Identification and Characterization of the Human Xylosyltransferase I Gene Promoter Region*

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Human xylosyltransferase I catalyzes the initial and rate-limiting step in the biosynthesis of glycosaminoglycans and proteoglycans. Furthermore, this enzyme has been shown to play a major role in the physiological development of bone and cartilage as well as in pathophysiological processes such as systemic sclerosis, dilated cardiomyopathy, or fibrosis. Here, we report for the first time the identification and characterization of the XYLT1 gene promoter region and important transcription factors involved in its regulation. Members of the activator protein 1 (AP-1) and specificity protein 1 (Sp1) family of transcription factors are necessary for the transcriptional regulation of the XYLT1 gene promoter region and important transcription factors involved in its regulation.

EXPERIMENTAL PROCEDURES

Cell Culture—SW1353 cells (human, chondrosarcoma) (ATCC, Manassas, VA) were grown in RPMI 1640 medium (Cambrex, Verviers, Belgium) supplemented with 10% (v/v) fetal calf serum (Biowest, Nuaillé, France), 1% l-glutamine (Cambrex, Verviers, Belgium) supplemented with 10% (v/v) fetal calf serum (Biowest, Nuaillé, France), 1% l-glutamine (Cambrex, Verviers, Belgium), and 1% antibiotic-antimycotic (Biowest). The cells were maintained in a humidified atmosphere containing 5% CO2 at 37 °C. Culture medium was changed twice a week. Where appropriate, cells were incubated with different concentrations of mithramycin A (10 nM to 1 μM).

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2 The abbreviations used are: GAG, glycosaminoglycan; AP-1, activator protein 1; EMSA, electrophoretic mobility shift assay; GlcA, glucuronic acid; RT, reverse transcription; siRNA, short interference RNA; Sp1/3, specificity protein 1/3; TGFβ1, transforming growth factor β1; XT-I, xylosyltransferase I; Xyl, xylose.
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(5'-GGA GAU AGU GUA) or recombinant c-Jun protein (Promega, Mannheim, Germany) was used as a positive control. Transfection of SW1353 cells was carried out as aforementioned (see “Transient Plasmid Transfections”) with a scrambled negative control (ID no. s13226, GAC GGA CAU UUG AUA GCU AUG GAU AGU). For internal control, transfection efficiency was determined by luciferase activity, using the Dual Luciferase Reporter assay system (Promega) on a Lumat LB9705 luminometer (EG&G, Berthold, Bad Wildbad, Germany). As a control for vector backbone-based luciferase expression, the promoterless pGL4.10 vector was used. Where appropriate, transfected cells were treated with medium supplemented with 5 ng/ml TGFβ1 24 h before promoter activity was assayed.

siRNA—Annealed siRNA oligonucleotides targeting human Sp1 (ID no. 143158; 5'-GGG ACC AGA GAA GTG ACT CAG TGA ACA CTT AG-3' and complement) and human Sp3 (ID no. 115337, 5'-GGT CTA GCA AAA TAG GCT GTC CC-3') were purchased from Ambion (Huntingdon, Cambridgeshire, U.K.) and used in accordance with the manufacturer’s guidelines. Transfection of SW1353 cells was carried out as aforementioned (see “Transient Plasmid Transfections”) with a final concentration of 15 nm siRNA in the cell culture supernatant.

Determination of Enzymatic Xylosyltransferase Activity—The enzymatic xylosyltransferase activity was detected in the cell culture supernatant by a radiochemical xylosyltransferase assay as described previously (7).

Statistical Analysis of Promoter Activity Assay Data and Real-Time Quantitative RT-PCR data—Promoter activity data were calculated as the ratio of the luminescence values for each XYLTI promoter firefly luciferase reporter construct and the corresponding value for the cotransfected pGL4.74 vector for normalization of transfection efficiency compared with the respective promoterless vector. All assays were performed in triplicate, and each experiment was repeated a minimum of
three times. Relative expression values and fold changes in expression of real-time quantitative RT-PCR data were calculated using the equation published by Pfaffl et al. (8), which considers the PCR efficiency. Activity and expression levels were compared by the Mann-Whitney U test. p values were considered significant below 0.05. All tests were calculated with GraphPad Prism 4.0 (GraphPad Prism software, San Diego, CA).

RESULTS

Identification of XYLT1 Promoter Region and Activity Analysis of Promoter Luciferase Constructs—Using Genomatix ModellInspector software (9) the putative XYLT1 gene promoter region was identified. A 1638-bp genomic fragment 5’ of the translation initiation start site was cloned upstream of a firefly luciferase reporter gene in a pGL4.10 vector, as described under “Experimental Procedures” (Fig. 1). Different 5’ and 3’ truncations of this putative promoter fragment were generated and transfected into SW1353 chondrosarcoma cells. The results of the dual luciferase assays are shown in Fig. 2A. The highest activities were observed for fragments spanning 1031 and 797 nucleotides of the 5’-flanking region, respectively, with an induction around 50-fold above control. A further truncation of 88 nucleotides dramatically decreases the promoter activity to a basal induction of about 5-fold above control. This residual activity is completely lost when the promoter is truncated to 218 nucleotides. Interestingly, a 3’ truncation of 218 nucleotides of the 797-bp and the 531-bp fragment nearly increases the promoter activity by 180% compared with the full-length construct (Fig. 2B) to 78.5 (±3.5)-fold and 8.3 (±0.2)-fold (mean ± S.E.) above control, respectively. On the basis of these findings, the sequence between 797 and 708 was investigated in detail. MatInspector analysis (9) highlighted one AP-1 binding site located at 730 bp. Therefore, this binding site was mutated and deleted in both the 797-bp full-length and in the 218-nucleotide 3’ truncated 797-bp fragment. In both cases the activity decreased dramatically to the level measured for the constructs between 218 and 709 nucleotides, which are all lacking the AP-1 binding site, to about 5-fold above control (Fig. 2C).

EMSA and Supershift Analysis—The results of the EMSA and supershift assay using a 32-bp biotin-labeled probe containing the AP-1 binding site of the XYLT1 promoter (bold letters in Fig. 1) are shown in Fig. 3, A and B. The probe was either incubated with SW1353 nuclear extracts or with recom
binant c-Jun protein, which is a component of the AP-1 transcription factor complex. In both cases, the same specific EMSA profile with a considerable single-shifted band of the 5'-biotinylated probe was observed (lanes 2, Fig. 3, A and B, respectively). The specificity of the transcription factor binding was proven by competition experiments, in which a molar excess ranging from 50- to 200-fold of unlabeled AP-1 probe was added to the binding reaction (Fig. 3A, lanes 3–6). The lowest concentration of unlabeled probe was sufficient to compete for the binding of the transcription factor to the biotin-labeled probe, demonstrating the specificity of protein binding. In experiments using a specific antibody against c-Jun protein, supershifted bands were observed accompanied by a considerable reduction of the shifted band (Fig. 3B, lanes 3 and 5).

AP-1 Inhibitor Treatment—To determine the effects of an AP-1 inhibition on the XT-I mRNA expression, SW1353 cells were treated with curcumin (20 µM) and tanshinone IIA (100 µM), respectively, for 6 h. In both cases, the XT-I mRNA expression was reduced significantly to 41.8% (±8.5%) and 13.8% (±4.1%), respectively (Fig. 4) as detected by real-time quantitative RT-PCR.

Mithramycin A Treatment—Because the region 5' of the XYLT1 translation initiation site is very GC-rich and contains several binding sites for transcription factors of the Sp1 family, as highlighted by MatInspector (Fig. 1), we determined whether members of this transcription factor family are involved in the regulation of the XYLT1 gene. Therefore, SW1353 cells were treated with different concentrations (10 nM to 1 µM) of mithramycin A for 24 h, which prevents the binding of transcription factors to GC-rich sequences (10). All concentrations used led to a highly significant decrease of XT-I mRNA levels compared with nontreated cells (Fig. 5). In fact, the lowest final concentration of 10 nm mithramy-
constructs. The equivalent 218-bp 3′ pGL4.10 vector (negative control). The underlined are investigated exhibited an increased promoter activity after measured by real time RT-PCR with and without TGF

FIGURE 2. Functional analysis of human XYLTI promoter constructs. A, functional analysis of human XYLTI promoter activity using 5′ deletion constructs in SW1353 cells. Bars show fold increase in luciferase activity for the XYLTI promoter constructs cloned into pGL4.10 vector compared with promoterless pGL4.10 vector (negative control). The numbers on the left indicate the 5′ ends of the constructs relative to the translation initiation start site. The values are the mean ± S.E. of triplicates from at least three independent experiments. B, effect of 218-bp 3′ deletions on XYLTI promoter activity for selected promoter constructs. C, comparison of the activities of the wild-type (wt) XYLTI promoter fragment ranging from −797 to the translation start site (−797 + 1 wt) and the equivalent 218-bp 3′ truncated form (−797 − 218 wt) to constructs in which the AP-1 binding site is either mutated (mut) or deleted (del). The mutated bases are underlined and printed in bold letters; the deletion is indicated by a gap.

FIGURE 3. Binding of nuclear proteins to AP-1 binding site of the XYLTI promoter. A, nuclear protein extracts (*) were incubated with biotin-labeled probe in the absence (lane 2) or presence of a 50–200-fold molar excess of unlabeled probe in competition experiments to verify the specificity of protein binding (lanes 3–6). A considerable shift of the probe incubated with protein extract could be observed (lane 2) compared with the free probe (FP, lane 1) without protein extract. B, EMSA and supershift assay with the same conditions used in A. The biotinylated probe was either incubated with nuclear protein extract (lane 2), protein extract and a specific c-Jun antibody (lane 3), with recombinant c-Jun (lane 4), or recombinant c-Jun and the specific c-Jun antibody, respectively. FP, free probe; ns, nonspecific protein bindings; ss, supershifted protein antibody complex.

Cin A in the cell culture supernatant was sufficient to reduce the XT-I mRNA expression to 14% (±0.45%).

TGFβ1 Treatment—To elucidate a putative TGFβ1-mediated induction of the XT-I promoter activity, cells transfected with the 1638-bp, 797-bp, and the 797-bp AP-1 site mutated XT-I promoter construct were treated with medium supplemented with TGFβ1 (5 ng/ml) for 24 h. None of the constructs investigated exhibited an increased promoter activity after TGFβ1 treatment compared with untreated controls as measured by the Dual Luciferase assay (data not shown). To ensure a TGFβ1 inducibility of the native XYLTI promoter in SW1353 cells, the XT-I mRNA expression of untransfected cells was measured by real time RT-PCR with and without TGFβ1 treatment for the same period of time. Here, an increase of 2.9 (±0.4) (mean ± S.D.) compared with controls was detected.

Effect of siRNA Treatment on XT-I mRNA Expression and Xylosyltransferase Activity—Based on the results obtained from the mithramycin A treatment, SW1353 cells were transfected with siRNA specifically targeting two members of the Sp1 family, Sp1 and Sp3, respectively. As shown in Fig. 6A, the mRNA level of both Sp1 and Sp3 was significantly reduced to less than 30% (p < 0.0001) compared with controls treated with a scram-

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ted siRNA for a total of 96 h after transfection. The siRNA-mediated knockdown of Sp1 had no detectable effect on the XT-I mRNA levels, whereas the knockdown of Sp3 led to a highly significant reduction to <40% compared with controls (p < 0.0001) (Fig. 6B). Similar results were obtained with two other siRNA oligonucleotides targeting Sp3. We also observed a considerable decrease of 51% in the enzyme xylosyltransferase activity in the cell culture supernatant of Sp3 siRNA-transfected cells (57 ± 9 dpm) compared with controls (116 ± 7 dpm) 96 h after transfection.

DISCUSSION

Human XT-I is nearly ubiquitously expressed in all tissues except for liver and a few cell lines (4, 6), albeit at comparatively low levels. The enzyme can be used as a diagnostic marker for systemic sclerosis (11, 12), participates in bone development (13), and was recently proven to exhibit a key role in cartilage repair (14). Additionally, Hurtado et al. (15) demonstrated that a knockdown of XT-I significantly improves axon growth through scar tissue in the adult rat spinal chord. However, to date, little is known about the transcriptional regulation and possibilities of modulating the expression of the XYLTI gene. To gain an insight into the transcriptional activation, we identified and characterized the promoter region of the XYLTI gene and determined important transcription factors involved in its regulation.

As a first step, the in silico analysis of the sequence immediately upstream of the XYLTI translation start site revealed that the investigated sequence does not contain a TATA box. In contrast, especially the first 300 nucleotides 5′ of the translation start exhibit a very high GC content of about 78% containing several GC boxes. The absence of a TATA box and the presence of CpG islands are often encountered within housekeeping gene promoters (16–18). This might be an explanation for the wide distribution of XT-I in different tissues, although the strength of the gene expression varies considerably in these tissues, also indicating tissue-specific regulation mechanisms (4, 6). As shown in Fig. 1, among the assumed transcription
factor binding sites are several belonging to the Sp1 and AP-1 families. These binding motifs were also found to be active in many other ECM protein-coding genes, i.e. collagens such as Col11a2 (19) and Col24a1 (20), proteoglycan core proteins such as biglycan (21, 22), decorin (23), glypican-3 (24) or murine glypican-4 (25). Binding sites for Sp1 and AP-1 are also involved in the transcriptional regulation of other enzymes participating in glycosaminoglycan biosynthesis, like UDP-glucose dehydrogenase (26, 27). Because XT-I catalyzes the initial step in GAG biosynthesis (3–5) and a similar regulation of genes involved in the same biosynthetic pathway is very probable, an important participation of members of the Sp1 and AP-1 protein family in the regulation of the XT-I expression is very likely.

The analysis of the activity of 5'H11032 and 3'H11032 truncated XT-I promoter constructs revealed that the highest transcriptional activity was detected for the AP-1 consensus sequence TGACT containing fragments /H110021 and /H11002797 (Fig. 2A). All larger promoter constructs exhibited only about half the activity. This might indicate the presence of negatively regulating cis-acting elements in this region. Further truncated promoter constructs, completely lacking the AP-1 binding site, even revealed a more dramatic decrease in promoter activity. By site-directed mutagenesis, EMSA, and supershift experiments, we additionally confirmed the participation of the AP-1 binding motif in driving the XT-I transcription. Using an antibody against c-Jun as a part of the AP-1 transcription factor complex, we clearly identified this protein as the binding species.

An interesting result was obtained for a 3’ truncation of 218 nucleotides of the −797 + 1 and −531 + 1 promoter constructs, which both nearly doubled the transcriptional activity of the respective fragment (Fig. 2B). This is possibly caused by the binding of negative regulatory elements within the first 218 nucleotides of the XT-I promoter.
nucleotides 5′ of the translation initiation site. Another possible reason might be the formation of distinct secondary structures caused by the high GC content, leading to a reduced transcriptional level in the full-length constructs. These assumptions are further supported by the fact that the −218 bp construct itself has no detectable promoter activity (Fig. 2B).

To investigate the participation of AP-1 on the native XYLT1 promoter, SW1353 cells were treated with the two different AP-1 inhibitors, curcumin and tanshinone IIA (28–31). As shown in Fig. 4, a significant reduction of XT-I mRNA compared with untreated controls could be observed for both inhibitors, again confirming an essential role of this transcription factor in driving the transcription of the XYLT1 gene.

Because transcription factors of the Sp1 family are frequently involved in the basal expression of extracellular matrix genes, as well as the transcription of many TATA-less promoters depending on Sp1 family proteins (18, 32), we investigated a possible participation of these proteins in the transcriptional regulation of the XYLT1 gene. Especially the first 650 nucleotides 5′ of the translation initiation site contain several predicted binding sites for Sp1 family members. In the majority of promoters containing binding sites for Sp1 family members, these transcription factors provide a basal level of gene expression (32, 33). This is in concordance with the observation that a basal transcriptional level was detected for promoter constructs spanning the first 531–709 nucleotides 5′ of the translation initiation site. Therefore, SW1353 cells were treated with mithramycin A, a well known GC-specific DNA-binding drug, which prevents the binding of Sp1 family transcription factors (10). Even very low concentrations at the nanomolar level of mithramycin A were sufficient to reduce the XT-I mRNA amount to less than 15%. To gain a more detailed insight into which member of the Sp1 family might participate in the transcriptional regulation of the XYLT1 gene, we used specific siRNA, targeting the ubiquitous Sp1 family members, Sp1 and Sp3. Interestingly, only the selective inhibition of Sp3 caused a significant reduction of the XT-I mRNA level to less than 40%, whereas the specific Sp1 inhibition showed no detectable effect. This indicates that the transcriptional regulation of XYLT1 may depend primarily on Sp3. Nevertheless, the transcriptional activity of Sp1 family proteins, and in particular that of Sp3, is thought to be dependent on the cellular context, the structure and arrangement of the recognition sites, as well as on the Sp1:Sp3 ratio within the cell (34, 35). Changes in the cellular environment and response to external stimuli have the potential to alter the respective levels of these transcription factors and may modulate their function (34–37). In addition, it cannot be excluded that a knockdown of Sp3 leads to overall changes in the transcription of the cell and its normal function, causing a down-regulation of XT-I gene expression in a manner unrelated to Sp3 directly regulating XT-I promoter activity. Therefore, an identification of the exact binding site has to be elucidated in future studies.

Although the mRNA data obtained in this study for SW1353 cells and a previously published study for human cardiac fibroblasts (12) provide evidence that the native XYLT1 promoter is inducible by TGFβ1, as detected by real time quantitative RT-PCR, we did not detect a TGFβ1-mediated induction of the transfected XYLT1 promoter constructs. This indicates that the TGFβ1, responsible element of the XYLT1 promoter is not included within the constructs analyzed and may be located upstream of the investigated fragments. The occurrence of such responsive elements in far distance to the proximal promoter is not uncommon and was reported among others for the COLIA2 or ILINB promoter regions, respectively (38, 39).

In conclusion, we have for the first time identified and characterized the proximal promoter region of XYLT1. We showed that a 531-nucleotide promoter fragment is sufficient to drive the transcription on a basal level. Furthermore, we demonstrated that the c-Jun/AP-1 transcription factor is essential for full XYLT1 promoter activity in SW1353 cells. We also provided the first hints that transcription factors of the Sp1 family, especially Sp3, are very likely involved in the regulation of the XT-I mRNA expression. Taken together, our findings present new insights into the regulation of XT-I and may contribute to understanding of the regulatory mechanisms of this enzyme and for extracellular matrix formation in health and disease. Moreover, these results might help to find novel ways of modulating the XYLT1 gene expression, especially in the development of therapeutic strategies for the treatment of fibrotic remodeling processes or cartilage repair.

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