Cloning, Golgi Localization, and Enzyme Activity of the Full-length Heparin/Heparan Sulfate-Glucuronic Acid C5-epimerase*

Brett E. Crawford, Sara K. Olson, Jeffrey D. Esko, and Maria A. S. Pinhal‡

From the Department of Cellular and Molecular Medicine, Glycobiology Research and Training Center, University of California, San Diego, La Jolla, California 92093-0867

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While studying the cellular localization and activity of enzymes involved in heparan sulfate biosynthesis, we discovered that the published sequence for the glucuronic acid C5-epimerase responsible for the interconversion of D-glucuronic acid and L-iduronic acid residues encodes a truncated protein. Genome analysis and 5'-rapid amplification of cDNA ends was used to clone the full-length cDNA from a mouse mastocytoma cell line. The extended cDNA encodes for an additional 174 amino acids at the amino terminus of the protein. The murine sequence is 95% identical to the human epimerase identified from genomic sequences and fits with the general size and structure of the gene from Drosophila melanogaster and Caenorhabditis elegans. Full-length epimerase is predicted to have a type II transmembrane topology with a 17-amino acid transmembrane domain and an 11-amino acid cytoplasmic tail. An assay with increased sensitivity was devised that detects enzyme activity in extracts prepared from cultured cells and in recombinant proteins. Unlike other enzymes involved in glycosaminoglycan biosynthesis, the addition of a c-myc tag or green fluorescent protein to the highly conserved COOH-terminal portion of the protein inhibits its activity. The amino-terminally truncated epimerase does not localize to any cellular compartment, whereas the full-length enzyme is in the Golgi, where heparan sulfate synthesis is thought to occur.

Heparan sulfate proteoglycans are located on the cell surface and in the extracellular matrix, where they play important roles in cell adhesion, differentiation, and growth in vitro and in vivo (1–3). To a large extent, these biological activities depend on the heparan sulfate chains attached to the core protein. Heparan sulfate, a type of glycosaminoglycan, initially assembles by the copolymerization of N-acetyl-D-glucosamine (GlcNAc) and D-glucuronic acid (GlcA). The backbone then undergoes extensive modification initiated by the N-deacetylation and N-sulfation of subsets of GlcNAc residues. Subsequently, D-GlcA residues adjacent to the N-sulfated sugars are converted to L-IdoUA† by a C5-epimerase and are sulfated at C-2 by a specific sulfotransferase. The glucosamine units also can be sulfated at C-6 and to a lesser extent at C-3. The blocklike arrangement of the modified residues confers specific binding properties to the chains for protein ligands, which in turn facilitate various biological activities.

Many of the enzymes involved in heparan sulfate and heparin formation seem to be members of multienzyme gene families. Two exceptions are the C5-epimerase that interconverts D-GlcA and L-IdoUA and the 2-O-sulfotransferase that adds sulfate to C-2 of IdoUA residues and to a lesser extent GlcA residues. The C5-epimerase has been partially purified from mouse mastocytoma (4) and purified to homogeneity from bovine liver (5). A bovine cDNA for the epimerase has been cloned as well (6). Kinetic studies have clarified the substrate specificity of the epimerase, showing that the enzyme will react with both D-GlcA (forward reaction) and L-IdoUA (reverse reaction) when these residues are located toward the reducing side of N-sulfated glucosamine residues, but it will not react with uronic acids that are O-sulfated or that are adjacent to O-sulfated glucosamine residues (7, 8). This specificity is consistent with the overall order of modification, suggesting that epimerization begins to occur after GlcNAc N-deacetylation and N-sulfation but before glucosamine residues undergo 6-O-sulfation and 3-O-sulfation (7, 9). The fact that the epimerase seems to be represented only once in vertebrate and invertebrate genomes suggests that the extent of uronic acid epimerization depends on the level of enzyme expression and production of the N-sulfated tracts.

In an attempt to study the cellular localization and potential interaction of the epimerase with other enzymes in the pathway, we discovered that the published bovine sequence encodes a truncated protein.‡ This report provides the full-length sequence from mouse and human, an improved set of conditions for assaying the epimerase in cell extracts, and a demonstration that the enzyme is localized to the Golgi in vertebrate cells.

EXPERIMENTAL PROCEDURES

Cell Culture—Chinese hamster ovary cells (CHO-K1) were obtained from the American Type Culture Collection (CCL-61, Manassa, VA). MST cells were derived from the Furth murine mastocytoma (10). CHO cells were grown in Ham’s F-12 medium (Life Technologies, Inc.), and MST cells were grown in RPMI 1640 medium. Both media were supplemented with 10% (v/v) fetal bovine serum (HyClone Laboratories), 100 µg/ml streptomycin sulfate, and 100 units/ml penicillin G. The cells were cultured at 37 °C under an atmosphere of 5% CO₂ in air at 100% relative humidity.

Cloning the Murine C5-Epimerase—A murine epimerase cDNA fragment was cloned into M13mp8 by an overlapping extension method. The enzyme was subcloned into plasmid Bluescript KS(−) vector (Stratagene) and sequenced. A fragment was inserted into the expression vector pEGFP-N1 (Clontech) for transient transfection studies. The cDNA was subcloned into pcDNA3 (Invitrogen) for expression in mammalian cells.

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In an attempt to study the cellular localization and potential interaction of the epimerase with other enzymes in the pathway, we discovered that the published bovine sequence encodes a truncated protein.‡ This report provides the full-length sequence from mouse and human, an improved set of conditions for assaying the epimerase in cell extracts, and a demonstration that the enzyme is localized to the Golgi in vertebrate cells.
The cDNA was designed to anneal to the 3' cleotides 1164 and 1183 of the mouse epimerase cDNA, whereas BC42). BC11 was designed to anneal between nucleotides 999-1019 of the murine coding sequence (GenBank™ accession number AC026992). This primer, 3EpiXhoI (5'-GGATCCGAGATTCCATGC-3') and 5EpiXhoI (5'-CTCGAGCCATGCGTTGCTTGGCAGCTCG-3')-end deduced from the human genomic DNA sequence (GenBank™ accession number AF003927) was cloned from an MST cDNA library. The clone used in these experiments actually contained two Myc tags in a tandem repeat at the amino-terminal c-myc amino acid 1-10, yielding values of 76 cpm/pmol GlcA (43 Ci/mol). Detection of epimerase activity was based on the release of 3H from 5'-5'Hglucose units in the polysaccharide and recovery as 3H2O (12). Initial assays were set up according to the published reaction conditions (“original”), which contained 50 mM HEPES, 15 mM EDTA, 100 mM KCl, and 0.015% Triton X-100, pH 7.4 (12). Protein, substrate, and various ancillary factors were adjusted to maximize the activity detected in normal and transfected cells. The “revised” assay consisted of 25 mM HEPES, pH 7.0, 0.1% Triton X-100, 300 pmol of 5'-sulfated heparan substrate, and 2 μg of cell protein in a total volume of 20 μl. Some assays contained 40 mM CaCl2, but divalent cations were later found not to be required. The reactions were incubated for 2 h at 37 °C and halted by the addition of 50 μl of cold 50 mM sodium acetate buffer, pH 4.0, containing 50 mM LiCl. The sample and a 100-μl rinse of the tube with buffer (25 mM HEPES, pH 7.0, and 0.1% Triton X-100) were transferred to a 0.4-ml column of DEAR-Sephasel (Amersham Pharmacia Biotech) that was equilibrated with the same buffer. The column was washed with 0.9 ml of assay buffer, and the 3H2O recovered in the elution buffer was quantitated by liquid scintillation spectrometry using Ultima Gold (Packard Instrument Co.). A reagent blank containing everything except a source of enzyme was included as a control. This yielded values of ~200 cpn, which were subtracted from the experimental values that ranged from 300 to 3000 counts. All assays were done in duplicate with comparable results from three or more independent experiments.

g of plasmid DNA was transformed into E. coli DH5α cells, which were grown on 24-well glass microscope slides (Corning). Cells were grown on 24-well glass microscope slides (Corning). The GlcA C5-epimerase was previously purified from murine mastocytoma and bovine liver (4, 5). The GlcA C5-epimerase was previously purified from murine mastocytoma and bovine liver (4, 5). Cells were harvested, and 25 μg of protein for each sample was analyzed by SDS-polyacrylamide gel electrophoresis on a 10% gel. The samples were transferred to a nitrocellulose membrane using the Bio-Rad Mini Protein II system. The membrane was blocked at 4 °C overnight with 4% BSA in Tris-buffered saline (10 mM Tris, pH 8.0, and 150 mM NaCl) with 0.1% Tween 20 (TBST). Mouse anti-GFP (CLONTECH) and mouse anti-Myc (Invitrogen) were diluted 1:1000 and 1:5000 in TBST, respectively, and incubated for 1 h with the membrane at room temperature with shaking. The membrane was washed three times with TBST before the application of the secondary antibody, goat anti-mouse horseradish peroxidase (Bio-Rad) diluted 1:3000 in TBST. The membrane was washed six times with TBST and developed with SuperSignal West Pico chemiluminescent substrate (Pierce).

RESULTS AND DISCUSSION

The GlcA C5-epimerase was previously purified from murine mastocytoma and bovine liver (4, 5). Sequencing the purified protein yielded proline as the amino-terminal amino acid, sug-
suggesting that the protein had been proteolytically cleaved during the purification process (6). Several internal peptides also were generated by controlled proteolysis, and the peptide sequences were used to design oligonucleotides for screening a bovine lung cDNA library. A cDNA sequence was obtained that was 3085 base pairs and contained an open reading frame corresponding to a 444-amino acid protein from the first in-frame ATG codon. The deduced amino acid sequence predicted a 49,905-Da protein with a potential transmembrane domain located near the amino terminus. Expression of this clone in the baculovirus system yielded the expected activity (6).

Further analysis of the epimerase sequence suggested that it was incomplete. First, computer-aided analysis using PSORT did not indicate the predicted transmembrane domain and in fact suggested that the protein was most likely soluble (14). As described below, this was confirmed in localization studies with GFP-tagged constructs. Second, alignment of the published bovine epimerase sequence with other orthologs in the GenBank database suggested that the bovine sequence was incomplete. First, computer-aided analysis using PSORT did not indicate the predicted transmembrane domain and in fact suggested that the protein was most likely soluble (14). As described below, this was confirmed in localization studies with GFP-tagged constructs. Second, alignment of the published bovine epimerase sequence with other orthologs in the GenBank database suggested that the bovine sequence was incomplete.

Based on this information, we cloned an extended cDNA from mouse mastocytoma mRNA using primers based on the human sequence. This fragment was further extended using 5'-rapid amplification of cDNA ends. The additional sequence added 753 nucleotides including 231 nucleotides in a 5'-UTR and 522 coding nucleotides. The 5'-UTR contained an in-frame termination codon (TGA) 21 base pairs upstream of the new initiation codon, suggesting that the cDNA encodes the full-length epimerase (Fig. 2). The context of the new start codon conforms to an “adequate” Kozak sequence (AATagC, consensus RNNatgY, where R = A or G and Y = T or C) (15), whereas the previously suggested start codon lacks A or G at -3 (tt-tatgt). Previous studies reported a 3'-UTR sequence of ~1.6 kilobases (6). Because the mRNA was found to be ~5 kilobases, an additional untranslated sequence of ~1.4 kilobases apparently exists, but its location is unknown.

The revised sequence adds 174 amino acids to the amino terminus of the previous sequence of the bovine epimerase for a total of 618 amino acids. The full-length epimerase predicts a protein of 70,099 Da with a relatively basic isoelectric point (pI = 8.95). The protein contains a stretch of 17 hydrophobic residues located 11 amino acids from the amino terminus. As expected, PSORT predicts this as a transmembrane domain, and therefore the protein would most likely have a type II transmembrane topology like other enzymes involved in polysaccharide biosynthesis. The full-length clone contains three potential N-linked glycosylation sites at residues 225, 304, and 394, consistent with previous studies suggesting that the protein contained one or more Asn-linked chains. Using the extended sequence to perform a BLAST search of the GenBank database did not reveal additional homologs, suggesting that there may be only one heparin/heparan sulfate C5-epimerase (16).

A comparison of C. elegans, D. melanogaster, mouse, bovine, and human epimerase sequences indicated weak homology in the amino-terminal domain (residues 1–171) followed by a region of high identity (62%, residues 172–223). However, neither of these regions seems critical for catalytic activity because the purified protein from bovine liver was truncated at residue 248 (6). The COOH-terminal domain was also highly conserved across phylogeny (60% identity, residues 497–618), suggesting that this may represent an important functional part of the protein.

Expression studies of the truncated and full-length epimerase were undertaken to determine whether the additional 174 amino acids had any effect on catalysis. Initial attempts to assay the basal enzyme activity in cell extracts prepared from CHO and MST cells met with limited success. Expression of the truncated or full-length enzyme gave variable results, suggesting that the assay originally described for the bovine enzyme might not be optimal for cultured cells or recombinant enzymes expressed in cultured cells (4, 5). Variation of each component improved the activity additively. Monovalent salts were inhibitory in contrast to previous findings (Fig. 3A) (5, 7). Divalent
cations, such as Ca\(^{2+}\) or Mg\(^{2+}\), and EDTA (up to 40 mM) had no effect (4, 7). The activity was highly dependent on detergent, even in sonicated extracts, with maximal effects obtained with 0.1% Triton X-100 (Fig. 3B). However, other detergents inhibited the reaction, suggesting that the effect was not merely because of solubilization of the protein from membranes. The pH optimum was ~7.0, which is in general agreement with previous findings (Fig. 3C) (4, 5, 7), but the activity showed marked sensitivity to the type of buffer (Fig. 3D). HEPES was found to be optimal. Under the revised conditions, the reaction was proportional with time for over 2 h and with protein concentration in the range of 1–30 \(\mu\)g. The \(K_m\) of the enzyme for the N-deacetylated/N-sulfated heparosan was estimated to be 25 \(\mu\)M GlcA equivalents (~500 pmol of GlcA/assay). With 300 pmol of GlcA/assay, a 4.5-fold increase in epimerase activity in MST cell extracts and a 6.5-fold enhancement in CHO cell extracts were observed compared with the original conditions (Fig. 3E). At lower concentrations of substrate, the difference was even more dramatic (data not shown).

Transient transfection of CHO cells revealed 4-fold greater activity associated with the full-length protein compared with the truncated enzyme in the revised assay (Fig. 4). Increasing the substrate 10-fold did not enhance the rate of reaction for either recombinant enzyme (data not shown). These findings indicated that the natural amino terminus was not a prerequisite to detect activity, which is consistent with previous findings.
ings showing that the truncated protein purified from liver and
mastocytoma had substantial activity (4, 5, 12, 17). Extracts
prepared from cells transfected with epimerase containing a
COOH-terminal GFP or YFP tag were analyzed by Western
blotting with an anti-GFP monoclonal antibody. As shown in
the inset of Fig. 4, the tagged full-length protein was present at
higher levels than the truncated enzyme. Both forms were
engineered into a near perfect Kozak sequence in the expres-
sion vector, suggesting that their expression was similar. Thus,
we believe that the lower amount of the truncated enzyme was
due to decreased stability. As shown below, the truncated
enzyme was also mislocalized, which may add to its instability.
Thus, the amino-terminal domain does not seem to enhance the
intrinsic activity of the enzyme.

Fusing c-myc or GFP to the COOH terminus resulted in a
dramatic reduction of enzyme activity (1 pmol/min/mg), but
when a c-myc tag was placed at the amino terminus, enzyme
activity was normal (23 pmol/min/mg versus 26 pmol/min/mg,
respectively). These findings suggested that the highly con-
nected epimerase activity under various reaction conditions. Initial conditions
consisted of 300 pmol of 3H-substrate and 50 µg of cell extract in
addition to the listed components. A, salt dependence was measured in
a reaction of 50 mM PIPES, pH 6.5, 15 mM EDTA, 0.015% Triton X-100,
and 0–150 mM salt. B, detergent preference was assayed in a reaction
of 50 mM PIPES, pH 6.5, 15 mM EDTA, and 0–0.3% detergent.
C, pH dependence was determined in the presence of 40 mM CaCl2 and 0.1%
Triton X-100 and buffer solutions of 25 mM MOPS/25 mM MES at pH
values ranging from 5.5 to 8.0. D, buffer specificity was measured in a
reaction of 40 mM CaCl2, 0.1% Triton X-100, and 0–100 mM of various
buffers at pH 7.0. E, parallel assays were run with MST and CHO cell
extract under original conditions (12) and the revised conditions de-
scribed under “Experimental Procedures.” Error bars represent stand-
ard error of the mean. All assays were done in duplicate.

FIG. 4. Enzymatic activity of truncated and full-length epime-
rase. CHO cells were transiently transfected with a truncated epime-
rase construct representing either amino acids 175–618 or a full-length
construct. After 48 h, cell extracts were prepared and assayed for
enzymatic activity under original and revised reaction conditions (de-
scribed under “Experimental Procedures”). Endogenous CHO C5-epi-
merase activities of 2.4 pmol/min/mg under the original assay condi-
tions and 10.2 pmol/min/mg under the revised conditions were
subtracted from the respective data points to obtain the activities of the
transfected proteins. Error bars represent the standard error of the
mean. Experiments were done in duplicate and were reproducible with
independent transfections. Inset, Western blot analysis of full-length
epimerase-GFP and truncated epimerase-YFP expressed transiently in
CHO cells.

FIG. 3. Effects of salt, detergent, pH, and buffer on epimerase
activity. Cell extracts prepared from MST cells (A–D) were assayed for

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The mislocalization of the truncated enzyme may act to destabilize its structure and activity (Fig. 4, inset).

Future studies of the epimerase will be greatly expedited by having the full-length sequence. Interestingly, very little information is available about the function of IdoUA in the biological activity of heparin and heparan sulfate. In general, it is assumed that the greater conformational flexibility of IdoUA will enhance the binding opportunities for heparin and heparan sulfate (19). The best studied example is the interaction of antithrombin with a heparin pentasaccharide, in which a critical IdoUA residue located to the reducing side of a central 3-O-sulfated glucosamine unit confers high affinity binding to antithrombin (20). Fibroblast growth factor-2 also apparently requires at least one IdoUA unit for binding and activation (21, 22). In the former case, the addition of the 2-O-sulfate group to the IdoUA residue seems to be dispensable (20, 23), whereas in the latter it is essential for binding (24–26). These findings suggest that in some cases the IdoUA may play a direct role in binding to the ligand, whereas in others it may simply serve as a scaffold for placement of a critical sulfate residue. In both cases, the epimerase plays an essential role in creating the preferred binding site for the ligand. With full-length recombinant enzyme now available, it should be possible to engineer binding sites in isolated oligosaccharides and to explore the function of epimerase in vivo by creating mutants in cells and model organisms.

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The Full-length Epimerase Localizes to the Golgi—To study the intracellular localization of epimerase, cDNAs encoding the truncated and full-length enzymes were fused to GFP or e-myc and expressed in CHO cells. Full-length epimerase was located in a juxtanuclear position, co-localizing with the Golgi marker, α-mannosidase II (Fig. 5, A–C). This localization was observed with tags on either the C or amino terminus, indicating that the location of the tag did not interfere with subcellular localization signals in the protein (Fig. 5E). When the truncated epimerase was expressed, it behaved as a soluble protein exhibiting diffuse cytoplasmic staining (Fig. 5D). This is not an unexpected result given that the protein lacks a signal peptide.
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