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**Biosynthesis of dermatan sulfate:  
Chondroitin glucuronate C5-epimerase is identical to SART2**  
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Running Title: Identification of the chondroitin glucuronate C5-epimerase

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We identified the gene encoding chondroitin glucuronate C5-epimerase (EC 5.1.3.19), that converts D-glucuronic acid to L-iduronic acid residues in dermatan sulfate biosynthesis. The enzyme was solubilized from bovine spleen, and a ~43,000-fold purified preparation containing a major 89 kDa candidate component was subjected to mass spectrometry analysis of tryptic peptides. SART2 (Squamous Cell Carcinoma Antigen Recognized by T cell 2), a protein with unknown function highly expressed in cancer cells and tissues, was identified by 18 peptides covering 26% of the sequence. Transient expression of cDNA resulted in 22-fold increase in epimerase activity in 293HEK cell lysate. Moreover, overexpressing cells produced dermatan sulfate chains with 20% of iduronic acid-containing disaccharide units, as compared to 5% for mock-transfected cells. The iduronic acid residues were preferentially clustered in blocks, as in naturally occurring dermatan sulfate. Given the discovered identity, we propose to rename SART2 (Nakao et al (2000), *Journal of Immunology* 164, 2565-2574) with a functional designation, chondroitin glucuronate C5-epimerase (or DS epimerase). DS epimerase activity is ubiquitously present in normal tissues, although with marked quantitative differences. It is highly homologous to part of the NCAG1 protein, encoded by the C18orf4 gene, genetically linked to bipolar disorder. NCAG1 also contains a

putative chondroitin sulfate sulfotransferase domain, and thus may be involved in dermatan sulfate biosynthesis. The functional relation between dermatan sulfate and cancer is unknown, but may involve known iduronic acid-dependent interactions with growth factors, selectins, cytokines or coagulation inhibitors.

Proteoglycans (PGs) consist of glycosaminoglycan (GAG) chains covalently linked to core proteins. The GAGs play important roles in mediating biological functions of PGs, mainly due to their ability to interact with a variety of proteins (1-5). Chondroitin sulfate (CS)/dermatan sulfate (DS) proteoglycans carry GAGs composed of alternating units of N-acetylgalactosamine (GalNAc) and glucuronic acid (GlcA), or L-iduronic acid (IdoA) in the case of DS. The chondroitin/CS/DS family has been ascribed a variety of physiological/developmental effects, that range from control of basic cellular processes such as cell division in *C.elegans*, scaffold functions in various types of connective tissue, to highly cell-type specific effects, as exemplified by the neurite outgrowth-promoting activity mediated by rare structures in cerebral CS/DS (6-9). Protein ligands targeted by CS/DS include growth factors, modulators of blood coagulation, selectins and chemokines. It is important to define the CS/DS structures involved in selective protein binding, and to understand how they are generated. The IdoA-containing

domains of DS chains are of particular significance in this regard, because IdoA residues endow conformational flexibility to the polymer, that is believed to facilitate protein interactions (10).

Biosynthesis of CS/DS involves initial formation of a precursor polysaccharide composed of alternating GlcA and GalNAc residues, which subsequently undergoes a series of modification reactions (11). Our previous studies established that the generation of IdoA units in DS (12;13), as well as in heparin/heparan sulfate (14), occurs by C5-epimerization of a portion of the GlcA residues previously incorporated into the polysaccharide chain. Moreover, CS/DS polysaccharides are O-sulfated at C2 of GlcA and IdoA, C4 and/or C6 of GalNAc (15). Notably, IdoA units are consistently found adjacent to 4-O-sulfated GalNAc residues. The extent of these modifications varies between tissues and seems to be influenced by the core protein structure (16). In addition, both epimerization and sulfation can be affected by growth factors (17).

The enzymes involved in biosynthesis of heparin/heparan sulfate and CS/DS have been cloned, all except the chondroitin glucuronate C5-epimerase required for IdoA formation in DS (in the following denoted DS epimerase). In the present study we therefore purified this enzyme ~43,000-fold from bovine spleen microsomes and identified by mass spectrometry a candidate protein, SART2 (Squamous Cell Carcinoma Antigen Recognized by T-cells 2) that had previously been cloned but not assigned any specific function. Transgenic expression of this protein in mammalian cell lines yielded a product with DS epimerase activity, capable of inducing IdoA formation in exogenous chondroitin substrate. Moreover, cells transfected with DS epimerase synthesized dermatan sulfate chains with increased IdoA content compared to mock-transfected control cells.

## EXPERIMENTAL PROCEDURES

### Materials

AH-Sepharose, Sephadex G-25, Red Sepharose and ConA Sepharose gels, Mono Q 5/5, Superose 12 HR 10/30, Mono Q PC 1.6/5 (operated with Smart system), Superdex Peptide 10/300 GL, and PD-10 columns were obtained from Amersham

Biosciences, as was D-[1-<sup>14</sup>C]glucose (57 mCi/mmol). D-[5-<sup>3</sup>H]glucose (20 Ci/mmol) was from Dupont/NEN, and inorganic carrier-free [<sup>35</sup>S]sulfate from Perkin Elmer. Chondroitinase ABC, AC-I, and B were from Seikagaku. Cell lines were from ATCC. Cell culture reagents were from Gibco. DNA oligonucleotides were synthesized by DNA Technology A/S, Denmark. Other chemicals were of reagent grade and were obtained from various commercial sources.

K4 polysaccharide was a gift from P.Oreste (Italfarmaco, Milan, Italy). After defructosylation (18), it was coupled to AH-Sepharose, by a carbodiimide-mediated procedure (19).

Protease inhibitors were obtained from Sigma, and applied at a final concentration of 1 mM PMSF, and 1 µg/ml each of aprotinin, leupeptin, and pepstatin.

### Preparation of <sup>3</sup>H- and <sup>14</sup>C-Labeled DS- and HS-Epimerase Substrates

Metabolically <sup>3</sup>H- or <sup>14</sup>C-labeled K4 polysaccharides (DS epimerase substrates) were prepared by growing a K4-producing *E.coli* strain in medium containing D-[5-<sup>3</sup>H]glucose or D-[1-<sup>14</sup>C]glucose, as described in (20). The resulting labeled K4 polysaccharides were defructosylated (dK4). HS epimerase substrate was generated in analogous manner, by incubating *E. coli* K5 bacteria with D-[5-<sup>3</sup>H]glucose, followed by N-deacetylation and N-sulfation of the resultant radiolabeled polysaccharide (21;22).

### Analytical Methods

DS epimerase was assayed by its ability to release <sup>3</sup>H-labeled water from chondroitin substrate containing [<sup>3</sup>H]GlcA residues, essentially as described (23), but with some modifications based on preliminary kinetics analysis of semipurified enzyme (*data not shown*). Enzyme samples, containing 3 mg BSA as carrier unless otherwise stated, were desalted at 4°C on Sephadex G-25 columns (0.7 cm X 3 cm), equilibrated with desalting buffer [20 mM MES (pH 5.5 at 37°C), 10% glycerol, 0.5 mM EDTA, 0.1% Triton X-100, 1 mM DTT, protease inhibitors]. Incubations were performed in 100 µl final volume of 0.8 X desalting buffer, 0.5 mg BSA, 2 mM MnCl<sub>2</sub>, 0.5% NP40, and 30,000 dpm [5-<sup>3</sup>H]dK4 or [1-<sup>14</sup>C]dK4 (approx 200 µM HexA for both substrates).

Incubations were done at 37°C for 2-14 h, stopped by boiling for 5 min, and samples were centrifuged. For quantitation of [<sup>3</sup>H]water, 90 µl of the incubations were transferred to a distillation tube containing 200 µl of (unlabeled) water. After distillation (24), 200 µl of the distillate was analyzed by scintillation counting. The assay is linear up to 3000 dpm of released <sup>3</sup>H.

Protein was estimated by the Bradford assay (BioRad), using BSA as standard. HexA content was determined by the carbazole reaction (25).

#### *Purification of DS Epimerase*

Bovine spleen was obtained fresh from the local slaughterhouse and processed immediately. All procedures were carried out at 4°C and all buffers contained 1 mM DTT as reducing agent.

*Step 1 – Microsomal preparation and extraction.* -Day 1 - Three spleens were freed from surrounding fat tissue, cut into 1- to 2-cm cubes, washed twice with cold distilled water and placed in homogenization buffer (20 mM MES pH 6.5, 250 mM sucrose, 5 mM EDTA, protease inhibitors). Batches of 350 g of tissue were first homogenized without any added buffer in a food processor and were then re-homogenized three times after step-additions of 350, 500, and 500 ml of buffer. The homogenate was centrifuged for 15 min at 8,000g. The resulting supernatant was centrifuged at 38,400g for 45 min. The 38,400g pellet was extracted with 90 ml of solubilization buffer (20 mM MES pH 6.5, 150 mM NaCl, 1 mM EDTA, 1% glycerol, 1% Triton X-100, protease inhibitors). The combined extracted material from 11 such preparations, in all corresponding to 3.9 kg of tissue, was subjected to two strokes with a 200-ml Potter device and centrifuged at 38,400g for 45 min. The supernatant was collected. *Day 2* – The final supernatant from day 1 was further centrifuged at 125,000g for 40 min. Approximately half of the resulting supernatant was clear and was collected, while the other half was turbid and was diluted 1:2 with solubilization buffer, re-centrifuged and the resulting clear supernatant collected.

*Step 2 - Red Sepharose.* The microsomal extract was applied at a flow rate of 1.5 ml/min to a Red Sepharose column (5 X 13 cm). After application, flow rate increased to 8 ml/min, the column was washed with 50 bed volumes of 10

mM MES pH 6.5, 1 mM EDTA, 1% glycerol, 150 mM NaCl, 0.1% Triton X-100, further washed with 4 bed volumes of 10 mM MES pH 6.5, 1 mM EDTA, 10% glycerol (Buffer A), containing 150 mM NaCl, and finally eluted with 4 L 10 mM MES pH 6.5, 0.1 mM EDTA, 10% glycerol, 2 M NaCl.

*Step 3 – ConA Sepharose.* The eluted material from Step 2 was adjusted to 1 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 0.1% Triton X-100 and applied to a column of ConA Sepharose (2.6 x 6 cm; gel not re-utilized) run at 2 ml/min. The gel was washed with 30 bed volumes of 10 mM MES pH 6.5, 0.1 mM EDTA, 10% glycerol, 1 mM MnCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 0.1% Triton X-100 (Buffer B)/ 0.5 M NaCl, and then with 6 bed volumes of buffer B/20 mM NaCl. To save elution buffer (buffer B/20 mM NaCl/0.5 M methyl- $\alpha$ -D-mannoside) and increase recovery, five 90-ml elutions were performed, each involving about 12 cycles each of 30 min buffer recirculation followed by 30 min without recirculation.

*Step 4 – dK4 Sepharose.* The eluate from Step 3 was applied to a dK4-Sepharose column (2.6 X 3 cm) at 2 ml/min. The column was washed with 10 bed volumes of buffer A/20 mM NaCl/10 mM CHAPS, further washed with 10 bed volumes of buffer A/20 mM NaCl, and finally eluted with buffer A/150 mM NaCl. Fractions with epimerase activity were pooled, dialyzed versus buffer A/10mM NaCl, and concentrated by applying them to a Mono Q 5/5 column, run at 0.5 ml/min. After application, the column was inverted, and eluted at a flow rate of 0.1 ml/min with buffer A/1 M NaCl.

*Step 5 - Superose 12.* The 1-ml concentrated material from Step 4 was injected to a Superose 12 column, that was subsequently eluted at 0.05 ml/min with buffer A/150 mM NaCl. Fractions of approx. 0.3 ml were collected (Fig. 2A) and analyzed for epimerase activity. Active fractions were pooled, diluted with buffer A to a final NaCl conc. of 20 mM, and concentrated on a Mono Q PC 1.6/5 column, operated as described above.

*Step 6 – Superose 12.* The 0.2-ml concentrated material from Step 5 was injected to a second Superose 12 column, operated as above.

*Step 7 – Red-Sepharose.* The most active fraction from the previous step was diluted with buffer A to 100 mM NaCl, CHAPS was added to

final 10 mM conc., and the sample was batch-incubated with 50  $\mu$ l of fresh Red Sepharose gel. After incubation for 2 h, the gel was washed 5 times with 500  $\mu$ l of buffer A/100 mM NaCl/10 mM CHAPS, further washed 5 times with 1 ml of the same solution without CHAPS, and finally eluted with 10 x 150  $\mu$ l buffer A/2M NaCl.

The ~0.6 million-fold purified analytical sample shown in Fig. 2B lane 2, was prepared as above, with one modification; an initial elution step with buffer A/1 M NaCl preceded the final elution with buffer A/2M NaCl. The final eluate contained ~10% of the epimerase activity incubated with the Red Sepharose gel.

#### *Tryptic Peptide Preparation*

Material from the last purification step was precipitated with TCA, and the pellet was resuspended in reducing Laemli buffer. SDS-PAGE was carried out on NuPage pre-made 10% acrylamide gels (Invitrogen) that were stained with Brilliant Blue G Colloidal staining solution (Sigma). Gel bands of interest were cut out and the proteins were digested with trypsin (Promega) as described (26).

#### *Peptide Mass Spectrometry*

ESI-based LC-MS/MS analyses were carried out using an Agilent 1100 series instrumentation (Agilent Technologies, Paolo Alto, CA, USA) on a 75- $\mu$ m x 10.5-cm fused silica microcapillary reversed phase column (5  $\mu$ m Magic C18 beads, Michrom Bioresources). The column was eluted at 200 nl/min using a gradient of 5-35% acetonitrile in 0.1% formic acid, over a 50-min period as described (27).

LC-MS/MS was performed using an LTQ iontrap (ThermoFinnigan, San Jose, CA, USA) with an electrospray voltage of 2 kV. The instrument was set up to perform one MS scan (400–1600 Da) followed by three MS/MS analyses in a data-dependent mode with an intensity threshold of 15,000 counts. The repeat count was set to 3, the repeat duration to 30 s, the exclusion duration was 60 s and the exclusion list size was 100.

Database search was carried out using SEQUEST (28) and the search results were further analyzed by Peptide and Protein Prophet as previously described (29;30). The identified peptides and corresponding proteins were stored and analyzed

in a MySQL data model, which was used to accommodate further analysis of the data (31).

#### *Expression of SART2/DS Epimerase*

SART2 human cDNA clone (IMAGE no. 5272885) was obtained from RZPD, Germany, and subcloned into pcDNA 3.1/myc-His vector (Invitrogen) using XhoI and AgeI restriction sites introduced by PCR using primers 5'-GATCCTCGAGATGAGGACTCACACACGGG G-3' and 5'-GATCACCGGTACACTGTGATTGGGAACAA GA-3', respectively. The insert was confirmed by sequencing. Ligation into the expression vector resulted in a construct with SART2 in frame with a C-terminal 6XHis tag. CHO-K1 cells, maintained in F12-K medium/10%FBS, HFL-1 cells and 293HEK cells, both maintained in MEM/10% FBS, were grown in 6-well plates and transiently transfected with pcDNA-His, or pcDNA SART2-His plasmid, using Lipofectamine 2000 reagent (Invitrogen), according to the manufacturer's instruction. After 48 h, cells were washed with PBS, and lysed in 20 mM MES pH 6.5, 150 mM NaCl, 10% glycerol, 2 mM DTT, 1 mM EDTA, 1% Triton X-100, protease inhibitors. After 30 min at 4°C, cell lysate was centrifuged for 1 h at 20,000g, and 200  $\mu$ l of the supernatant was desalted (without carrier BSA added prior to desalting), followed by determination of protein content and enzyme activity.

#### *Preparation of Rat Tissue Lysates*

DA strain rats, 6 weeks old, were sacrificed and the organs were put in ~3-fold excess (v/w) lysis buffer (see above for buffer composition and lysate preparation). The predominantly muscular tissues heart, uterus, and skeletal muscle were ground, and further homogenized by 3 Potter strokes. For the remaining soft tissues the use of the Potter homogenizer alone was adequate.

#### *Product Analysis of [ $^{14}$ C]dK4 Incubated with DS Epimerase*

Two strategies were adopted to analyze the reaction products. In one experimental set, samples of 15,000 dpm of [1- $^{14}$ C]-labeled dK4, recovered after incubation with cellular lysate as described in Fig. 4A, were digested with chondroitinase AC-I and the split products were

analyzed by gel chromatography, as described in the following section. Alternatively, the overall composition of enzyme-incubated [ $1\text{-}^{14}\text{C}$ ]-labeled dK4 was determined by analysis of labeled HexA-Tal<sub>R</sub> disaccharides, generated by N-deacetylation followed by deaminative cleavage and reduction of the products essentially as described (20). Briefly, samples were subjected to hydrazinolysis (64% hydrazine/36% H<sub>2</sub>O/1% hydrazine sulfate) at 100 °C for 16 h, and the product was reisolated on a PD-10 column eluted with water, and then treated with HNO<sub>2</sub> at pH 3.9. The resultant disaccharides, representing 64% of the total radioactivity, were reduced with NaBH<sub>4</sub> and recovered by gel chromatography on a Superdex Peptide column, eluted at 0.3 ml/min with 0.2 M NH<sub>4</sub>HCO<sub>3</sub>. The labeled disaccharides were separated by paper chromatography (20).

#### *Analysis of Cellular CS/DS*

Transient transfection of 293HEK cells, grown in 75-cm<sup>2</sup> flasks was performed as described above, with pcDNA-His, or pcDNA SART2-His plasmid. After transfection, cells were grown for 24 h in ordinary MEM, washed once in sulfur-deprived DMEM, maintained for 2 h in 10 ml of sulfur-deprived DMEM/10% FBS, and finally 100 µCi/ml <sup>35</sup>SO<sub>4</sub> was added. After an additional 24-h culture period, medium was collected and applied to a 2-ml DE52 column, equilibrated with 50 mM acetate pH 5.5, 0.1% Triton X-100, 6 M urea. The column was washed with 30 bed volumes of equilibration buffer, then with 5 volumes of water, 5 volumes of 0.2 M NH<sub>4</sub>HCO<sub>3</sub>, and finally eluted with 10 volumes of 2 M NH<sub>4</sub>HCO<sub>3</sub>. Ten µg of cold DS were added, the samples were lyophilized, and then subjected to alkaline β-elimination (50 mM KOH, 1 M NaBH<sub>4</sub>, 45°C for 16 h). GAG chains were re-isolated using the DE52 column, operated as described above. The GAG chains were deaminated at pH 1.5 (32), and intact CS/DS chains were recovered after gel filtration. <sup>35</sup>S -Labeled CS/DS chains (15,000 dpm) were digested for 4 h at 37°C in 200 µl 0.1 M Tris-HCl/sodium acetate pH 7.3, with 36 mU chondroitinase ABC or 10 mU chondroitinase AC-I. Chondroitinase B digestions were performed with 8 mU enzyme in 200 µl 20 mM Tris-HCl/sodium acetate pH 7.3, 50 mM NaCl, 4 mM CaCl<sub>2</sub>, 0.01% BSA. Digests were heated at 100°C for 2 min, mixed with 100 µg heparin, and applied

to a Superdex Peptide column, operated at 0.3 ml/min in 0.2 M NH<sub>4</sub>HCO<sub>3</sub>.

## RESULTS

*Purification of DS Epimerase* – Initial database searches for clues to the DS epimerase coding sequence based on similarity with the HS or alginate C5-epimerases were unsuccessful, and we therefore decided to isolate the DS epimerase protein. Screening various rat tissues for enzyme activity pointed to spleen as the richest source of DS epimerase (Fig. 1). A survey of bovine tissues gave similar results (*data not shown*), and bovine spleen was therefore selected as starting material for purification of DS epimerase. The overall purification process (see “Experimental Procedures”) is summarized in Table I. Solubilized microsomes were first applied to Red Sepharose gel, yielding a 10-fold purification with excellent recovery. A ConA Sepharose column efficiently removed contaminants, but allowed only 30-40% recovery of epimerase activity. dK4 Sepharose provided consistent 3-fold purification, with high recovery. After concentration, the partially purified enzyme was applied to a Superose 12 column. Most of the protein emerged as high-molecular-weight complexes, while the epimerase fraction was more retarded and relatively homogeneous in size, being eluted 4-fold purified with the peak of activity at the position of a 67-kDa marker (Fig. 2A). Size fractionation was refined by reapplication of the active pool to the same column, thus yielding a sharp peak with 80% of the eluted activity in two effluent fractions, again with a 4-fold purification. The most active fraction was incubated, batch-wise, with a small amount of Red Sepharose gel. Most of the activity was recovered, 14-fold further purified. The complexity of this sample, purified altogether ~43,000 fold, was assessed on SDS-PAGE (Fig. 2B, lane 1). An 89-kDa band was considered of particular interest, because the (Colloidal Blue) staining intensity of this band in the eluted fractions from Superose 12 and Red Sepharose correlated with epimerase activity (*data not shown*). Furthermore, by modifying the elution conditions of the last purification on Red Sepharose (see “Experimental”), ~0.6 million-fold purified active material was obtained, at the expense of recovery. Silver staining after SDS-



PAGE showed the 89-kDa band as a major component (Fig. 2B, lane 2).

**Gene Identification** - Seven SDS-PAGE Colloidal Blue-stained bands (Fig. 2B, lane 1) were trypsinized and subjected to ESI-based LC-MS/MS analysis. Database search of the results against the NCBI non-redundant (NRP.nci.fasta.20041115) database yielded 56 proteins identified with more than one peptide matched and with a protein prophet probability of 1.0 (29;30). Several proteins were identified in more than one band, decreasing the number of unique proteins identified to 33. Four proteins were identified from the 89 kDa gel band, believed to contain the DS epimerase: human SART2 (UPTR:Q9UL01; 6 peptides), lactotransferrin (gi.7428768; 11 peptides), hu-k4 (Q92853; 2 peptides), and pld3 protein (O35405; 2 peptides). The seven LC-MS/MS runs were then searched against a database generated from the merged output from two genome-wide gene-finder programs, GenScan and GeneId (downloaded from <http://genome.uscs.edu>) and protein sequences from Bos Taurus at NCBI (<http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi>). Again, the 89 kDa band yielded four proteins with more than one peptide and a protein prophet score of 1.0: SCAFFOLD270174.1 (11 peptides; SART2), gi.7428768 (12 peptides; lactotransferrin), SCAFFOLD796601.1 (2 peptides; ATPase), and SCAFFOLD172907.1 (2 peptides; prolylcarboxypeptidase). The identities of these scaffolds (indicated above in parenthesis) were assessed by blast searches. In all, 18 peptides (14 unique peptides; see Fig. 2B, lane 1, and Fig. 2C) with a peptide prophet probability score of  $\geq 0.93$  for all but one, and ranging from 8 to 24 amino-acid residues, matched SART2 or corresponding scaffold, and are shown in Table II and supplemental data. The resulting protein coverage was 8% for human SART2 and 26% for the corresponding scaffold. Out of the 33 unique proteins identified, SART2 was the only one without a known function.

**SART2/DS Epimerase Expression** - Full-length human SART2 cDNA (Fig. 2C for amino-acid sequence) was subcloned into an expression vector in frame with a C-terminal His tag, and transiently

transfected into three different cell lines. SART2/DS epimerase transfection increased the DS epimerase specific catalytic activity 2-, 3-, and 22 -fold in cellular lysates of HFL-1, CHO, and 293HEK cells, respectively, compared with mock transfections (Fig. 3A). Assays of culture media showed essentially background levels of DS epimerase activity for SART2/DS epimerase-transfected HFL-1 and CHO cells, whereas medium from 293HEK cells displayed activity ~15-fold higher than that from mock-transfected cells (Fig. 3B). The transfection data unambiguously indicated that SART2 DNA encodes a protein that can release a volatile radiolabeled component from the C5- $^3\text{H}$ -labeled dK4 polysaccharide substrate. Gel chromatography of similar incubations containing equal amounts of cell lysates from either mock-transfected or SART2/DS epimerase-transfected 293HEK cells showed that 3% and 46%, respectively, of the total  $^3\text{H}$  emerged at the elution position of  $^3\text{H}_2\text{O}$  (Fig. 4A). Since the  $^3\text{H}$  label was about equally distributed between the C-5 positions of the GlcA and GalNAc units (20), this result indicates that essentially all GlcA residues had been targeted by the transfected epimerase.

**Effect of DS Epimerase on dK4 Incubated In Vitro** - The  $^{14}\text{C}$ -labeled polysaccharide products were recovered following incubation with lysates of either mock-transfected or SART2/DS epimerase-transfected cells, and their HexA composition was analyzed in two different ways. One approach involved depolymerization of the polysaccharides by chondroitinase AC-I, that cleaves N-acetylgalactosaminidic linkages to GlcA but not to IdoA, and the digests were analyzed by gel chromatography (Fig. 4B). The AC-I products of dK4 incubated with mock-transfected 293HEK cell lysate consisted to 99% of disaccharides, ~1% of tetrasaccharides, and  $\leq 0.2\%$  of hexasaccharides. The corresponding proportions for a sample incubated with epimerase-transfected cell lysate were 85% disaccharides, 12% tetrasaccharides, and 2.5% hexasaccharides (Fig. 4B). The IdoA contents calculated from these analyses were 0.6% of total HexA in a sample incubated with mock-transfected lysate, and 8% after incubation with epimerase-overexpressing cell lysate. In a second strategy, GalNAc residues in dK4 polysaccharide, incubated with mock-transfected or epimerase-

transfected cell lysates, were N-deacetylated by hydrazinolysis, and were then deaminated with nitrous acid (pH 3.9 procedure) to cleave the chains at the sites of N-unsubstituted GalN units. Disaccharides, recovered by gel chromatography, were separated into GlcA-anhydro-D-talitol and IdoA-anhydro-D-talitol species by paper chromatography (designated *GT* and *IT*, respectively, in Fig. 4C). The IdoA-containing disaccharide barely exceeded background in samples that had been treated with endogenous epimerase (mock-transfected), but increased to 9% in a sample incubated with over-expressed epimerase.

To exclude that DS epimerase could have a depolymerizing activity on the substrate, <sup>3</sup>H-labeled dK4 was incubated with ~200-fold purified enzyme and its size verified on a Superose 6 column. The elution position of dK4 was unchanged after incubation with the enzyme (data not shown).

*Effects of DS Epimerase Overexpression on DS Biosynthesis* - 293HEK cells were grown in the presence of inorganic [<sup>35</sup>S]sulfate, and the isolated GAGs were treated with nitrous acid to eliminate any heparan sulfate present (see "Experimental"). The GAG resistant to cleavage, ~60% of initial labeled polysaccharide, was quantitatively converted into disaccharides upon digestion with chondroitinase ABC (*data not shown*), and thus was identified as galactosaminoglycan (CS/DS). This material was digested with chondroitinase B lyase, that cleaves -GalNAc-IdoA- sequences in DS chains but leaves -GalNAc-GlcA- sequences intact. DS chains isolated from DS epimerase-transfected cells yielded a conspicuous disaccharide peak, that amounted to 18% of the total label, with smaller but significant proportions of tetra- and hexasaccharides (Fig. 5A). Together, these fractions accounted for 23% of the total label of the digest. The corresponding sample derived from mock-transfected cells was more resistant to chondroitinase B digestion, the disaccharide peak representing 4%, and the sum of di-, tetra-, and hexasaccharides amounting to 7% of the total label. Disregarding the possible occurrence of nonsulfated disaccharide units, lacking radiolabel, calculations based on the di-, tetra- and hexasaccharides result in IdoA contents of 5% in CS/DS from control cells, and 20% in CS/DS from

epimerase-overexpressing cells. The large proportion of disaccharides compared to tetra- and hexasaccharides generated by digestion with chondroitinase B (Fig. 5A) suggested that the IdoA residues were preferentially introduced in blockwise fashion by the transgenic DS epimerase. This conclusion was corroborated by digestion of the <sup>35</sup>S-labeled polysaccharide with chondroitinase AC-I, that cleaves -GalNAc-GlcA- but not -GalNAc-IdoA- sequences. Enzyme-resistant blocks, corresponding to the general structure, -GlcA-GalNAc-(IdoA-GalNAc-)<sub>2-3</sub> in the parental chains, constituted 5% of the labeled CS/DS chains modified by endogenous DS epimerase and 22% of the chains produced by overexpressing cells (Fig. 5B). Notably, the ratio of tetrasaccharides (indicative of isolated IdoA residues in intact polysaccharide) to larger oligosaccharides (≥3 consecutive IdoA-containing disaccharide units, *i.e.* block sequences) was reversed between the two samples. Chondroitinase AC-I-generated oligosaccharides larger than octasaccharides were quantitatively converted to disaccharides by treatment with chondroitinase B (*data not shown*), confirming their IdoA content. In summary, analysis of DS chains produced by transfected cells conclusively established that the SART2 gene encodes a DS epimerase that is active also in the context of the biosynthetic cellular machinery.

## DISCUSSION

DS epimerase was purified from bovine spleen, using the previously established radiochemical assay procedure to monitor the process. MS analysis of peptides derived from partially purified enzyme identified SART2 (Squamous Cell Carcinoma Antigen Recognized by T cell 2) as a candidate protein. Overexpression of human SART2 cDNA in 293HEK cells resulted in >20-fold and >10-fold increase of DS epimerase specific activity in cellular lysate and culture medium, respectively, compared to mock-transfected control cells. The product of the reaction catalyzed by the recombinant enzyme, acting on a chondroitin substrate, was confirmed to be IdoA residues, by specific enzymatic digestion and identification of chemical degradation products. Based on these findings we conclude that the protein encoded by the SART2

gene is a DS epimerase. The designation SART2 relates to the previous observation, that this protein is recognized by a subset of cytotoxic T lymphocytes in certain tumors (33), but does not define any function. We therefore propose to rename SART2 with the functional term: chondroitin glucuronate C5-epimerase (EC 5.1.3.19) or, in short, DS epimerase.

Three classes of epimerases are known to act on HexA residues in polysaccharides: HS epimerase, alginate epimerases, and DS epimerase. DS epimerase shows no significant homology with either one of the other two types of epimerases. In addition, it does not display the “consensus sequence” of 6 spaced amino-acid residues revealed by hydrophobic cluster analysis in alginate epimerases and in the HS epimerase (34). It therefore appears likely that the DS and HS epimerases have evolved to similar functions by convergent evolution. The human DS epimerase gene (located at 6q22) includes 6 exons, and the HS epimerase gene (15q23) 3 exons (33;35). The DS epimerase is conserved between species; compared with the human sequence, the bovine (XP\_591812.2, predicted sequence) protein shows 91% amino-acid identity, mouse (NP\_766096.1) 93%, chicken (XP\_419777.1; predicted sequence) 86%, and zebrafish (CAI20604.1; predicted sequence) 65%. The 958aa-residue human DS epimerase has one putative transmembrane region at its N-terminus (aa 8-30), and two at its C-terminus (aa 901-923 and 931-952; see Fig. 2C).

An unexpected and highly interesting observation links the human DS epimerase with NCAG1 (SwissProt Q8IZU8), the protein product of the C18orf4 gene cloned in 2003 (36), genetically linked to bipolar disorder. The DS epimerase sequence from aa 43 to aa 673 thus shares 50% identity and 66% similarity with NCAG1. Moreover, NCAG1 is recognized by protein family databases, *e.g.* Pfam, as a chondroitin sulfate sulfotransferase with conserved 5'- and 3'-PAPS binding sites. These homology observations suggest that that NCAG1 may be an enzyme with dual epimerase and O-sulfotransferase activities involved in DS biosynthesis. We currently attempt to express the C18orf4 gene.

DS epimerase shares significant homology (21% identity and 36% similarity between aa 24 and aa 584) with a second protein, a bacterial

putative alginate oligo-lyase (SwissProt Q8UBJ1). We have shown in this report that DS epimerase has no lyase, *i.e.* depolymerizing, activity. However, comparison of the two proteins might provide clues to the mode of action of DS epimerase. In fact, lyase and epimerase reactions are mechanistically related and are even expressed by a single protein, the alginate AlgE7, a single amino-acid residue being essential for both activities (37).

The quantitative comparison of DS epimerase specific activities in different organs (Fig. 1) showed that spleen was the richest site. Stomach, uterus, and ovary had ~one-third of the specific activity of spleen, and kidney, lung, liver ~one-tenth. Skeletal muscle, heart, and brain contained low but detectable activity, whereas serum contained none. Specific activities of the enzyme in lysates from different rat organs generally correlated with the ubiquitous but variable expression of a 4.0-kb SART2/DS epimerase human mRNA transcript (33).

Exhaustive treatment of chondroitin (dK4) substrate with recombinant DS epimerase resulted in conversion of only 8-9% of the GlcA to IdoA residues, largely as isolated units (Fig. 4). This result agrees with previous studies of solubilized microsomal enzyme, which showed a freely reversible reaction with favored retention of *D-gluco* configuration (20). Similar findings applied to solubilized HS epimerase (21), which, however, was found to act in essentially irreversible mode in the intact cell (22). The intracellular epimerase reactions are presumably closely associated with O-sulfation steps (12;38-40) that preclude “back-epimerization” and thus promote generation of IdoA-containing structures. Accordingly, transfection of DS epimerase into cultured cells resulted in formation of DS with sulfated IdoA-containing disaccharide units in blockwise arrangement (Fig. 5).

SART2 was originally identified through its association with certain cancer forms. Western blot analysis thus showed expression of SART2 as a 100-kDa protein in squamous cell carcinoma (33), glioma (33), gynecological cancer (41), pancreatic cancer (42), colorectal carcinoma (43), and prostate cancer (44), but not in breast cancer cell lines or in any normal tissues (33). We assume that the apparent lack of the protein in normal tissues reflected the relative insensitivity of the

Western blot method, such that only tissues with overexpressed SART2/DS epimerase would be detected. Nakao *et al.* (33) identified two peptides in the N terminus and one in the C terminus of SART2 which elicited a cytotoxic reaction in a HLA-A24-restricted CTL cell line. The authors hypothesized that SART2 may be targeted by cancer immunotherapy. Indeed, the three SART2 peptides originally identified were tested in phase I clinical trials in patients with hormone-refractory prostate cancer (44).

The overexpression of DS epimerase in various cancers raises questions about the pathophysiological role of DS chains (although we can not exclude effects of the DS epimerase protein as such, that are independent of its function in DS biosynthesis). A tumor may be

regarded as a specialized organ, in which growing cancer cells are embedded in a supportive stroma composed of cellular components and of a specialized extracellular matrix (45). Various DS-carrying core proteins have been reported overexpressed in different human cancers (46-48), and several DS-dependent processes may be hypothetically related to tumor biology. Potentially relevant candidate protein ligands include heparin cofactor II (49), several growth factors [e.g. HGF (50), FGF-2 and FGF-7 (51)], and chemokines/cytokines [e.g. RANTES and IFN- $\gamma$  (9)]. If DS epimerase can indeed be functionally linked to tumor biology, it might become a target for therapy.

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## FOOTNOTES

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Abbreviations used: aTal<sub>R</sub>, 2,5-anhydro-D-talitol (formed by the reduction of anhydrotalose units, obtained by deamination of galactosamine residues); CS, chondroitin sulfate; CTL, cytotoxic T lymphocytes; dK4, defructosylated K4; DS, dermatan sulfate; ESI, electrospray ionization; GalNAc, 2-acetamido-2-deoxy-D-galactose (*N*-acetyl-D-galactosamine); GlcA, D-glucuronic acid; HexA, hexuronic acid; HS, heparan sulfate; IdoA, L-iduronic acid; LC, liquid chromatography; MS, mass spectrometry; PG, proteoglycan; SART2, Squamous Cell Carcinoma Antigen Recognized by T cell 2

## FIGURE LEGENDS

**Fig. 1. DS Epimerase activity in rat tissues.** Tissue lysates were prepared, and 80 µl of desalted lysates were assayed for epimerase activity (see "Experimental") for 14 h. Specific activities based on protein contents are shown as means ± 2 S.D. of triplicate assays.

**Fig. 2 Isolation of DS epimerase and identification of SART2.** **A**, Gel chromatography of partly purified enzyme. Material derived from bovine spleen microsomal preparation, purified ~200-fold (see Step 5 in Table I), was applied to a Superose 12 column, and effluent fractions were assayed for epimerase activity and protein. *Arrows* indicate elution positions of molecular-weight markers. Active fractions were pooled as indicated and further purified. **B**, SDS-PAGE analysis of purified DS epimerase. Lane 1, the ~43,000-fold most purified preparative sample (see Table I) was separated and stained with Colloidal Blue. Seven bands were excised as indicated, and their tryptic peptides subjected to LC-MS/MS. For each band, the number of proteins identified by MS/MS and the number of SART2-derived peptides are indicated. Lane 2, the ~0.6 million-fold purified analytical sample (see Step 7 of Purification in "Experimental Procedures") was separated and silver stained. The *arrow* indicates the predominant 89 kD band. **C**, Alignment of human SART2 (SwissProt Q9UL01) with bovine SCAFFOLD270174.1. Unique MS/MS peptides matched to the two sequences are underlined. The three transmembrane regions, as defined by hydrophobicity analysis using the SOSUI program, and in agreement with (33), are *boxed*.

**Fig. 3. Expression of SART2/DS epimerase.** Cells were transiently transfected with empty pcDNA 3.1/His expression vector (empty bars) or with the vector containing SART2/DS epimerase in frame with a C-terminal His tag (filled bars). After 48 h, **A**, cell lysates were prepared and 40 µg of desalted protein assayed or **B**, 80 µl of desalted medium was assayed. Data are represented as the means ± 2 S.D. of triplicate assays. Repeated transfections gave similar results.

**Fig. 4. Product analysis after *in vitro* epimerase reaction.** **A**, Gel chromatography of radiolabeled dK4 after epimerase incubation. Desalted cellular lysate (400 µg of protein) from mock-transfected or

SART2/DS epimerase-transfected 293HEK cells were incubated with either 5-<sup>3</sup>H- or 1-<sup>14</sup>C-labeled dK4, in a final volume of 1 ml. After 20 h incubation, the reaction was stopped by addition of NaCl to final 1 M conc., centrifuged at 20,000g x 20 min, and applied to a Superose 12 column, operated at 0.2 ml/min in 0.2M NH<sub>4</sub>HCO<sub>3</sub>. Fractions of ~0.5 ml were collected and 50 µl were counted for radioactivity. <sup>3</sup>H-dK4 incubated with mock-transfected (□) or DS epimerase overexpressing (■) lysate; <sup>14</sup>C-dK4 incubated with mock-transfected (○) or DS epimerase overexpressing (●) lysate. The intact <sup>14</sup>C-labeled polysaccharides were pooled as indicated and subjected to structural analysis. **B**, Size analysis of chondroitinase AC-I split products. <sup>14</sup>C-labeled dK4, reisolated after incubation as in (A), was digested with chondroitinase AC-I and applied to a Superdex Peptide column, eluted at 0.3 ml/min with 0.2 M NH<sub>4</sub>HCO<sub>3</sub>. Fractions were collected every minute and analyzed for radioactivity. <sup>14</sup>C-dK4 non-incubated control (▲), incubated with mock-transfected (○) or DS epimerase-overexpressing (●) cell lysate. Elution positions of di-, tetra- and hexasaccharides derived from dK4 are indicated by *arrows*. **C**, Paper chromatography of disaccharides. <sup>14</sup>C-Labeled dK4, reisolated after incubation as in (A), was extensively deacetylated, followed by deaminative cleavage at pH 3.9. The resulting disaccharides were recovered by gel chromatography and analyzed by paper chromatography. <sup>14</sup>C-dK4 incubated with mock-transfected (○) or DS epimerase-overexpressing (●) cell lysate. Positions of GlcA-aTal<sub>R</sub> (GT) and IdoA-aTal<sub>R</sub> (IT) are indicated by *arrows*

**Fig. 5. Formation and distribution of IdoA residues in DS epimerase-transfected cells.** 293HEK cells were mock- or transiently DS epimerase-transfected and then grown in the presence of inorganic [<sup>35</sup>S]sulfate (see “Experimental”). Labeled CS/DS chains were purified from the culture medium and subjected to chondroitinase B (**A**), or chondroitinase AC-I (**B**) digestion. Split products were applied to a Superdex Peptide column, eluted as in Fig.4. Fractions were collected every minute and analyzed for radioactivity. DS derived from mock-transfected (○) or DS epimerase-transfected (●) cells. Elution positions of di-, tetra-, hexa-, and octasaccharides derived from dK4 are indicated by *arrows*.



TABLE I  
*Purification of DS epimerase*

	Total protein (mg)	Total activity (dpm/h x 10 <sup>-6</sup> )	Recovery (%)	Specific activity (dpm/h/mg x 10 <sup>-3</sup> )	Purification -fold
Microsomal preparation (step 1)	12,102	92	100	8	1
Red Sepharose (step 2)	1,033	77	84	75	10
ConA Sepharose (step 3)	54	28	30	520	68
dK4 Sepharose (step 4)	10.9 (6.5)*	18 (12)*	19 (13)*	1,650	220
Superose12 (step 5)	1.53 (0.32)*	9.3 (6.6)*	10 (7)*	6,080	800
Superose12 (step 6)	0.067	1.8	2.0	26,900	3,500
Red Sepharose (step 7)	~0.004**	1.3	1.4	~330,000	~43,000
Red Sepharose (step 7)					
- analytical sample -***	~1 ng ****	0.005	n.a.	~5,000,000	~600,000

\* Values in parentheses after concentration.

\*\* Evaluation from Colloidal Blue staining after SDS-PAGE.

\*\*\* See purification - Step 7 - in “Experimental Procedures”.

\*\*\*\* Evaluation from silver staining after SDS-PAGE.

TABLE II  
Unique MS/MS peptides identifying human and bovine SART2

Peptide Sequence <sup>a</sup>	Hit <sup>b</sup>	Mass Da (M+H) <sup>+</sup>	Probability <sup>c</sup>	x corr <sup>d</sup>	delta Cn <sup>e</sup>	Ions <sup>f</sup>
AEVAELQLR	h	1028.574	0.99	2.3	0.18	11/16
AIYDIVHR	h	986.5423	0.97	2.6	0.16	12/14
AIYDIVHR	b	986.5423	0.98	2.5	0.27	13/14
EVTDGSLYEGVAYGSYTTR	h	2067.951	1	3.3	0.42	20/36
EVTDGSLYEGVAYGSYTTR	b	2067.951	0.99	3.3	0.36	21/36
GEGVGAYNPQLHLR	b	1510.777	1	3.3	0.49	19/26
GTFASVTYPR	b	1098.558	1	3.7	0.44	15/18
HFDINHFGHPWLK	b	1647.819	1	3.8	0.52	19/24
MAAQPSWLK	h	1130.603	0.98	2.9	0.27	16/18
MYWMDDTGYSEK	h	1525.598	1	3.8	0.46	20/22
MYWMDDTGYSEK	b	1525.598	1	3.8	0.46	20/22
QVLTIMEK	b	961.5392	0.74	1.5	0.23	6/14
SCFSPWEGQVTEDCSSK	b	2003.769	0.99	4.3	0.42	23/32
SLFQYMFLVQR	h	1431.746	0.97	3.3	0.21	15/20
SLFQYMFLVQR	b	1431.746	0.98	3.3	0.34	16/20
TVAIADSNYNWFYGPESQLVFLDK	b	2777.346	0.96	2.6	0.41	13/46
VVAAVEKNGVVFIR	b	1500.89	1	2.1	0.21	20/26
YTFENNVLMFSPAASK	b	1836.9	0.93	2.8	0.23	15/30

<sup>a</sup> All peptides were derived from consensus trypsin cleavage sites.

<sup>b</sup> h, human SART2 (UPTR:Q9UL01); b, bovine SCAFFOLD270174.1.

<sup>c</sup> Peptide prophet probability score (28).

<sup>d,e</sup> Database search peptide probability scores.

<sup>f</sup> Number of matched ions over observed ions.

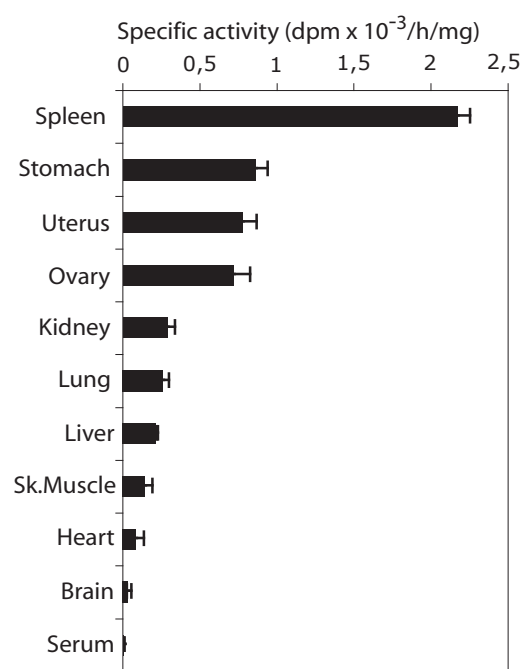


Figure 1

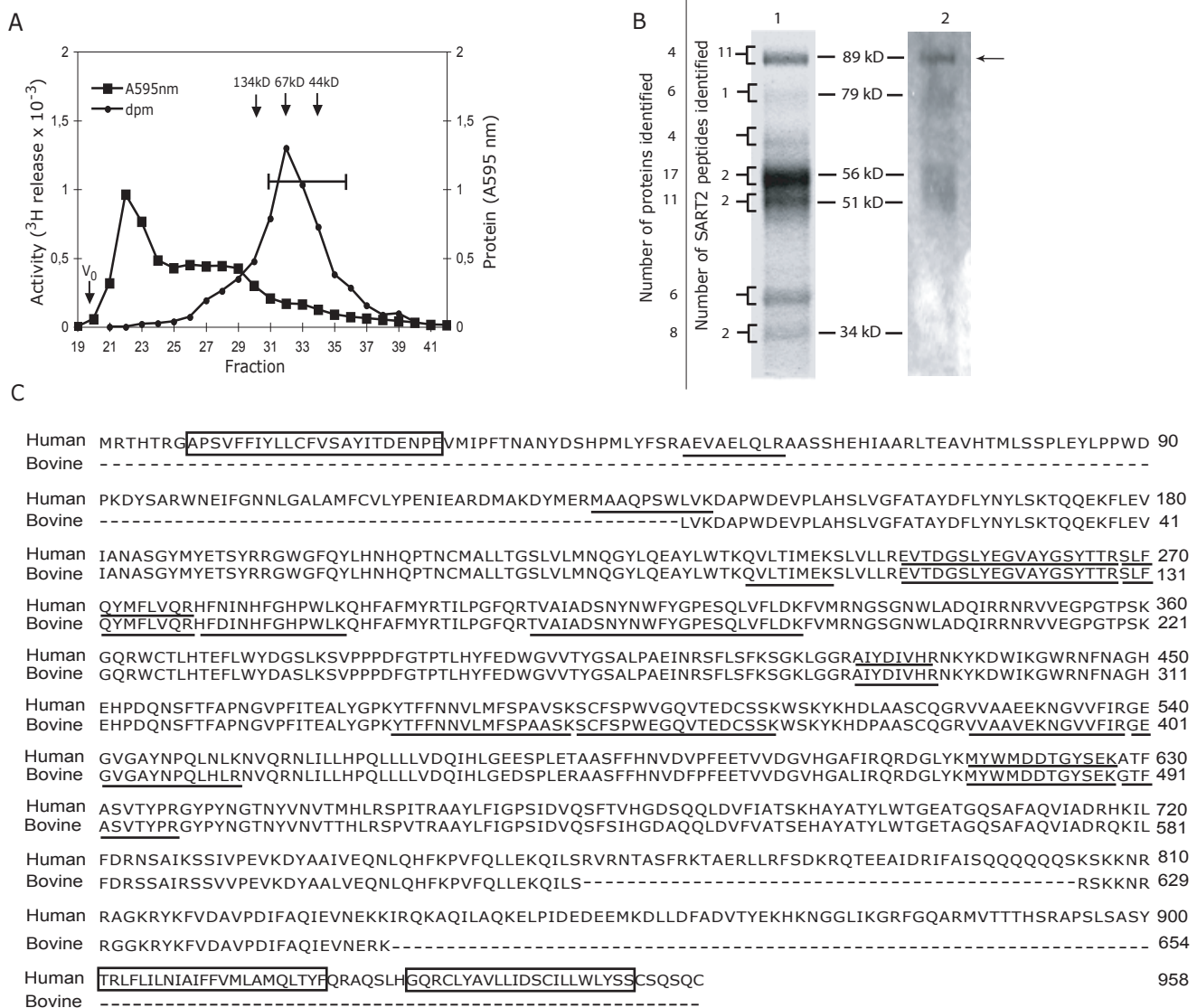


Figure 2

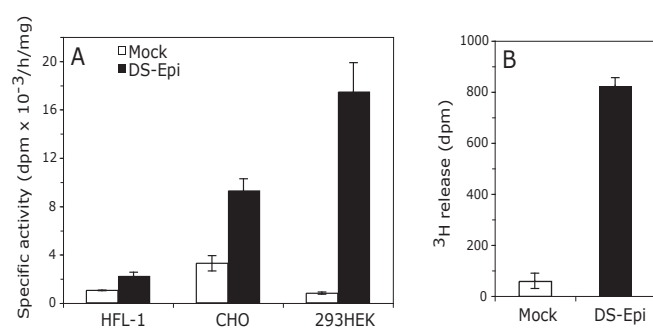


Figure 3

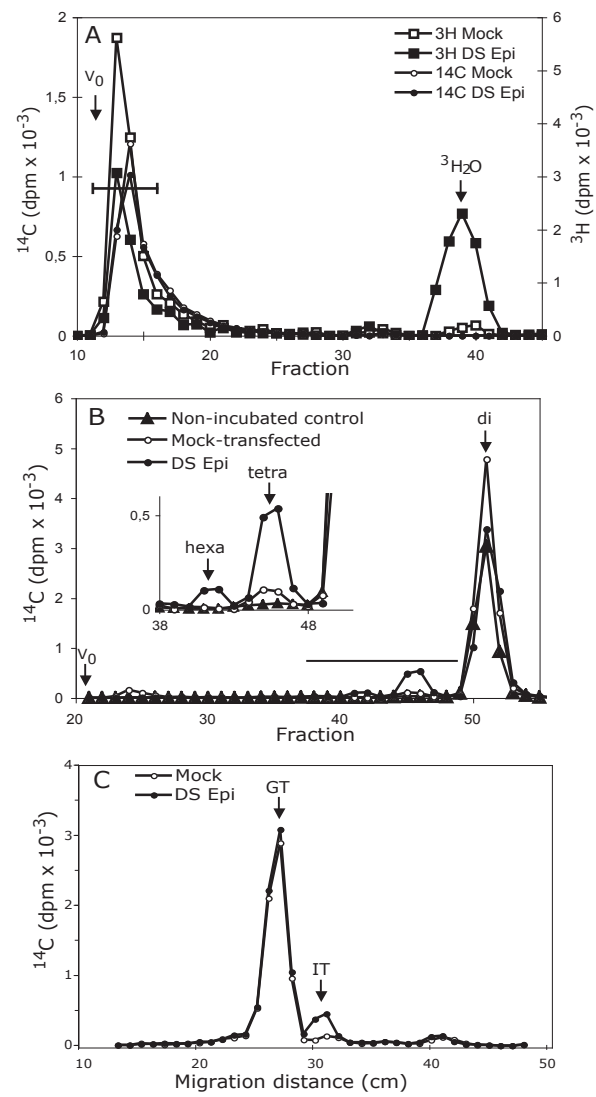


Figure 4

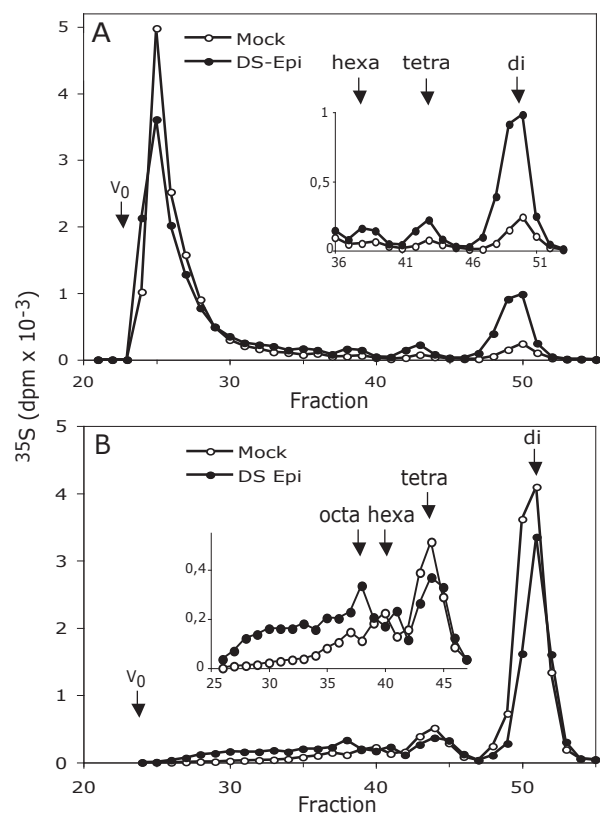


Figure 5