required for the response to PDGF-BB; a promoter fragment containing repressor activity resides at -611/-716

Top panel. M6P/IGFIIIR promoter-luciferase constructs (shown to the left) were transfected into stellate cells which were then placed into three conditions: serum-deprived for 48 h, serum-activated and 48-h serum-deprived plus 16-h PDGF-BB (15 ng/ml). Transfections used luciferase (Luc) constructs containing the full-length promoter (-2824wt), or the promoter truncated to -2490 (-2490del) or -144 (-144del). Luciferase activity was adjusted to efficiency of co-transfected β -galactosidase reporter and expressed per mg of protein extract. Data are means $\pm$ S.D., $n = 6$. Bottom panel. Truncated promoter-CAT constructs illustrated on the left were transfected into HSCs. As in the top panel, truncations were made at the indicated nucleotide. Transfection results are shown on the right; data are means $\pm$ S.D., $n = 6$.

a 20-bp oligomer containing the distal E-box plus the flanking 7 bp were used in a gel shift with nuclear extracts from culture-activated stellate cells and from stellate cells grown without serum for 48 h. There is a marked increase in E-box binding in extracts from activated stellate cells (Figure 2, bottom panel, lanes 4-6). The protein bound to the larger 130 bp fragment (Figure 2, bottom panel, lanes 7-9) gives the same result except that the band migration is higher in the gel. That this is the same protein bound to the 20-bp E-box probe is shown by the unlabelled E-box probe being able to compete with the 130-bp probe for protein binding (Figure 2, bottom panel, lane 9), and so the higher shift is due to a larger probe rather than a change in protein binding. For comparison, a gel shift of one of the proximal E-box probes was also performed, resulting in a markedly different band migration to that of the distal E-box shift, suggesting that different proteins are binding to the proximal and distal E-boxes (Figure 2, bottom panel, lanes 1-3). A super-shift was carried out to determine if any of the known CACGTG-binding proteins were binding to the distal E-box. Nuclear extracts were preincubated with either anti-USF (upstream stimulatory factor), anti-Max or anti-Myc [30-32]. None of these antibodies super-shifted the distal E-box-binding protein (results not shown).

Ribonuclease protection assay

Cultured stellate cells were treated with three stimulants that activate differing signalling pathways in stellate cells relevant to fibrogenesis: PDGF-BB [33], UV irradiation [27] and the bile-salt cholate [34]. Whole RNA (5 μ g) from each condition was hybridized to a M6P/IGFIIIR cRNA probe (Figure 3). When adjusted for loading with the cyclophilin control, PDGF-BB treatment results in a 2.4-fold increase in IGFIIIR mRNA transcript steady-state level, as assessed by densitometry of a PhosphorImage of the radioactivity, whereas cholate and UV have little to no effect (Figure 3).

PDGF-BB-treated cells: transfections and gel-mobility shifts

Three M6P/IGFIIIR promoter-luciferase constructs were transfected into stellate cells. Each construct contained the promoter truncated to nucleotides -2824 (full length, -2824wt), -2490 (distal 334 bp deleted, -2490del) or -144 (distal -2680 bp deleted, -144del). All three constructs also contained the initial 109 bp of the first exon. Cultures were serum-deprived for 48 h after transfection and then treated with either 20% fetal bovine serum or 15 ng/ml PDGF-BB for 16 h, or left in 0.4% serum (Figure 4, top panel). The full-length M6P/IGFIIIR promoter

Figure 5 Gel-shift assay showing that PDGF-BB causes a major increase in distal E-box protein binding (left-hand panel), decreases proximal E-box protein binding and has only a borderline effect on protein binding to a putative repressor fragment (right-hand panel)

Nuclear extracts are from stellate cells grown in 20% serum (ser act), serum-deprived for 48 h (ser dep, s.d.) or PDGF-BB-treated after 48 h of serum deprivation. Left-hand panel. Extracts were probed with the distal E-box. Right-hand panel. Extracts were probed with the proximal E-box (lanes 1–3) or the –611/–716 promoter fragment (lanes 4–7). Arrows indicate shifted bands. Representative ($n = 3$) shifts are shown.

responded equally to both serum and PDGF-BB when compared with its response in serum-deprived cells (Figure 4, top panel, –2824wt transfections). When the distal 334 bp were deleted (–2490del), the promoter was unable to respond to either serum or PDGF-BB (Figure 4, top panel, –2490del transfections). Interestingly, the –144del construct (Figure 4, top panel) had a strong response to serum, indicating the presence of a strong repressor element residing between –144 and –2490, as well as an inducer between –144 and +109. However, –144del was unable to respond to PDGF-BB, indicating the presence of a serum factor, other than PDGF-BB, that mediates M6P/IGFIIIR expression. A promoter fragment was found subsequently to have repressor activity. The M6P/IGFIIIR promoters truncated to –611, –523 and –292 all had substantial promoter activity (Figure 4, bottom panel). However, when the promoter was extended to –716, there was a dramatic decrease in promoter activity, indicating the existence of a repressor element within the –611/–716 fragment.

Gel-mobility shifts were used to determine whether PDGF-BB was the major component of serum-mediated changes in protein binding to both the distal E-box and the proximal E-boxes, and to the putative repressor fragment at –611/–716 (Figure 5). Similar to serum, PDGF-BB induced an increase in protein binding to the distal E-box (Figure 5, left-hand panel). PDGF-BB had an opposite effect on P75 repressor binding to the proximal E-box, a decrease in protein binding, but to a lesser magnitude (Figure 5, lanes 1 and 2), similar to serum's effect on P75 repressor binding ([16] and Figure 2, bottom panel, lanes 1 and 2). Lanes 4–6 of Figure 5 show a protein bound to the –611/–716 fragment of the M6P/IGFIIIR promoter. The binding activity does not change significantly as a result of serum

or PDGF-BB treatment. Figure 4 (bottom panel) shows that promoter activity of the –611del construct is dramatically reduced with the addition of the next 105 bp, the –611/–716 fragment. Thus the protein binding to the –611/–716 fragment is likely to be a repressor which is constitutively bound to the M6P/IGFIIIR promoter in stellate cells. An inspection of the –611/–716 sequence revealed two consensus protein-binding sites, that of CCAAT-enhancer-binding protein (C/EBP) and xenobiotic response element (XRE) [35]. Two 20-mer double-stranded oligonucleotides, derived from the native promoter sequence that included the C/EBP and XRE elements plus flanking nucleotides, were used both as gel-shift probes and as unlabelled competitors for a –611/–716-probed gel shift. The results were negative (results not shown); neither probe produced a gel shift nor could they compete with –611/–716 binding. Therefore, neither XRE nor C/EBP constitute the protein-binding site present in the –611/–716 fragment.

DISCUSSION

This investigation was undertaken to extend analysis of the regulation of M6P/IGFIIIR gene expression during stellate cell activation. Several promoter elements appear to be responsible for control of M6P/IGFIIIR-promoter activity in activated stellate cells. We had previously analysed two proximal E-boxes that played an important role in M6P/IGFIIIR expression [16]. Here we analysed the function of a distal E-box and investigated the effect of PDGF-BB on gene expression in activated stellate cells. PDGF was specifically studied because it is the most potent HSC mitogen present in serum and its *de novo* receptor expression in HSCs may occur during M6P/IGFIIIR expression and thereby

may play a major role in the progression of fibrogenesis [5]. A distal promoter fragment, found to be required for M6P/IGFIIIR gene expression, contained a CACGTG E-box that was responsive to PDGF-BB treatment. PDGF-BB increased M6P/IGFIIIR mRNA transcript abundance and also increased protein binding to the distal E-box, indicating its function as an enhancer. In cultured HSCs the promoter was not responsive to either PDGF-BB or serum without the distal E-box. Additionally, the proximal E-box is modestly responsive to PDGF-BB and a putative repressor binding to a -611/-716 fragment is refractory to both serum and PDGF-BB.

The gel shifts in Figure 2 clearly show proteins of widely varying gel migration that bind to the proximal and distal E-boxes, suggesting that the protein binding to the distal E-box is not the P75 repressor that binds to the proximal E-boxes. Serum was able to down-regulate binding to the proximal E-box and simultaneously up-regulate binding to the distal E-box, which is further indication of their separate identities. Additionally, the proximal E-box was able to bind to the P75 repressor in a Southwestern blot [16], whereas the distal E-box did not bind to any protein on the Southwestern blot (results not shown). Because E-box-binding proteins are helix-loop-helix dimers [30], it is not surprising that no binding occurs under the denaturing conditions of the Southwestern blot, which separates dimers in SDS/PAGE. There are several known proteins that bind the CACGTG E-box, which indicates the importance of nucleotides flanking this hexamer in determining binding specificity. The nucleotides flanking the distal E-box, TGcactgTT, shown in upper case, are apparently not part of the extended consensus for any of the known vertebrate CACGTG-binding proteins [23,30,31], further indication of the importance of the flanking nucleotides in conferring specificity.

Figure 3 shows that PDGF-BB increases M6P/IGFIIIR mRNA abundance but that cholate and UV irradiation have little to no effect on mRNA level when adjusted for the cyclophilin loading control. PDGF activates ERK (extracellular signal-related protein kinase) 1 and ERK2 and cholate activates ERK1, ERK2 and Egr [34,36]. Cholate affects M6P/IGFIIIR mRNA level only slightly and therefore PDGF-BB may be controlling M6P/IGFIIIR gene expression primarily through a signalling pathway that does not involve ERK1, ERK2 and Egr. Recently it was found that phosphatidylinositol 3-kinase is required for signalling through the PDGF β -receptor in HSCs [6], and therefore it is possible that this pathway is mediating M6P/IGFIIIR expression. This notion is supported by another recent study finding that blocking phosphatidylinositol 3-kinase with wortmannin inhibits the movement of endosomal M6P/IGFIIIR to the cell surface [37]. UV irradiation specifically activates the stress signalling pathway (JNK) in HSCs and up-regulates expression of the fibrogenic collagen $\alpha 1(I)$ [27], but has no effect on M6P/IGFIIIR transcript abundance. The stress (JNK) pathway therefore does not regulate M6P/IGFIIIR expression in stellate cells. It is possible that the fibrogenic signals altering HSC gene expression and producing hepatic fibrogenesis are separate for M6P/IGFIIIR and collagen $\alpha 1(I)$.

The data presented here provide a partial model for control of M6P/IGFIIIR gene expression in HSCs. We have defined three elements responsive to PDGF-BB (the distal E-box and two proximal E-boxes), and a fourth element which may be constitutively repressor-bound (-611/-716 fragment). Also, the presence of an additional activating element 3' to -144 is suggested by the ability of -144del to drive luciferase expression efficiently in the presence of serum. The -144del construct does not respond to PDGF-BB, indicating that there is some constituent in serum other than PDGF-BB that may be exerting

Figure 6 Proposed scheme for fibrogenesis after liver injury involving PDGF-BB up-regulation of M6P/IGFIIIR

The Figure consists of a series of molecular interactions involving altered gene expression and several signalling pathways, which are probably self-perpetuating and result in overexpression and accumulation of matrix proteins such as collagen $\alpha 1(I)$. MPR, M6P/IGFIIIR.

influence on M6P/IGFIIIR gene expression. However, it is PDGF-BB that is primarily required for this expression, as demonstrated by the fact that the -2490del construct, which removes the distal E-box but leaves the rest of the promoter intact, is unable to respond to serum. Even though the proximal E-box-binding protein is probably responding to PDGF-BB and serum in the -2490del construct, the -611/-716 repressor is apparently strong enough to overcome any downstream changes resulting from serum stimulation. It is only when the distal element is present that PDGF-BB (and serum) up-regulates M6P/IGFIIIR gene expression. These results indicate that PDGF-BB plays a prominent role in cytokine regulation of M6P/IGFIIIR gene expression during stellate cell activation.

We propose that the linking of PDGF to M6P/IGFIIIR up-regulation completes a molecular loop in which the fibrogenic events that occur during injury become self-sustained (Figure 6). PDGF is secreted by Kupffer cells upon hepatic injury [5]. In stellate cells PDGF-BB up-regulates total M6P/IGFIIIR mRNA transcript abundance (this study) and may be involved in recycling M6P/IGFIIIR surface protein [37]. HSC surface M6P/IGFIIIR protein promotes the activation of TGF- β [10], which then up-regulates collagen $\alpha 1(I)$, resulting in matrix overproduction [5]. TGF- β also up-regulates PDGF β -receptor, resulting in increased PDGF-BB-receptor binding within the activated stellate cell [38], which perpetuates the fibrogenic cycle. This autocrine pathway probably plays a key role in hepatic fibrogenesis.

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