Supporting information for

The specificity of the malarial VAR2CSA protein for chondroitin sulfate depends on 4-*O*-sulfation and ligand accessibility

Running Title

The VAR2CSA tropism

Authors and affiliations

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Materials

Bare fused silica capillary for capillary zone electrophoresis (CZE) (360 µm o.d. × 50 µm i.d.) was purchased from PolyMicro Technologies (Phoenix, AZ), borosilicate glass capillaries (1.0 mm OD × 0.75 mm i.d.) and pulled electrospray emitters (1.0 mm OD × 0.75 mm i.d.) were obtained from CMP scientific (Brooklyn, NY). Coating reagent N-(6-aminohexyl) aminomethyltriethoxysilane (AHS, Gelest, Morrisville, PA) was prepared in toluene as described previously (57).

Methods

CZE Samples. All samples were desalted with 3 kDa Amicon Ultra centrifugal filter (Millipore, Temecula, CA) prior to CZE separation and MS analysis. Filters were conditioned with water, and the sample washed with two filter volumes of HPLC grade water (14,000 × g for 25 min each). Before analysis, GAG samples were diluted in 30 µL of water.

Instrumentation. CZE separations were conducted with an Agilent HP 3D capillary electrophoresis instrument (Wilmington, DE) with a -30 kV potential applied. A 60 cm cation coated bare fused silica capillary was used for CZE, and the inner surface was modified with AHS to reduce migration times. Ammonium acetate (25 mM in 70% methanol) was used as both the background electrolyte (BGE) and the sheath liquid (SL) for reverse polarity experiments. Aqueous GAG samples were injected for 3 s at 950 mbar followed by a background electrolyte (BGE) injection for 10 s at 10 mbar.

An EMASS-II (CMP Scientific, Brooklyn, NY) CZE-MS interface was employed to couple the CZE with a Thermo Scientific Velos Orbitrap Elite mass spectrometer (Bremen, Germany) (58-60). The separation capillary was positioned inside a glass emitter tip at 0.6 mm from the tip of the sheath emitter orifice. An external power supply provided a voltage of -1.75 kV to the sheath liquid for nano-electrospray. MS detection was performed in negative ion mode. Prior to CZE-MS experiments, a semi-automatic optimization of source parameters was performed using sucrose octasulfate to improve sensitivity of sulfated GAGs and reduce sulfate decomposition during ion transfer. The Orbitrap Elite was scanned from m/z 150-2000 in negative ion mode for GAG oligosaccharides with a specified resolution of 120,000 for full MS and 60,000 for MS/MS experiments. Tandem mass spectrometry experiments were performed using collision induced dissociation (CID). Each peak from the electropherogram was averaged to obtain a tandem mass spectrum with mass accuracy of 5 ppm or better. Data analysis was performed using Glycoworkbench (61). Fragments were assigned based on the Domon-Costello nomenclature (62).

Supporting tables

Table S1. MS/MS signature ions for [$^{12}C_6$]aniline tagged dp4 oligosaccharides

Ion	Composition	Calculated m/z	Observed m/z
Y_1	$C_{14}H_{21}N_2O_8S$	377.10	377.09
Y_3^{2-}	$C_{28}H_{41}O_{22}N_3S_2$	417.58	417.56
$^{0,2}X_4^{2-}$	$C_{30}H_{43}O_{23}N_3S_2$	438.59	438.57
$[M\text{-}SO_3]^{2-}$	$C_{35}H_{50}O_{23}N_3S$	456.12	456.04
B_3	$C_{20}H_{26}NO_{19}S$	616.08	616.06
B_3Z_3/B_2	$C_{14}H_{18}O_{13}NS$	440.05	440.03

Table S2. MS/MS signature ions for underivatized dp6 oligosaccharides

Ion	Composition	Calculated m/z	Observed m/z
Z_1	$C_8H_{12}O_8NS$	282.03	282.03
Y_1	$C_8H_{14}O_9NS$	300.04	300.04
Y_3^{2-}	$C_{22}H_{34}O_{23}N_2S_2$	379.05	379.05
Y_5^{3-}	$C_{36}H_{54}O_{37}N_3S_3$	405.39	405.39
$[M\text{-}3H\text{-}SO_3]^{3-}$	$C_{14}H_{18}O_{13}N$	418.08	418.08
$[M\text{-}3H]^{3-}/Z_2/Z_4/C_2/C_4$	$C_{14}H_{20}O_{14}NS$	458.06	458.06
B_5^{2-}	$C_{34}H_{46}O_{33}N_2S_2$	537.07	537.07
B_3	$C_{20}H_{28}O_{19}NS$	618.10	618.08

Supplemental Figure Legends

Supplemental Figure S1. LC-MS2 analysis of A-ChABC resistant dp4. We have previously reported that iduronic acid- and glucuronic acid-containing dp4 can be distinguished by MS2 analysis (28). Here, resistant tetrasaccharide products resulting from the enzymatic digestion of placental CS and R-ofCS using A-ChABC, along with the CS (D0a4/6-G0a4/6) and DS (D0a4-I0a4) dp4 standards, were analyzed by MS2 after collision-induced dissociation (CID). Generic dp4 structures and possible fragment ions are shown in A and Table S1. (B) The placental CS dp4 appeared as three co-eluting peaks, appearing at 67.0 min (component 1), 68.0 min (component 2), and 69.0 min (component 3). (C-E) The extracted ion chromatogram obtained for the three components, gated for *m/z* = 496.59. As previously reported, the three components can be distinguished by the ratio of the Y_1 ion to the desulfated precursor ion [M-SO_3-2H] (28). The first component had a Y_1 ion as the predominant product ion as well as a B_3 ion, Y_3 ion, $^{0,2}X_4$ cross-ring cleavage ion, and neutral losses of sulfates from the precursor ion (Y_1:[M-SO_3-2H] = 1:1.4) (C). Component 2 had Y_3 as the predominant product ion, along with $^{0,2}X_4$ cross-ring cleavage ion, neutral losses of sulfates from the precursor ion, and a Y_1 ion (Y_1:[M-SO_3-2H] = 1:0.5) (D). Component 3 had similar product ions as component 1, but more neutral losses of sulfates from the precursor ion (Y_1:[M-SO_3-2H] = 1:0.8) (E). (F-I) The R-ofCS dp4 species also appeared as three co-eluting species, with similar elution times and fragmentation patterns as the placental CS dp4 species (B-E), but with a higher proportion of component 1 than 2. (J) The CS dp4 standards consisted of one distinct peak eluting at 67.0 min (component 1) and two co-eluting peaks at 69.0 min (component 2) and 69.5 min (component 3). (K-M) The respective ratios of Y_1 ion to the desulfated precursor ion [M-SO_3-2H] were 1:0.3, 1:1.4, and 1:0.7. This suggests, that the placental CS and R-ofCS component 1 (C, G) may have the same structure as the CS component 1 (K), while the placental CS and R-ofCS component 3 (E, I) may have the same structure as CS component 2 or 3 (L, M). (N) The DS dp4 standard eluded as a single broad peak at 68.0 min. Similar to the placental CS component 2 (D) and R-ofCS component 2 (H), the predominant DS dp4 product ion was Y_3 and had a Y_1:[M-SO_3-2H] ratio of 1:0.2 (O). These data suggest that placental CS contains a high abundance of iduronic acid, which are also found in R-ofCS.

Supplemental Figure S2. MS2 analysis of A-ChABC resistant dp6. To further characterize the resistant hexasaccharides resulting from the enzymatic digestion of R-ofCS by A-ChABC (Figure 2*C*), underivatized R-ofCS digest was separated by capillary zone electrophoresis (CZE) (Supplemental Methods). (A) The dp6 species eluded as two separate peaks (1 and 2). (B) MS2 was performed using collision-induced dissociation (CID) on CS (B) and DS (C) dp6 standards and compared to the R-ofCS component 1 (D) and 2 (E). A generic CS/DS dp6 structure and possible fragment ions are shown in F and Table S2. (B) The extracted ion chromatogram of the CS dp6 showed an abundance of B_5 and Y_1 ions, along with B_3, Y_3, and a [M-3H]/Z_2/Z_4/C_2/C_4 product ion, similar to the fragmentation pattern of the R-ofCS component 1 (D). (C) The DS dp6 standard contained additional product ions, including a Y_5 ion and neutral losses of sulfate from the precursor

ion, which was similar to the fragmentation pattern of the R-ofCS component 2 (E). These data suggest that the A-ChABC resistant R-ofCS dp6 species contain a mixture of glucuronic acid- and iduronic acid-containing structures.

Supplemental Figure S3. DNA sequences of HeLa (A) *CHST14*, (B) *CHPF*, and (C) *CHPF2* mutant alleles.

Supplemental Figure S4. (A) Disaccharide composition of HeLa WT and HeLa *CHST14*$^{-/-}$ clone C4 was analyzed by LC-MS (*N*=3). Clone C4 was significantly enriched in 4-*O*-sulfated CS, and expressed slightly reduced cell surface CS compared to WT (B, *N*=3). (C) Similar to clone C3 (Figure 2*F*), clone C4 had significantly less DS/CSC dp4 generated by A-ChABC digestion, suggesting a reduction in iduronic acid residues. This was confirmed by monosaccharide analysis, which showed that the uronic acid content of clone C4 was 79% glucuronic acid and 21% iduronic acid, whereas WT consisted of 41% glucuronic acid and 59% iduronic acid. (D) Titration of rVAR2 to HeLa WT and *CHST14*$^{-/-}$ C4, as measured by flow cytometry, showed significantly higher binding to clone C4 than WT (*N*=3). Curve fitted with nonlinear 4PL regression. Error bars indicate mean ± SEM. ***p*<0.01, **p*<0.05, and #*p*<0 by unpaired t-test.

Supplemental Figure S5. CS disaccharide analysis of intact ofCS (I-ofCS) derived from 50 µg of human (A) liver, (B) muscle, (C) lung, (D) lymph node, and (E) spleen CS (*N*=1), analyzed by reverse-phase high performance-liquid chromatography, as previously described (23). (F) Percentage of I-ofCS purified from input CS (*N*=1).

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