Chapter 6: Generation of Chondroitin Sulfate Antibodies*

Background

The biological activities of chondroitin sulfate glycosaminoglycans (CS GAGs) are potentially regulated by a "sulfation code," through which distinct sulfation patterns serve as molecular recognition elements for growth factors and modulate cellular growth (see Chapters 3, 4, and 5). CS sulfation motifs are tightly regulated *in vivo*, supporting this hypothesis, and sequence expression is tissue specific and developmentally controlled.[1,2] For example, differentially sulfated CS variants have been found in cartilage, connective tissues, defined brain regions and along axonal growth tracts, and specific sulfotransferases regulate the sulfation profile of CS chains in the embryonic brain and in areas of central nervous system injury.[3,4,5]

Four major approaches have been used to monitor patterns of sulfation: RT-PCR (reverse transcription polymerase chain reaction) for analysis of CS sulfotransferase mRNA levels, *in situ* hybridization to probe for the expression of CS sulfotransferases,[5] isolation and examination of CS chains,[1,6] and immunohistochemistry or immunocytochemistry with CS antibodies,[2,3,7-12] but these current methods do have limitations. RT-PCR can be used to determine the amounts of known sulfotransferase mRNA in specific tissues, such as the brain or cartilage, but with *in situ* hybridization, it is possible to pin point the exact location of expression through staining slices from the regions of interest. These techniques imply the potential sulfation patterns being

* Neuronal staining was done in collaboration with Cristal I. Gama, a graduate student in the Hsieh-Wilson laboratory. Dr. Susan Ou, Director of the Monoclonal Antibody Facility at Caltech, assisted with antibody generation.

generated, but do not confirm that they are present, as it is unclear how the sulfotransferases may be regulated after expression. In addition, while many CS sulfotransferases have been identified, it is possible that not all of them have been characterized, thus prohibiting determination of their mRNA levels.[13]

To isolate and analyze CS side chains, CS proteoglycans (PGs) from tissues are isolated and digested with chondroitinase ABC, an enzyme that cleaves CS polysaccharides to generate disaccharides. The disaccharides are then purified by high-performance liquid chromatography (HPLC), and compared to known CS disaccharide standards to determine the sulfation motifs present. This approach is tedious, and in order to obtain enough material for HPLC analysis, only the sulfation profiles of entire organs or tissues can be established, not the profiles of distinct sections, slices, or cells.

The final methods to elucidate cellular or tissue specific distribution of CS GAGs are through use of immunocytochemistry or immunohistochemistry with antibodies for defined CS sulfation motifs. These are the most direct approaches for studying the localization of sulfation motifs, and enable systematic study of distinct cell types and regions of the brain or embryo. Several commercial CS antibodies exist, and are summarized in Table 6.1. A broad range of antigens have been used to generate CS antibodies including proteoglycans,[14-17] bone proteins,[2,18,19] collagen,[20] cell extracts,[21-23] glycoproteins from the mouse brain,[24] and chondroitinase ABC-digested CSPGs.[3,25] Extensive chondroitinase ABC digestion cleaves CS side chains and leaves behind disaccharide “stubs” on the protein core, and antibodies 1B5, 2B6, and 3B3 were raised against these “stubs.” Mixtures of sulfation patterns are present on all of these haptens, making generation of an antibody that recognizes a distinct sulfation sequence difficult.

Typically, the specificities of these antibodies have been determined by competition assays through which binding of the haptens to the antibodies is inhibited by unsaturated disaccharide standards or NMR characterized hexa- and octasaccharides with a variety of sulfation patterns from digested chondroitin sulfate polysaccharides.[2,3,14-26] Due to the size and non-native structure of the unsaturated disaccharides and the mixed sulfation of the hexa- and octasaccharides, it is difficult to fully elucidate the specificities of these antibodies. With our synthetic CS molecules (see Chapters 3 and 4), we saw the potential to generate and characterize antibodies with CS oligosaccharides of known sulfation sequence, creating the first CS antibodies made with structurally-defined haptens.

Antibody	Antigen	Specificity
1B5	Chondroitinase ABC digested rat chondrosarcoma proteoglycan	Unsaturated unsulfated disaccharides
2B6	Chondroitinase ABC digested bovine articular cartilage proteoglycan	Unsaturated CS-A disaccharides
3B3	Chondroitinase ABC digested rat chondrosarcoma proteoglycan	Unsaturated CS-C disaccharides
LY111	Chicken-type IX collagen containing chondroitin-4-sulfate	CS-A polysaccharides
MC21C	Partially purified adult rat bone proteins	CS-C polysaccharides
MO225	Chick embryo limb bud proteoglycan	CS-D polysaccharides, some CS-C and CS-E, little CS-A
473HD	"rest-L2" glycoprotein fraction from adult mouse brain	CS-D polysaccharides, DSD-1 proteoglycan
CS-56	Ventral membranes of chicken gizzard fibroblasts	CS-A, CS-C, and CS-D polysaccharides
mCS6	M14 human melanoma cell extract	CS-C polysaccharides
mCS4	Mouse proteoglycan	CS-A polysaccharides
2H6	Rat brain proteoglycans	CS-A polysaccharides

Table 6.1: Commercially available chondroitin sulfate antibodies.

Development of antibodies to synthetic chondroitin sulfate molecules

Synthetic tetrasaccharides **79**, **85**, and **88**, representing three major CS sulfation patterns found *in vivo*,[27] conjugated to keyhole limpet hemocyanin (KLH) were used as antigens to generate mouse monoclonal antibodies (Figure 6.1). Ozonolysis of the anomeric allyl group of the tetrasaccharides was followed by treatment with KLH and $NaBH_3CN$ in H_2O adjusted to pH 9.5 with 5% K_2CO_3. The same method was used to generate bovine serum albumin (BSA) conjugates needed for later dot blot and enzyme-linked immunosorbent assay (ELISA) analyses. For each KLH conjugate, three Balb/c female mice, 4-6 weeks old, were primed and boosted at 2-week intervals for a total of 5 intraperitoneal injections (5 µg per injection). Bleeds were taken 1 week after each injection, monitored by dot blot analysis, and the most responsive mouse was boosted and sacrificed after three days.[28] Strong, specific responses were observed in the pre-bleeds for the CS-A and CS-E antibody producing mice, but the mice treated with the CS-C KLH conjugate made antibodies that recognized CS-C and CS-E (Figure 6.2). Spleen cells were fused with HL-1 murine myeloma cells as described previously by Lebron *et al.*,[28] and multiclonal and monoclonal cell lines were screened via ELISA and dot blot analysis.

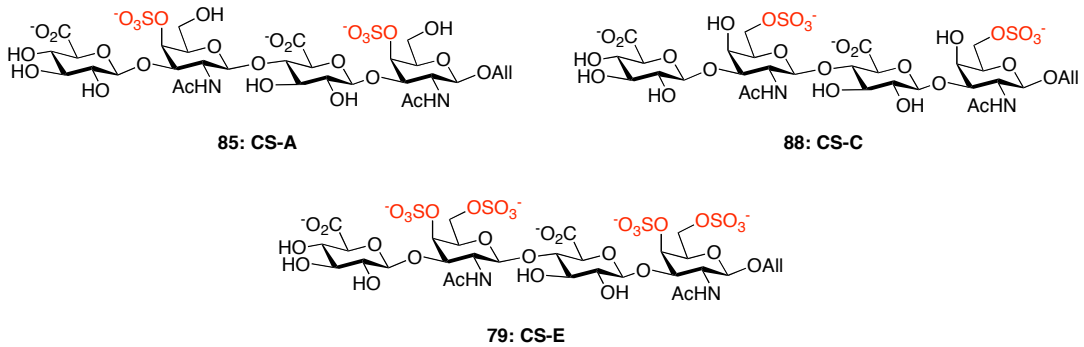

Figure 6.1: Compounds for antibody generation.

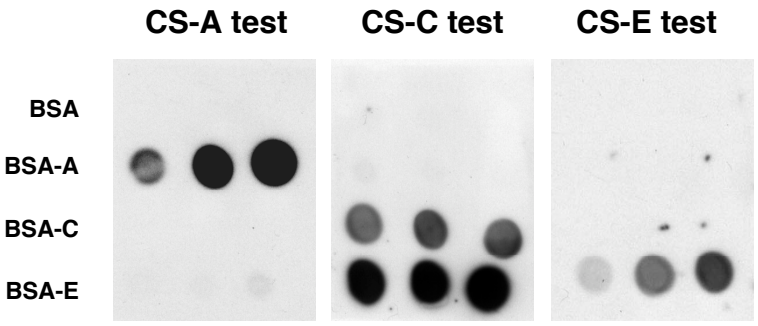

Figure 6.2: Dot blots of antibody pre-bleeds. The left blot shows the CS-A pre-bleed with 25, 50, and 100 ng BSA, BSA-C, and BSA-E spots and 1, 5, and 10 ng BSA-A spots. The central blot shows the CS-C pre-bleed with 25, 50, and 100 ng BSA, BSA-A, and BSA-E spots and 1, 5, and 10 ng BSA-C spots. The right blot shows the CS-E pre-bleed with 25, 50, and 100 ng BSA, BSA-A, and BSA-C spots and 1, 5, and 10 ng BSA-E spots. Pre-bleeds were diluted 1:2000.

Hundreds of multiclonal and monoclonal cell lines were screened for each antibody, and five CS-A, thirteen CS-C, and eight CS-E specific monoclonal antibodies were identified after ELISA and dot blot assessments (Table 6.2).[29,30] Despite the response of the mice injected with CS-C-KLH to both CS-C and CS-E, CS-C selective monoclonals were isolated.

anti-CS-A monoclonal antibodies	anti-CS-C monoclonal antibodies	anti-CS-E monoclonal antibodies
10G9-2B5	7A11-2E8	2D11-1D11
10G9-2C3	7A11-2G6	2D11-1A10
10G9-1D5	7A11-2G9	2D11-1E6
10G9-1F2	7A11-1H10	2D11-1F2
11D6-1F5	7A11-2E11	2D11-1C4
	5D2-2C2	2D11-1C5
	5D2-2F5	2D11-1H8
	5D2-1D2	2D11-2D12
	5D2-2F8	
	5D2-1F10	
	5D2-1H3	
	5D2-2G12	
	8B9-2F4	

Table 6.2: List of anti-CS-A, -C, and –E monoclonal antibodies generated from the synthetic chondroitin sulfate oligosaccharides **85: CS-A**, **88: CS-C**, and **79: CS-E**.

Characterization of monoclonal antibodies 10G9-2B5, 5D2-1D2, and 2D11-2A10

The specificities of anti-CS-A monoclonal 10G9-2B5, anti-CS-C monoclonal 5D2-1D2, and anti-CS-E monoclonal 2D11-2A10 were further tested by microarray analysis (see Figure 5.13, Chapter 5) and dot blots with CS-BSA conjugates and CS polysaccharides using supernatants from the monoclonal cell line cultures. 10G9-2B5 showed exquisite specificity for the CS-A tetrasaccharide motif by microarray and dot blot analysis, with a small amount of the CS-E tetrasaccharide recognized at high concentrations (Figure 6.3). CS-A, CS-B, CS-C, CS-D, CS-E, heparan sulfate (HS), and heparin polysaccharides were also tested, and the CS-A and CS-B polysaccharides were recognized. CS-B is a dermatan sulfate variant structurally similar to CS-A, with sulfation at the C-4 hydroxyl of *N*-acetylgalactoamine, but the uronic acid subunit is iduronic acid instead of glucuronic acid.[27] Since the polysaccharides are mixtures only 30 – 90% concentrated in a specific sulfation motif, it is possible the antibody is binding CS-A sulfated units contaminating the CS-B preparation, an example of the difficulty of fully-characterizing antibodies using polysaccharide mixtures. Despite recognizing the CS-E tetrasaccharide at high concentrations, the CS-E polysaccharide was not bound by 10G9-2B5.

Anti-CS-C monoclonal 5D2-1D2 selectively identified the CS-C tetrasaccharide on microarrays and dot blots, however, the antibody did not bind the CS-C polysaccharide on the dot blot (Figure 6.3). Interesting, polylysine microarray analysis did demonstrate recognition to the CS-C polysaccharide, suggesting display of the molecule on the dot blot was not optimal. Commercially available anti-CS-A and anti-CS-C antibodies mCS4 and mCS6 did not recognize the CS-A and CS-C

tetrasaccharides, but bound the respective polysaccharides with little to no binding of the other motifs, indicating a longer sequence than a tetrasaccharide is required (Figure 6.4).

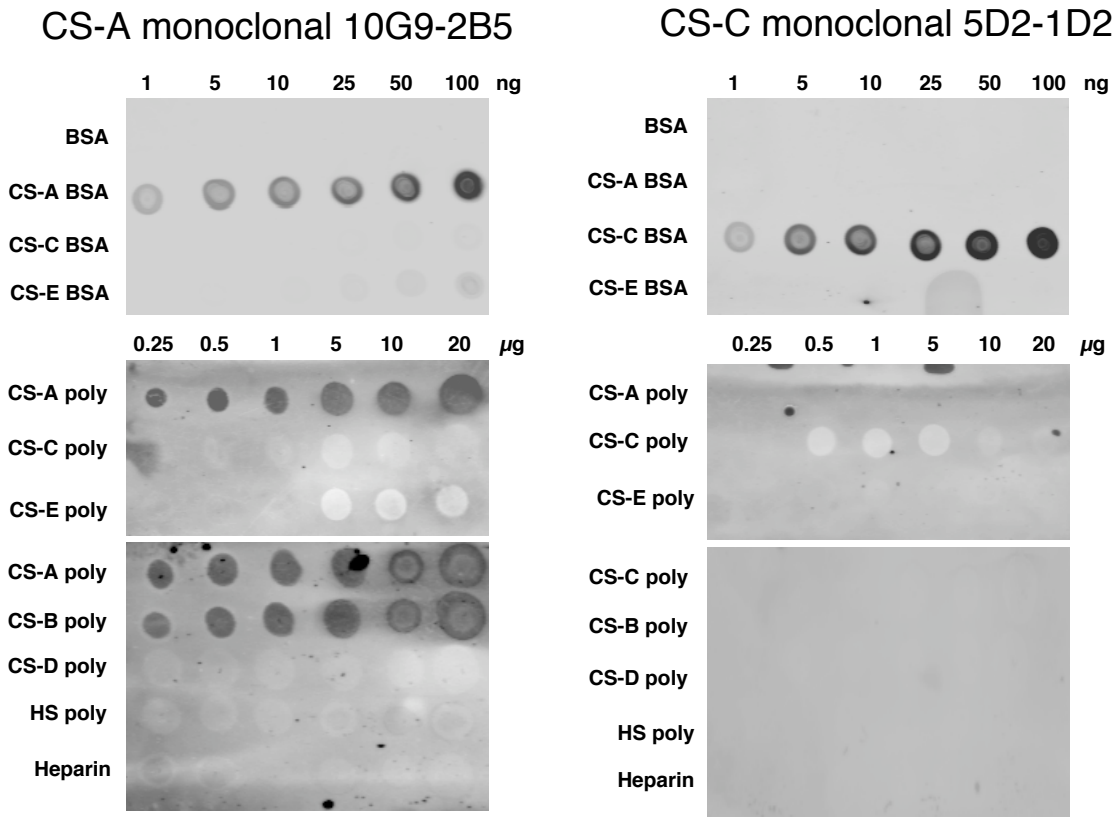

Figure 6.3: Dot blot characterization of anti-CS-A 10G9-2B5 and anti-CS-C 5D2-1D2 monoclonal antibodies.

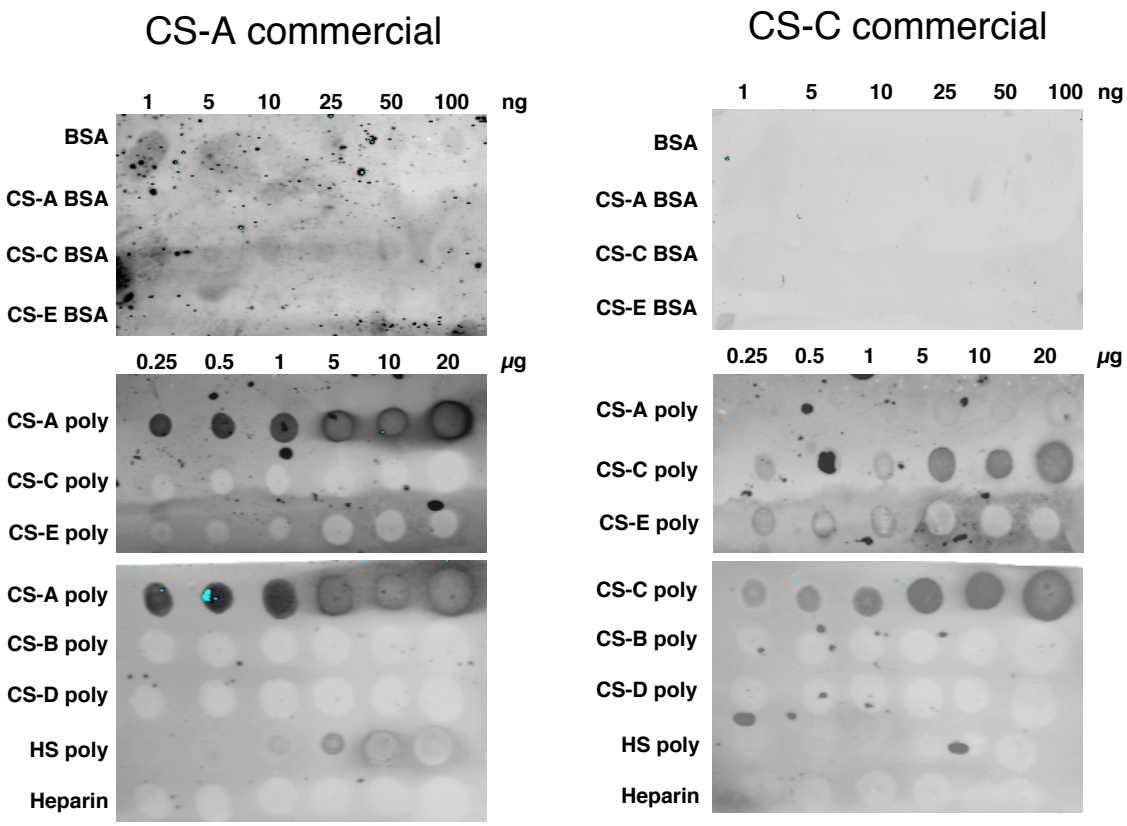

Figure 6.4: Dot blot characterization of commercial anti-CS-A and anti-CS-C antibodies.

Monoclonal antibody 2D11-2A10 for the CS-E tetrasaccharide beautifully bound CS-E with only a small amount of CS-C binding observed, and the CS-E polysaccharide was exclusively observed by dot blot analysis (Figure 6.5). We decided to analyze binding of a commonly used anti-CS antibody, CS-56, and found it bound CS-A, -C, and –D polysaccharides, but had no recognition of the CS-E polysaccharide or the tetrasaccharides (Figure 6.5), so to our knowledge, 2D11-2A10 is the first CS-E specific antibody reported in the literature. With all of the antibodies fully characterized, we produced and purified ascites for further studies including Western blotting of brain lysates, immunocytochemistry, and immunohistochemistry.

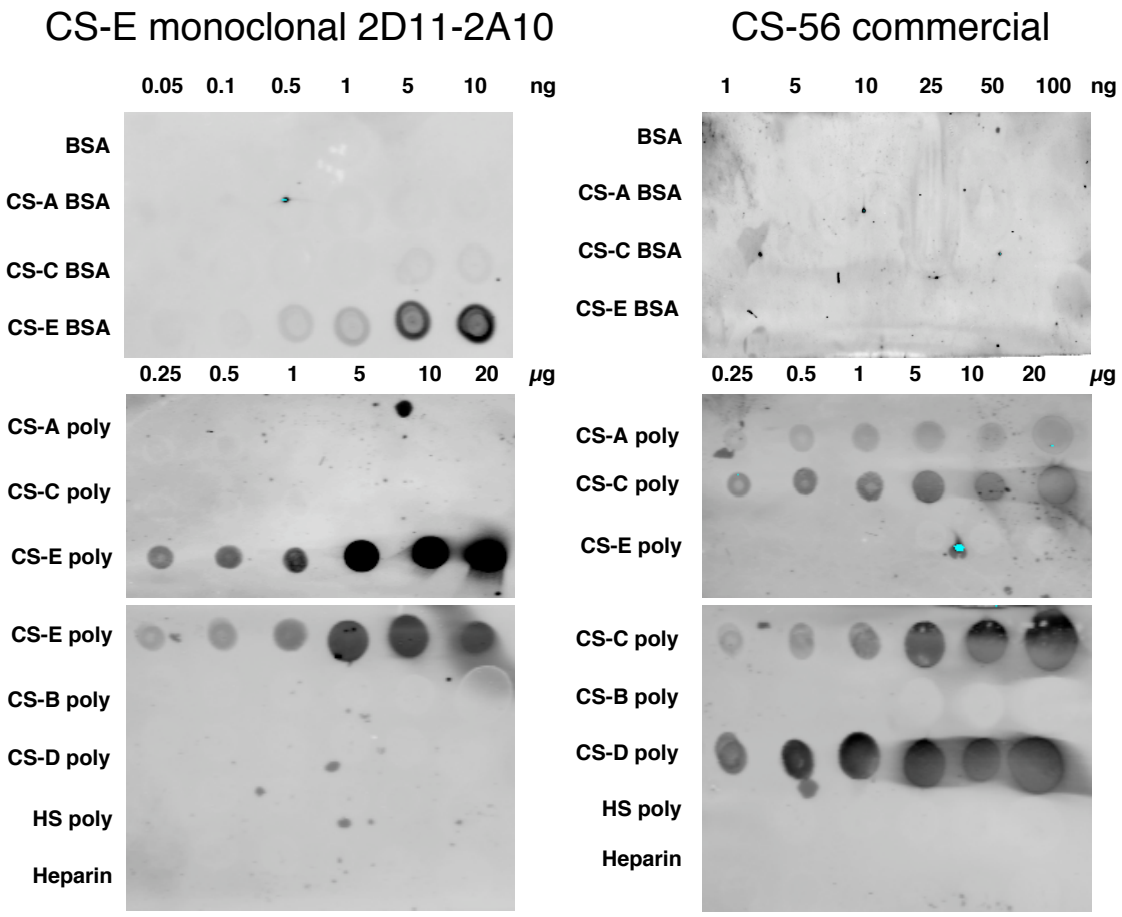

Figure 6.5: Dot blot characterization of anti-CS-E monoclonal 2D11-2A10 and commercially available CS-56.

Staining with the chondroitin sulfate antibodies

Cerebellum, cortex, hippocampus, and striatum were isolated from the adult rat brain, the sections were homogenized, and the resulting lysates treated or not treated with chondroitinase ABC were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred to nitrocellulose, and the Western blots probed with the three CS monoclonal antibodies, 10G9-2B5, 5D2-1D2, and 2D11-2A10 (Figures 6.6 – 6.8). A small fraction of the proteoglycans in the adult rat brain contained the CS-A and CS-E sulfation motifs, but CS-C was the predominantly

expressed form. Chondroitinase ABC digestion enhanced the signal, presumably because the smaller binding motifs generated after treatment are better recognized by the antibodies raised against tetrasaccharide haptens.[6] The cerebellum appeared to be particularly enriched in CSPGs, agreeing with previous studies.[7,11]

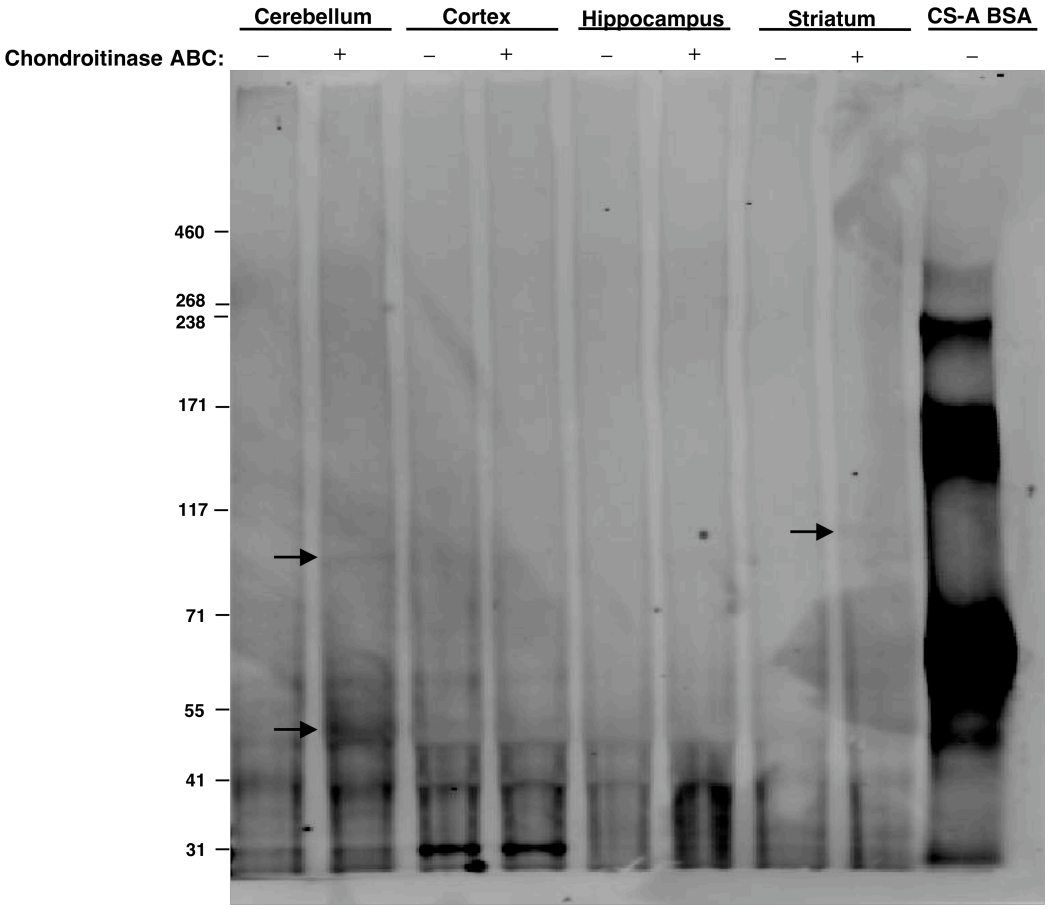

Figure 6.6: Western blot of adult rat brain lysates probed with anti-CS-A monoclonal antibody 10G9-2B5. 50 μg of lysate and 2 ng CS-A BSA were added to lanes of a 3 – 8% Tris-acetate minigel. Indicated samples were treated with 100 mU of chondroitinase ABC for 2 h at 37 °C.

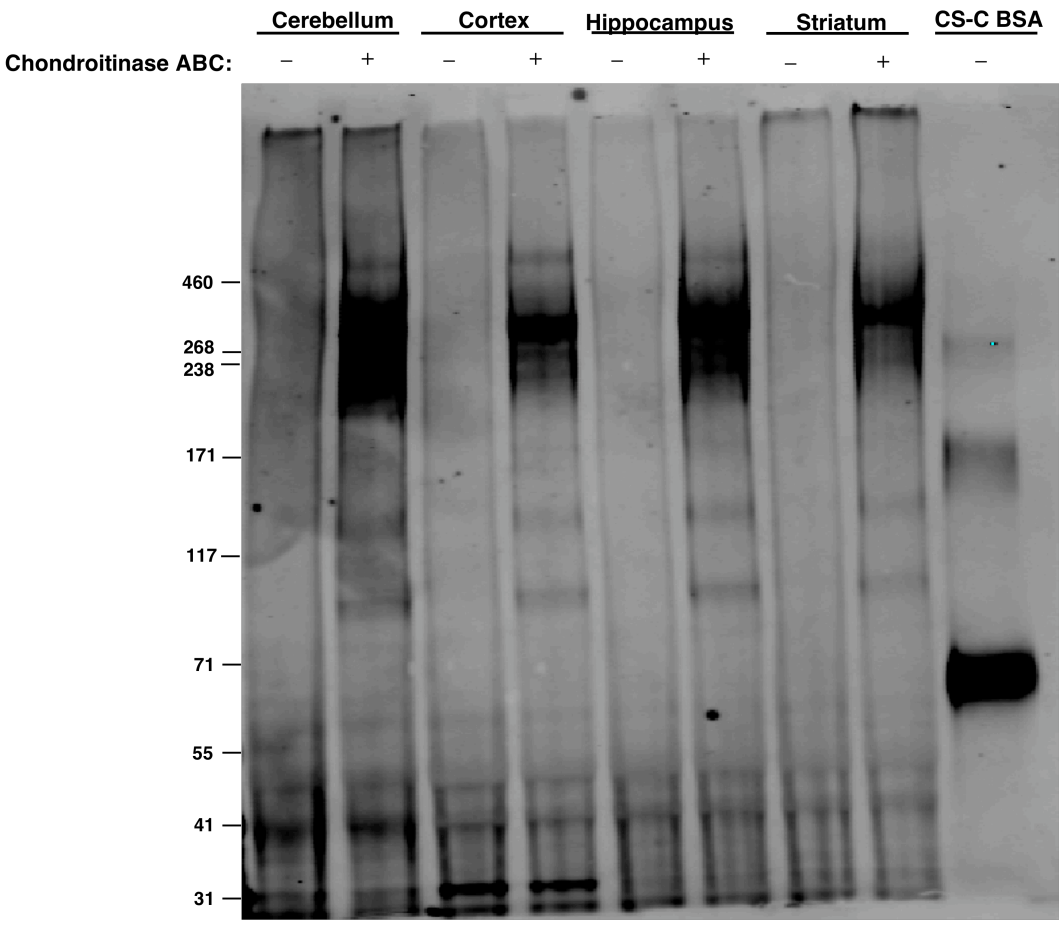

Figure 6.7: Western blot of adult rat brain lysates probed with anti-CS-C monoclonal antibody 5D2-1D2. 50 μg of lysate and 2 ng CS-C BSA were added to lanes of a 3 – 8% Tris-acetate minigel. Indicated samples were treated with 100 mU of chondroitinase ABC for 2 h at 37 °C.

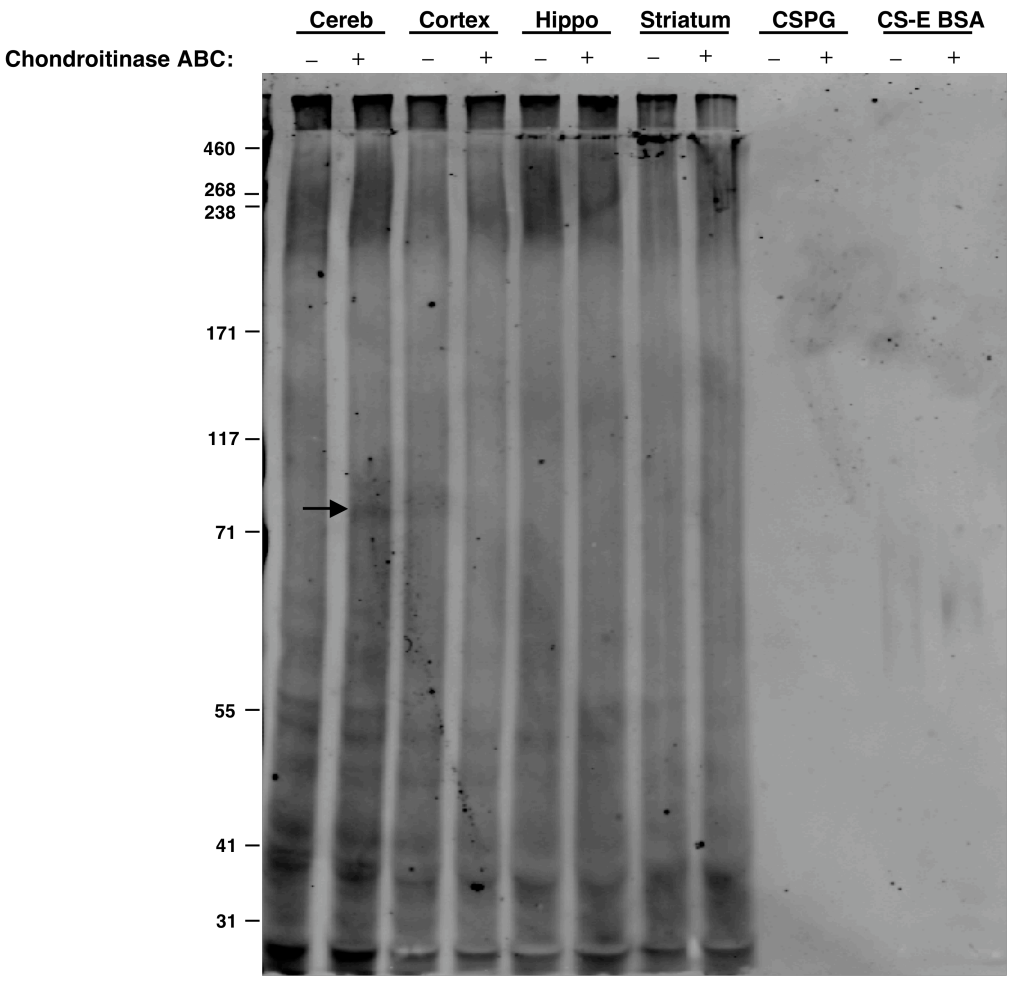

Figure 6.8: Western blot of adult rat brain lysates probed with anti-CS-E monoclonal antibody 2D11-2A10. 200 μg of lysate, 5 μg of CSPG and 2 ng of CS-E BSA were added to lanes of a 6% SDS-PAGE. Indicated samples were treated with 100 mU of chondroitinase ABC for 2 h at 37 °C. Cereb = cerebellum, Hippo = hippocampus, CSPG = chondroitin sulfate proteoglycan, a mixture of chick brain proteoglycans.

Cristal Gama isolated embryonic rat brain lysates, treated them with chondroitinase ABC, resolved the lysates by SDS-PAGE, transferred the proteins to nitrocellulose, and probed the Western blot with the anti-CS-E antibody 2D11-2A10 (Figure 6.9). Strong binding was observed in the hippocampus, olfactory bulb, cerebellum, and midbrain, and recognition increased upon chondroitinase ABC treatment. Comparison of the embryonic and adult rat brain lysate Western blots indicates that CS-E proteoglycans are up-regulated in the embryonic brain, demonstrating that sulfation

patterns are modulated throughout development and implicating the CS-E motif as important in regions of growth.

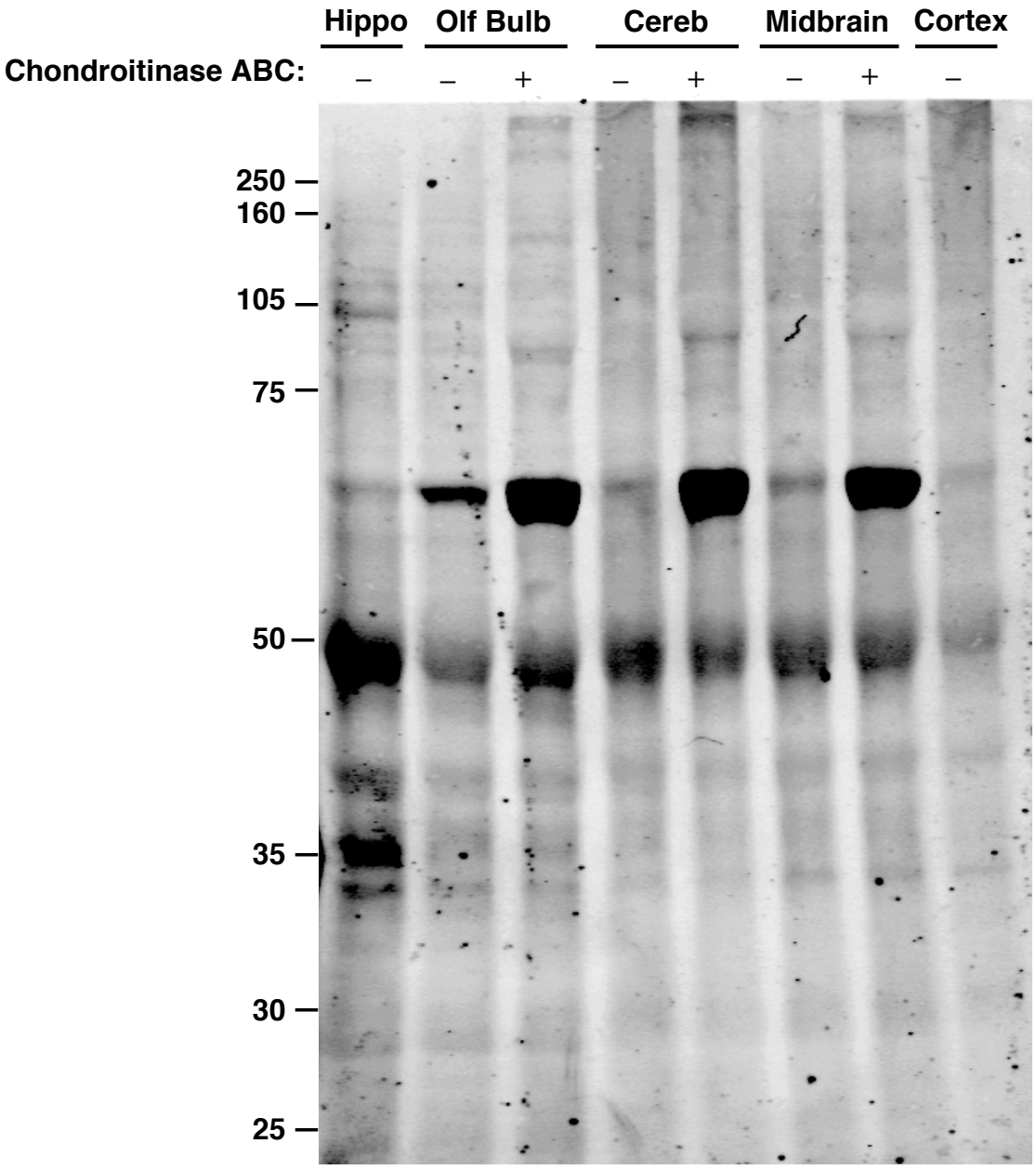

Figure 6.9: Western blot of embryonic day 20 rat brain lysates probed with anti-CS-E monoclonal antibody 2D11-2A10. 200 μg of lysate were added to lanes of a 10% SDS-PAGE. Indicated samples were treated with 100 mU of chondroitinase ABC for 2 h at 37 °C. Cereb = cerebellum, Hippo = hippocampus, Olf Bulb = olfactory bulb.

Additionally, Cristal Gama cultured dorsal root ganglion (DRG) neurons, a good model for the spinal cord, and treated the cells with 2D11-2A10 (Figure 6.10). Intense staining was observed in the axons and neurite processes with weaker staining in the cell body, indicating the presence of CS-E proteoglycans in those areas.

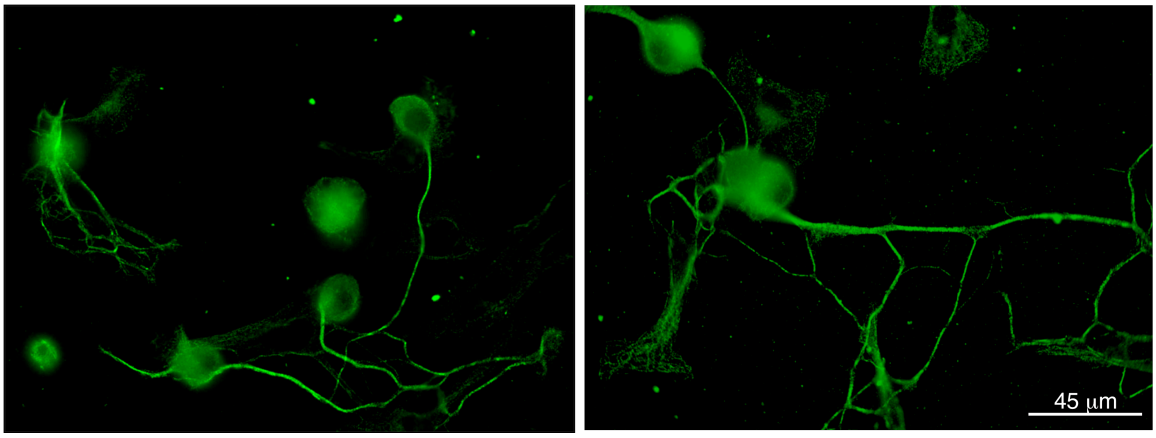

Figure 6.10: Staining of DRG neurons from embryonic day 20 rat spinal cord cultured for 15 hours *in vitro* with the anti-CS-E antibody 2D11-2A10 (1:750).

Discussion

With our synthetic CS tetrasaccharide library, we were able to generate and characterize the first antibodies to be made against structurally-defined CS antigens for three of the major sulfation patterns *in vivo*, CS-A, CS-C, and CS-E. The tetrasaccharide haptens and polysaccharides bearing the same respective motifs were strongly and specifically recognized by the antibodies, making them useful tools for studying CS sulfation in the developing and adult brain, and in isolating and characterizing CSPGs.

Western blot analysis of adult rat brain lysates showed the presence of CS-A, -C, and –E containing proteoglycans, with the predominant sulfation pattern being CS-C. Up-regulation of monosulfated CS disaccharides in the adult brain has previously been reported, agreeing with our results, but the low amount of CS-A containing proteoglycans we observed was unexpected, and indicates a potential need to optimize the use of these antibodies.[6,27] The Western blots might be improved by elucidating the ideal amount of antibody needed for probing the blot, adding more lysate to the lanes of the gel, and purifying the proteoglycan fraction from the tissue of interest to eliminate excess proteins. Intriguingly, the highest amounts of CSPGs found were in the cerebellum, where CS has been shown to be important in Purkinje cell and glial cell interactions. Purkinje cells are one of the largest neuron types in the brain and are important in inhibitory and excitatory responses.[7,11]

A small fraction of the proteoglycans of the adult rat brain displayed CS-E sulfation, but this pattern was dramatically up-regulated in the embryonic brain, demonstrating the presence of oversulfated motifs in areas of development. CS-E

proteoglycans were found in the embryonic hippocampus, olfactory bulb, cerebellum, midbrain, and to a lesser extent, the cortex. The olfactory bulb is of particular interest as the CSPGs neurocan and phosphacan are important in promoting axonal growth and patterning in this structure.[31] In addition, in Chapter 5 we discovered that CS-E selectively bound to Nogo-A, a protein highly present in the developing olfactory bulb, but weakly expressed in the adult olfactory system. Nogo-A may be involved in axonal growth through the regulation of microtubule dynamics,[32] and perhaps some of CS-E's growth promoting properties are mediated through a pathway involving Nogo-A. Further investigation of this possibility is required, as well as identification of the proteoglycans resolved by SDS-PAGE through use of mass spectrometry or by probing Western blots with both the CS monoclonal antibodies and commercially available proteoglycan antibodies.

Staining of DRG neurons with 2D11-2A10 revealed CS-E sulfation on axons, and confirmed the presence of this motif on membrane bound CS-containing proteins. Growth factors, as discussed in Chapters 4 and 5, may interact with these cell-surface proteoglycans and are then brought into the optimal alignment for binding to their receptors. Cristal Gama plans to investigate this possibility by examining embryonic and adult brain slices with both CS and BDNF antibodies, and determining whether this growth factor co-stains with the CS-E sulfation motif during development. Through use of our antibodies, we can systematically investigate growth factor and CS co-expression, monitor the appearance and disappearance of specific CS sulfation patterns in the embryo and adult, and determine the CS motifs up-regulated after central nervous system injury.

Experimental Procedures for Chapter 6

CS antibody development

The anti-CS-A, anti-CS-C, and anti-CS-E monoclonal antibodies were generated according to standard immunological techniques, using the CS-A, CS-C, and CS-E tetrasaccharides conjugated to keyhole limpet hemocyanin (KLH) as the antigens.[28] The conjugation of tetrasaccharides **79**, **85**, and **88** to KLH were performed as follows. Ozonolysis of the anomeric allyl group of the tetrasaccharide (0.51 μmol) as described in Chapters 4 and 5 was followed by treatment with KLH (0.44 mg, 0.0063 μmol) and $NaBH_3CN$ (0.5 mg) in H_2O (pH 9.5 with 5% K_2CO_3) for 2 d at rt. The product was then exhaustively dialyzed against 0.01 M Na_2HPO_4, 0.15 M NaCl, pH 7.4 at 4 °C and the protein concentration determined by BCA assay (Pierce). The epitope density was determined by comparing the conjugated proteins to the unconjugated proteins using the Habeeb assay.[33] In short, to the protein solution (10 μL) in PBS (40 μL) were added 0.1% trinitrobenzenesulfonic acid (50 μL) and 4% $NaHCO_3$, pH 9.5 (50 μL). The mixture was incubated at 40 °C for 2 h, quenched with 10% SDS (50 μL), 1 M HCl (25 μL), and H_2O (500 μL), and the absorbance at 363 nm. The epitope densities were as follows: CS-A conjugate = 15, CS-C conjugate = 15, CS-E conjugate = 14.

Three Balb/c female mice, 4-6 weeks old, were primed and boosted at 2-week intervals for a total of 5 intraperitoneal injections (5 μg per injection). The CS-A, CS-C, and CS-E KLH conjugates were mixed with RIBI adjuvant (RIBI Immunochem) for the first two injections, and a final series of 3 boosts was performed without adjuvant. Bleeds were taken 1 week after each injection and monitored by dot blot analysis. The most responsive mouse was boosted and sacrificed after three days. Spleen cells were

fused with HL-1 murine myeloma cells (Ventrex) using polyethylene glycol (PEG 1500, Boehringer-Mannheim) as described previously by Lebron *et al*.[28] Multiclonal and monoclonal cell lines were screened via ELISA and dot blot analysis.

ELISA analysis

CS tetrasaccharides **79**, **85**, and **88** were conjugated to bovine serum albumin (BSA) as follows. Ozonolysis of the anomeric allyl group of the CS-A, -C, and -E tetrasaccharides (0.51 μmol) as described in Chapters 4 and 5 was followed by treatment of each compound with BSA (0.34 mg, 0.0051 μmol) and $NaBH_3CN$ (0.5 mg) in H_2O (pH 9.5 using K_2CO_3) for 2 d at rt. The CS-BSA conjugates were then exhaustively dialyzed against 0.01 M Na_2HPO_4, 0.15 M NaCl, pH 7.4 at 4 °C, and the protein concentrations were determined using the BCA assay (Pierce). The epitope densities were measured by comparing the conjugated proteins to the unconjugated proteins using the Habeeb assay,[33] and the epitope densities were as follows: CS-A conjugate = 14, CS-C conjugate = 16, CS-E conjugate = 14.

The BSA conjugates (1 μg/mL in 50 mM Na_2CO_3, pH 9.6) were added to a 384-well NUNC Maxisorp clear plate (25 μL per well), and the plate was sealed and incubated for 12 h at 4 °C. The wells were aspirated, washed four times with PBS containing 0.05% Tween-20 (PBST, 75 μL/wash), and blocked for 2 h at rt with 10% horse serum (Gibco) in PBS (75 μL). After the blocking step, the plate was washed four times with PBST, and the supernatants from the anti-CS-A, anti-CS-C, and anti-CS-E antibody producing cultures (25 μL) were added to the wells and incubated at rt for 2 h. Following aspiration, the wells were washed four times with PBST and treated with a

horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody (Pierce; 1:10,000, 25 μL/well) in blocking buffer for 1 h at rt. The wells were again aspirated, washed four times with PBST, and then developed with ABTS liquid substrate solution (Sigma; 25 μL/well, solution at rt) for 30 min at rt. Color development was monitored on a Victor plate reader (PerkinElmer) at 405 nm. Only clones specific for the hapten tetrasaccharide and with absorbance values greater than 1.0 were kept for subsequent dot blot screening.

Dot blot analysis

Immunoblotting analysis was performed by spotting solutions of the BSA conjugates (relative epitope density adjusted, 1-100 ng, 1 μL/spot) in 10 mM Tris•HCl, 0.02% Nonidet P-40, pH 7.5 onto 0.45 μm nitrocellulose (Schleicher and Schuell) or commercially available HS (Neoparin) or CS polysaccharides (Seikagaku; calibrated with the carbazole assay, see Chapter 5) in 10 mM Tris•HCl, 0.02% Nonidet P-40, pH 7.5 onto Immobilon NY^+ (Waters), allowing the spots to air dry, and fixing the blots with 40% MeOH, 10% AcOH, 50% H_2O for 15 min at rt. The dot blots were then blocked for 30 min in 5% non-fat milk containing 50 mM Tris•HCl pH 7.4, 150 mM NaCl, 0.05% Tween-20 (TBST) followed by treatment with appropriate dilutions (1:500, 1:1000, and 1:2000) of the antibody serum in blocking buffer, commercially available CS-A (mCS4), CS-C (mCS6; RDI), and CS-56 (Sigma; all 1:100 in blocking buffer) or supernatant from the monoclonal cell cultures (1:1 in blocking buffer) for 2 h at rt. The blots were then washed with TBST three times for 10 min and treated with an HRP-conjugated goat anti-mouse antibody (Pierce; 1:10,000) in blocking buffer for 1 h at rt. The dot blots were

washed with TBST three times for 10 min and visualized by chemiluminescence (SuperSignal West Pico, Pierce).

Blots could also be developed through use of the Odyssey system. For the secondary antibody, the blots were incubated with AlexaFluor 680 goat anti-mouse (Molecular Probes, 1:10,000) in TBST with 10% SDS (1:50) for 1 h at rt in the dark. The dot blots were then washed with TBST three times for 10 min and analyzed using the Odyssey system (LI-COR Biosciences).

Generation and purification of CS antibody ascites

To generate ascites, 2 x 10^6 hybridoma cells in 0.5 mL culture medium were intraperitoneally injected into Balb/c mice that had been primed with 0.2 mL of pristine (Sigma; 2,6,10,14-tetramethylpnetadecane) 8 days prior to injection.[28] Ascites were removed by needle and 20 – 25 mL of ascites were generated for monoclonals 10G9-2B5, 5D2-1D2, and 2D11-2A10. 1 – 1.5 mL aliquots were purified with an ImmunoPure (A/G) IgG purification kit (Pierce) according to the manufacturer's instructions. The fractions with the highest A_{280} were combined, aliquoted, and stored at – 80 ºC. 10G9-2B5, CS-A antibody = 1.55 mg/mL (Figure 6.11) 5D2-1D2, CS-C antibody = 1.71 mg/mL (Figure 6.12); 2D11-2A10, CS-E antibody = 1.18 mg/mL (Figure 6.13). Concentrations determined by the A_{280}.

To confirm the purity of the ascites, 3 – 8% Tris-acetate minigels (Invitrogen) were performed according to the manufacturer's instructions, stained with Coomassie [100 mg in 100 mL 40% MeOH, 10% AcOH, and 50% H_2O (destain)], and washed with destain until bands were clearly visible.

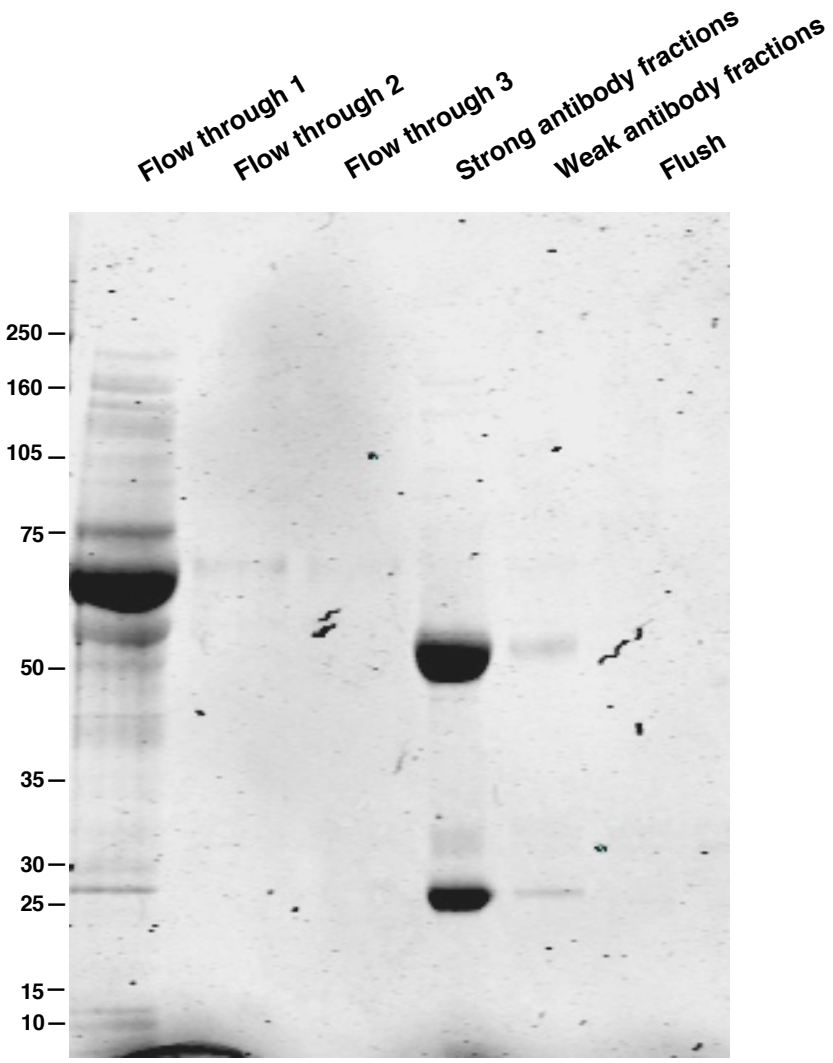

Figure 6.11: Coomassie stained 3 – 8% Tris-acetate minigel of the column fractions from the 10G9-2B5 anti-CS-A monoclonal antibody purification.

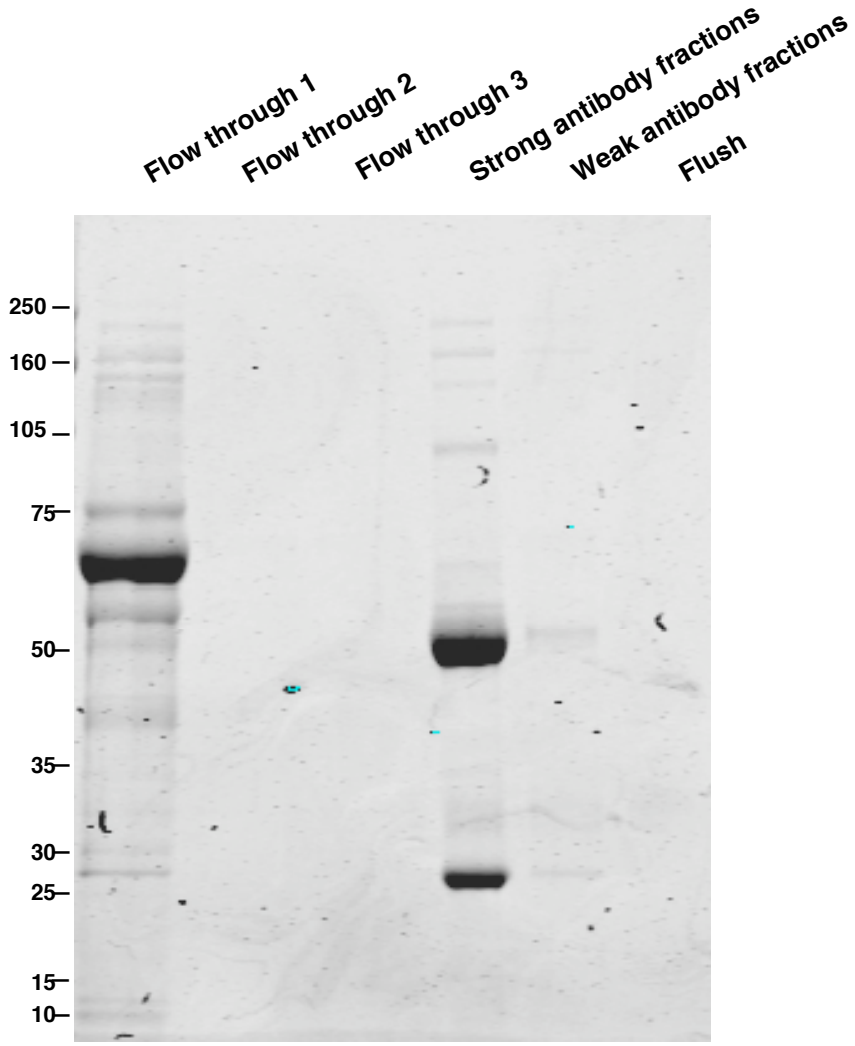

Figure 6.12: Coomassie stained 3 – 8% Tris-acetate minigel of the column fractions from the 5D2-1D2 anti-CS-C monoclonal antibody purification.

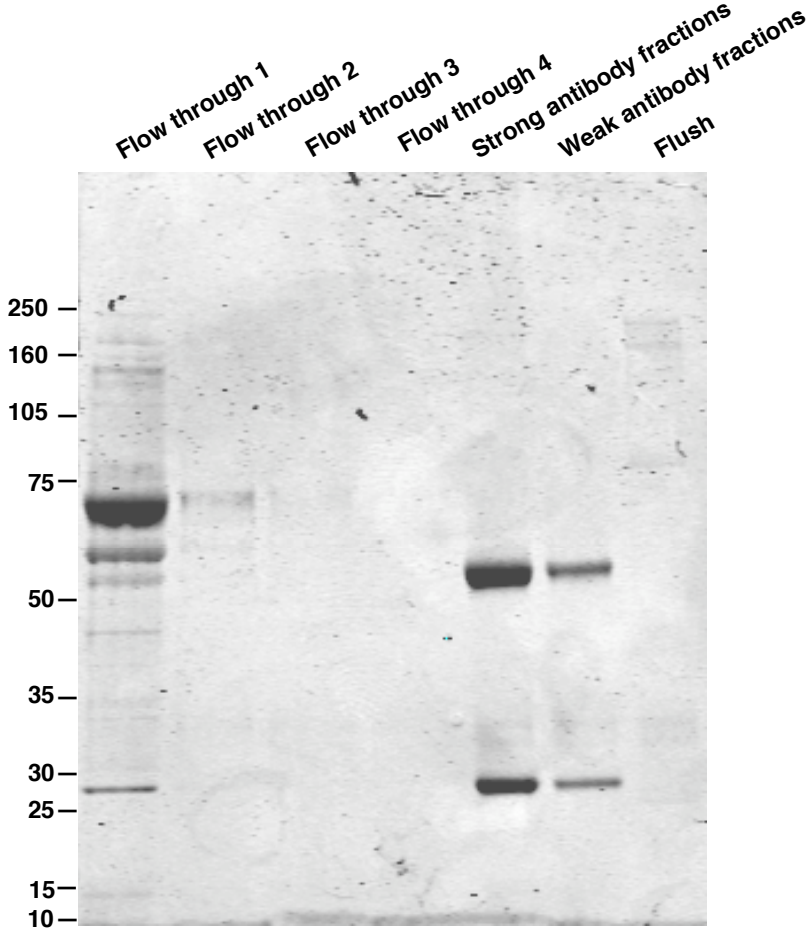

Figure 6.13: Coomassie stained 3 – 8% Tris-acetate minigel of the column fractions from the 2D11-2A10 anti-CS-E monoclonal antibody purification.

SDS-PAGE

The brains of embryonic and adult Sprague Dawley rats (Charles River Laboratories) were dissected on ice, and the regions were homogenized in 1% SDS containing Complete Protease Inhibitor Cocktail Tablets™ (Roche), boiled for 5 min, and frozen at –80 ºC for later use. Chondroitinase ABC (Seikagaku) digestion of the lysates was performed for 2 h at 37 ºC in 0.4 M Tris•HCl, 0.5 M NaOAc, pH 8.0 buffer with 1 μL of 100 mU/μL chondroitinase ABC added to each 50 or 200 μg sample.[6] A mixture of chondroitin sulfate proteoglycans (CSPG, Chemicon) or the CS-BSA conjugates were used as controls. Protein lysates were resolved using SDS-PAGE [6% and 10% gel with a 5% stacking gels or 3 – 8% Tris-acetate minigels (Invitrogen)] and transferred to nitrocellulose membranes (Schleicher and Schuell). The blots were then blocked for 30 min in TBST containing 5% non-fat milk followed by treatment with purified 10G9-2B5, 5D2-1D2, or 2D11-2A10 ascites (1:500) for 2 h at rt. The blots were then washed with TBST three times for 10 min and treated with an AlexaFluor 680 goat anti-mouse secondary (Molecular Probes, 1:10,000) in TBST with 10% SDS (1:50) for 1 h at rt in the dark. The blots were then washed with TBST three times for 10 min and analyzed using the Odyssey system (LI-COR Biosciences). HiMark™ protein standards (Invitrogen) ranging from 31 – 460 kDa or Full-range rainbow™ molecular weight markers (Amersham) ranging from 10 – 250 kDa were used.

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Chapter 7: Chondroitin Sulfate as a Modulator of Tumor Necrosis Factor-alpha Activity[*†]

Background

Proteoglycans are major constituents of the extracellular matrix and cell membranes, modulating cell-cell and cell-matrix interactions, contributing to the maintenance of normal tissue architecture and function, and participating in cell adhesion and growth control.[1-5] Rapid remodeling of the extracellular matrix and changing of its proteoglycan composition is a characteristic of inflammation.[6,7] After injury, chondroitin sulfate proteoglycans (CSPGs) are major components of wound fluid[8] and bind several factors involved in leukocyte adhesion, including L-selectin, P-selectin, and CD44.[9] The binding appears to be sulfation dependent as L-selectin and P-selectin bind only to motifs enriched in CS-E and CS-B, but CD44 is capable of binding all CS sulfation motifs.[10] Versican, a CSPG containing high amounts of CS-E and CS-B, has been shown to bind to the inflammatory chemokines SLC, IP-10, and SDF-1β.[9,10] The highly sulfated CS motifs antagonize the binding of these proteins to their receptors[9,10] and perhaps bind the chemokines to create reservoirs of them *in vivo*.

Many cytokines and chemokines contribute to extracellular matrix remodeling,[11,12] including tumor necrosis factor-alpha (TNF-α).[13-15] TNF-α is a proinflammatory cytokine involved in numerous diseases, including rheumatoid arthritis, Crohn's disease and psoriasis.[16-18] It acts at sites of inflammation and binds to

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[*] Computational modeling studies were done in collaboration with Peter M. Clark, a graduate student in the Hsieh-Wilson laboratory.

[†] Portions of this chapter were taken from S.E. Tully *et al*. (2006) *J. Am. Chem. Soc.* **128**, 7740-7741.

extracellular matix molecules such as fibronectin and laminin to increase their abilities to bind to leukocytes.[19,20] During inflammation, it has been shown that TNF-α can induce apoptosis, cause changes in the sulfation of heparan sulfate proteoglycans (HSPGs) and alter the expression of HS sulfotransferases.[14] In addition, TNF-α is a known binder of HS and dermatan sulfate, both members of the glycosaminoglycan (GAG) family.[21,22] Using microarrays, enzyme-linked immunosorbent assays (ELISA), and cellular assays, we decided to investigate the binding of CS polysaccharides and tetrasaccharides to TNF-α, to elucidate whether binding is sulfation pattern dependent and to discover if CS can regulate TNF-α activity.

Chondroitin sulfate binding to TNF-α

Microarrays generated in Chapter 5 were utilized to investigate CS recognition of TNF-α. The CS microarray was incubated with TNF-α, and binding was detected using an anti-TNF-α antibody followed by a secondary Cy3-labeled antibody. We found that TNF-α selectively recognized only the CS-E tetrasaccharide (Figure 7.1).[23] Binding was sulfation pattern dependent, not charge dependent, as CS-R did not bind strongly to TNF-α.

Peter Clark performed docking studies to understand the CS-E tetrasaccharide/TNF-α interaction. The TNF-α trimer was found in the protein database, the structure minimized, and the binding site for the CS-E tetrasaccharide found through use of ScanBindSite, a program that references DOCK4.0 (Figure 7.2a).[24,25] Three potential binding sites were isolated, and the tetrasaccharide was docked into the binding sites using MSCDOCK.[24,25] Each of the binding sites corresponds to a region of the TNF-

α trimer that binds the TNF-α receptor, TNF-α receptor 1 (TNFR1), suggesting that the CS-E tetrasaccharide could disrupt TNF-α binding to this receptor (Figure 7.2b). Intriguingly, the sites do not overlap with amino acids on TNF-α implicated in binding to TNFR2, indicating that the CS-E tetrasaccharide may not prevent this interaction.[26-28]

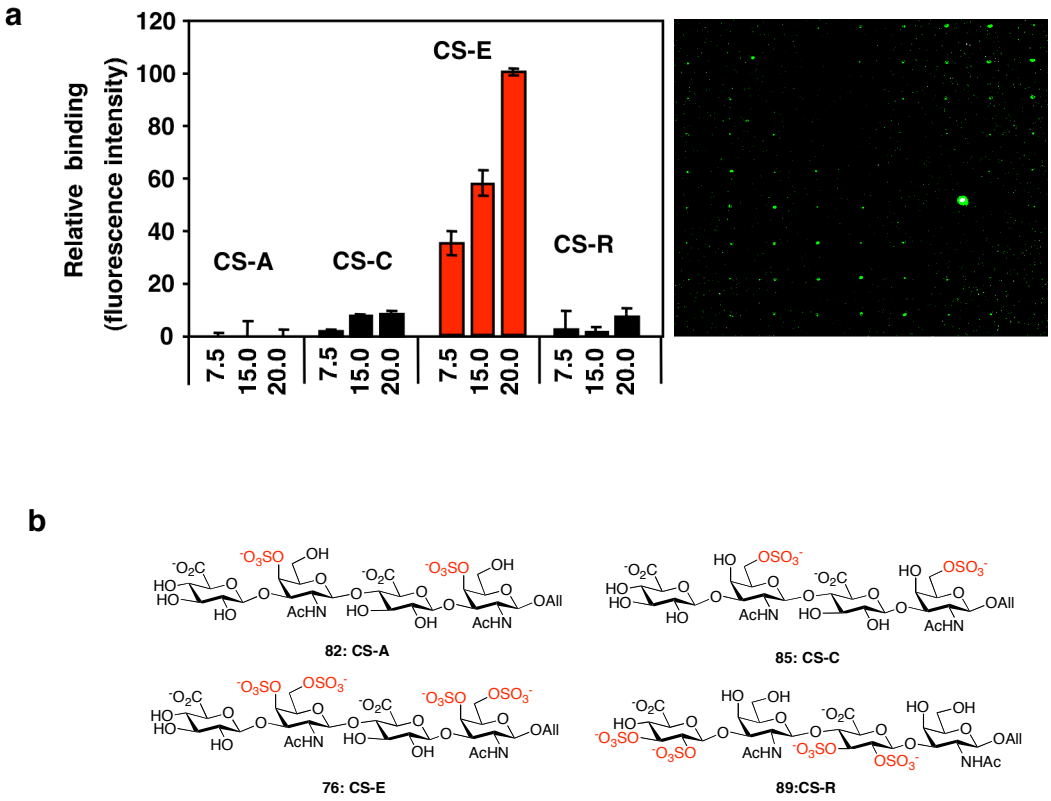


Figure 7.1: TNF-α binding to chondroitin sulfate microarrays. (**a**) Microarray analysis of TNF-α binding. The right panel shows a bar graph depicting the data and the left panel is a selected grid of the microarray. A key for the grid is shown in the Experimental Procedures for Chapter 7. (**b**) Structures of the tetrasaccharides on the microarray.

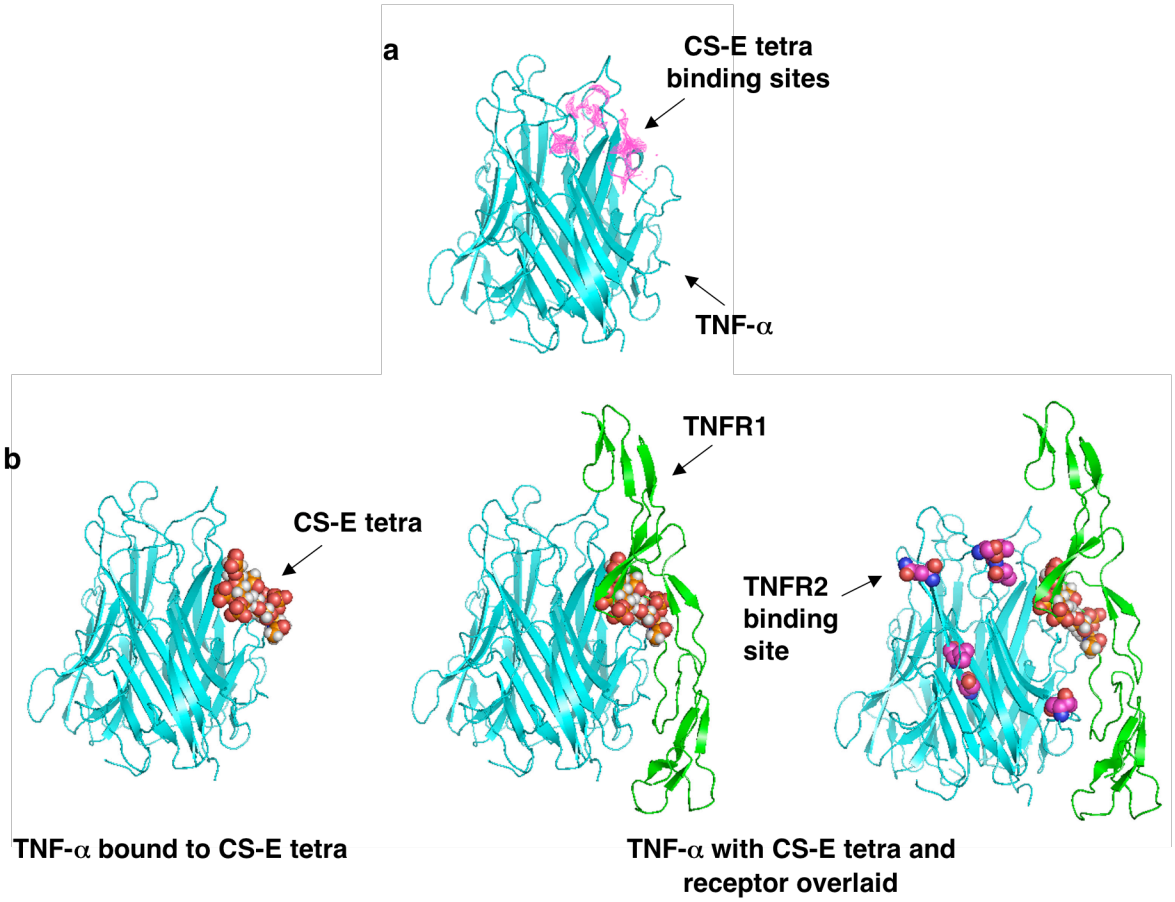


Figure 7.2: Computational modeling of the TNF-α/CS-E tetrasaccharide interaction. (**a**) The three potential CS-E tetrasaccharides (in pink). TNF-α is light blue. (**b**) Structures showing the binding of TNF-α with the CS-E tetrasaccharide (orange and white), TNF-α with the CS-E tetrasaccharide and TNFR1 (green) overlaid, or the CS-E tetrasaccharide, TNF-α, TNFR1 and amino acids important in binding TNFR2 highlighted (pink and dark blue).

ELISA analysis of the TNF-α/TNFR1 or TNF-α/TNFR2 interactions

We next examined whether CS-E could agonize or antagonize the binding of TNF-α to the cell surface receptor, TNFR1, which modulates TNF-α induced apoptosis and nuclear factor kappa B (NF-κB) activation through a cytoplasmic death domain.[29-31] TNFR1 was immobilized on a microtiter plate, and binding of TNF-α to the receptor was measured in the presence of varying concentrations of the CS-E tetrasaccharide or naturally occurring CS polysaccharides (Figure 7.3). Both CS-E tetrasaccharide **79** and polysaccharides enriched in the CS-E sulfation motif inhibited the interaction between TNF-α and TNFR1. In contrast, polysaccharides enriched in the CS-C or CS-A motifs could not antagonize the TNF-TNFR1 interaction. Potency measurements showed a median inhibitory concentration (relative IC_{50}) for the CS-E polysaccharide of 13.7 ± 2.5 μM, which is comparable to a recently reported small molecule inhibitor of TNF-α.[32] Although the potency of the tetrasaccharide (relative IC_{50} of 343.9 ± 37.8 μM) was reduced relative to the polysaccharide, the activity of the two compounds is comparable (25-fold difference) given that the polysaccharide is estimated to contain 37 CS-E tetrasaccharide epitopes. Notably, the IC_{50} values are within the physiological concentration range of CS, which is estimated to be at least 60 μM in the brain and may exist at 5- to 10-fold higher local concentrations at the cell surface and in the extracellular matrix.[33]

Binding to the other TNF-α cell surface receptor, TNFR2, was assayed in a manner similar to that described above, but only the CS-E polysaccharide was tested. TNFR2 has been less studied than TNFR1, presumably due to the fact that most TNF-α signaling is modulated through TNFR1.[34,35] Interestingly, we found no inhibition of

binding of TNF-α to TNFR2 in the presence of CS-E polysaccharide (Figure 7.4) indicating that TNF-α binds TNFR2 differently than TNFR1, a model that has been previously supported. While TNFR2 activation can lead to apoptosis,[36] this receptor lacks the death domain present on TNFR1 and has been found to initiate cell proliferation in some cell lines.[37] Potentially, CS-E containing proteoglycans bring TNF-α to the cell surface to promote cell proliferation through TNFR2 and inhibit the apoptotic effects of TNFR1 by blocking TNF-α binding.

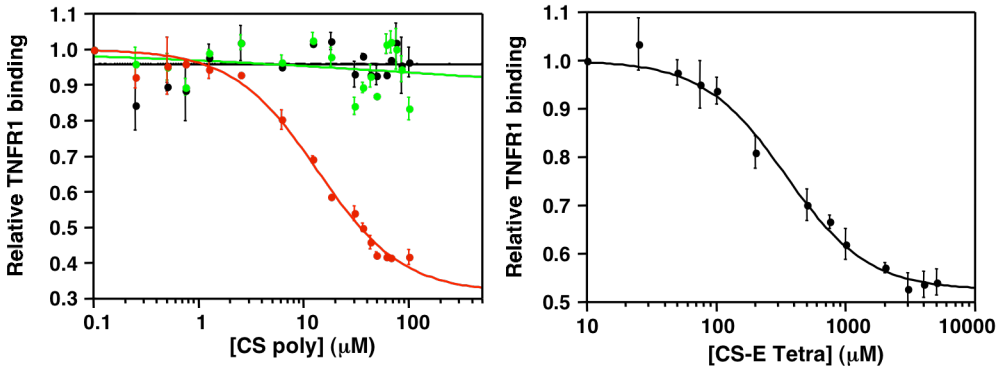


Figure 7.3: ELISA analysis of TNF-α/TNFR1 binding in the presence of chondroitin sulfate. CS polysaccharides enriched in the CS-E motif (left) and CS-E tetrasaccharide **79** (right) inhibit TNF-α binding to TNFR1. Polysaccharides enriched in CS-A (black), CS-C (green) and CS-E (red).

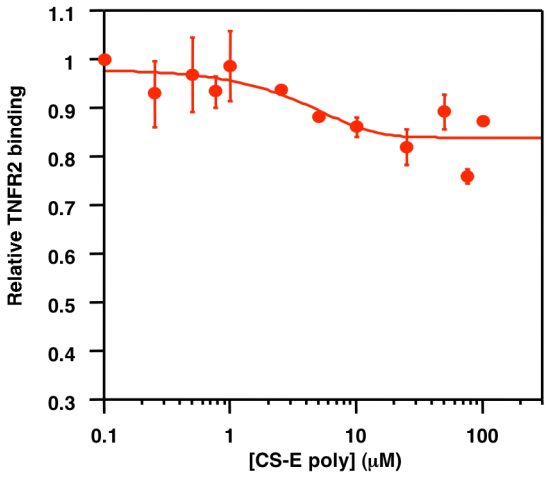


Figure 7.4: ELISA analysis of TNF-α/TNFR2 binding in the presence of chondroitin sulfate. CS polysaccharides enriched in the CS-E motif do not inhibit TNF-α binding to TNFR2.

Protective effects of the CS-E tetrasaccharide

The ability of CS-E to disrupt the TNF-TNFR1 interaction suggested that CS-E might inhibit TNF-α-induced cell death. Histiocytic lymphoma U937 cells, known to be sensitive to TNF-α stimulated apoptosis,[36,38] were treated with TNF-α and varying concentrations of tetrasaccharide **79** or CS polysaccharides enriched in the CS-E sulfation motif. The extent of apoptosis was determined by monitoring the production of caspases 3 and 7 using a fluorescent rhodamine-DEVD peptide substrate. Caspases 3 and 7 are indicators of apoptotic activity, and TNF-α is known to activate caspase 3.[39] Both compounds prevented the cells from undergoing apoptosis, effectively blocking cell death (Figure 7.5). Interestingly, treatment of the cells with high concentrations of either compound reduced the extent of cell death compared to cells not treated with TNF-α, suggesting that the compounds may exert a protective function. The protective effect is only observed in the presence of TNF-α, as cells cultured with CS-E tetrasaccharides alone were essentially unaffected, with only a small increase in apoptosis over the cell control observed (Figure 7.6).

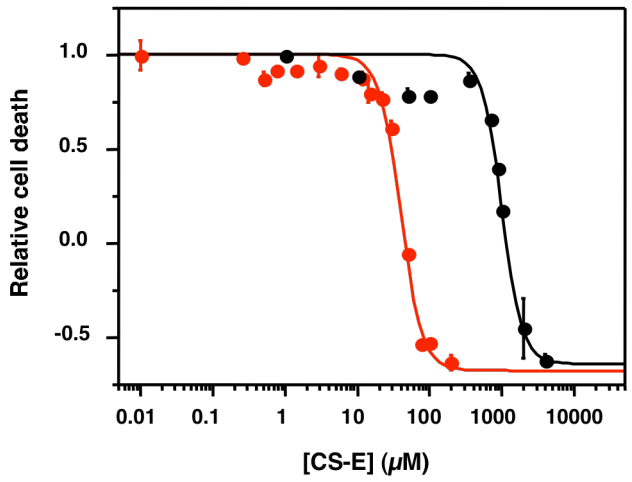


Figure 7.5: CS polysaccharides enriched in the CS-E motif (red) and CS-E tetrasaccharide **79** (black) inhibit TNF-α-induced apoptosis.

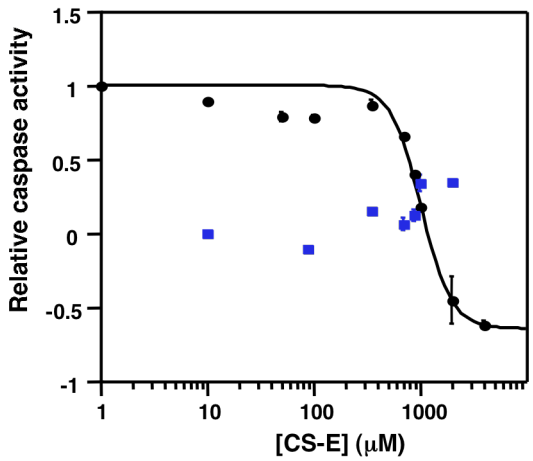


Figure 7.6: CS-E tetrasaccharide **79** (black) inhibits TNF-α-induced apoptosis and a protective effect is observed. In the absence of TNF-α, the tetrasaccharide **79** (blue) has no protective effect.

Discussion

Using carbohydrate microarrays, we discovered a novel interaction between CS and TNF-α and demonstrated that CS-E tetra- and polysaccharides can antagonize the activity of this therapeutically important cytokine. The specificity of this molecular interaction is intriguing given the lack of small molecule inhibitors of TNF-α and the prevalence of CS GAGs at sites of inflammation.[6,18,32] As previously hypothesized, it is possible that CS-E containing proteoglycans generate extracellular pools of TNF-α *in vivo* for later release.[9,10] The ability of CS-E to inhibit TNF-α/TNFR1, but not TNF-α/TNFR2 binding suggests that it may be used to regulate the activities of these two receptors, and potentially acts as a switch between cell proliferation or apoptosis. Peter Clark plans to further investigate these receptor interactions.

Much is known about the importance of TNF-α in inflammation, but less is understood about the other functions it serves. Recently, it has been discovered that TNF-α produced by glia plays an important part in synaptic plasticity, and through modulation

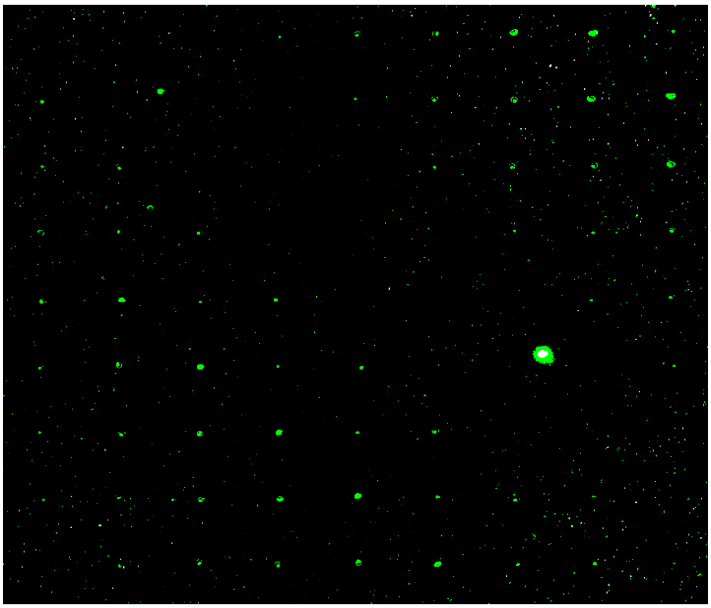
of the levels of TNF-α in the hippocampus, glia regulate synaptic connectivity.[40] In development, TNF-α is believed to regulate embryonic apoptosis, cell growth and differentiation, and the composition of the extracellular matrix,[7] and it is possible that CSPGs affect TNF-α activity during these processes. Additionally, investigation into whether TNF-α directs CS sulfotransferases and sulfation patterns in a manner similar to how it controls HS and HS sulfotransferases[14] would provide insight into how CS sulfation patterns are regulated *in vivo*, a growing field of interest in the study of GAGs.

Experimental Procedures for Chapter 7

Carbohydrate microarrays

Solutions of the aminooxy oligosaccharides (see Chapter 5; in 300 mM NaH_2PO_4, pH 5.0, 10 μL/well in a 384-well plate) were arrayed on Hydrogel Aldehyde slides (NoAb Biodiscoveries) by using a Microgrid II arrayer (Biorobotics) to deliver sub-nanoliter volumes at rt and 50% humidity. Concentrations of carbohydrates ranged from 0 – 500 μM. The resulting arrays were incubated in a 70% humidity chamber at rt for 12 h and then stored in a low humidity, dust-free desiccator. Prior to use, an array was outlined with a hydrophobic pen (Super Pap Pen, Research Products International) to create a boundary for the protein treatments and rinsed three times with H_2O. The slide was then blocked by treatment with $NaBH_4$ (125 mg) in 140 mM NaCl, 2.7 mM KCl, 5.4 mM Na_2HPO_4, and 1.8 mM KH_2PO_4 (phosphate buffered saline, PBS, 50 mL) at rt for 5 min with gentle rocking and washed five times for 3 min with PBS. For all incubations, the slide was placed in a covered pipette tip box. Human TNF-α (Peprotech; reconstituted to 2 μM in 0.1% Triton X-100 in PBS) was spotted onto the slide in 200 μL quantities, and incubated statically at rt for 2 h. The slide was then washed as previously described and incubated with anti-TNF-α (Peprotech; 1:1000 in 0.1% Triton X-100 in PBS) for 2 h at rt with gentle rocking. Following the incubation, the slide was washed as previously described and treated in the dark at rt with a secondary IgG antibody conjugated to Cy3 (Amersham; 1:5000 in 0.1% Triton X-100 in PBS) at rt for 1 h with gentle rocking. The slide was washed three times for 2 min with PBS, two times for 1 min with H_2O, and dried under a gentle stream of N_2. Microarrays were analyzed at 532 nm using a GenePix 5000a scanner, and fluorescence quantification was performed using

GenePix 6.0 software after correction for local background. Each protein was analyzed in triplicate, and the data represent an average of at least five spots for a given carbohydrate concentration. All solutions used for the carbohydrate microarrays were sterile-filtered through a 0.2 μm syringe filter prior to use. A grid of the TNF-α array is shown in Figure 7.7.



60A	100A	500A	50C	10E	20E	35E	70E	25Di
50R	60A	100A	500A	50C	10E	20E	35E	70E
25Di	50R	60A	100A	500A	50C	10E	20E	35E
70E	25Di	50R	60A	100A	500A	50C	10E	20E
35E	70E	25Di	50R	60A	100A	500A	50C	10E
20E	35E	70E	25Di	50R	60A	100A	500A	50C
10E	20E	35E	70E	25Di	50R	60A	100A	500A
50C	10E	20E	35E	70E	25Di	50R	60A	100A
500A	50C	10E	20E	35E	70E	25Di	50R	60A

Figure 7.7: Representative portion of the microarrays illustrating spot morphology and fluorescence intensity after incubation with TNF-α. The panel below indicates the corresponding oligosaccharides and their concentrations for each spot shown. Values are in μM. A = CS-A tetrasaccharide, C = CS-C tetrasaccharide, Di = CS-E disaccharide, E = CS-E tetrasaccharide, R = CS-R tetrasaccharide. Each microarray contained 1500 spots.

TNF-α ELISA analysis

Human recombinant TNFR1 or TNFR2 (Peprotech; 1 μg/mL in 50 mM Na_2CO_3, pH 9.6) was added to a 384-well NUNC Maxisorp clear plate (25 μL per well), and the plate was sealed and incubated for 12 h at 4 °C. The wells were aspirated, washed four times with PBS containing 0.05% Tween-20 (PBST, 75 μL/wash), and blocked for 2 h at rt with 1% BSA in PBS (75 μL). During this time, biotinylated TNF-α (PerkinElmer; 25 μL/well of a 1 nM solution in 0.05% Tween-20, 0.1% BSA in PBS) was pre-incubated with the indicated concentrations of polysaccharides enriched in the CS-A, CS-C, or CS-E motifs (Seikagaku; 0.1 – 100 μM in H_2O) or CS-E tetrasaccharide **79** (0.01 – 5 mM in H_2O). A 1 nM solution of biotinylated TNF-α gave the optimal absorbance value at 405 nm in a standard curve with biotinylated TNF-α concentrations ranging from 0.01 – 25 nM (Figure 7.8). After the blocking step, the plate was washed four times with PBST, and the solutions of CS and TNF-α were added to the wells and incubated at rt for 2 h. Following aspiration, the wells were washed four times with PBST and treated with streptavidin-HRP (Pierce; 25 μL/well, 1:2000 in 0.05% Tween-20, 0.1% BSA in PBS) for 30 min at rt. The wells were again aspirated, washed four times with PBST, and then developed with ABTS liquid substrate solution (Sigma; 25 μL/well, solution at rt) for 30 min at rt. Color development was monitored on a Victor plate reader (PerkinElmer) at 405 nm. Each carbohydrate concentration was analyzed in triplicate, and the absorbance values were corrected for background in the absence of carbohydrate and normalized with respect to the absorbance value at the lowest carbohydrate concentration.

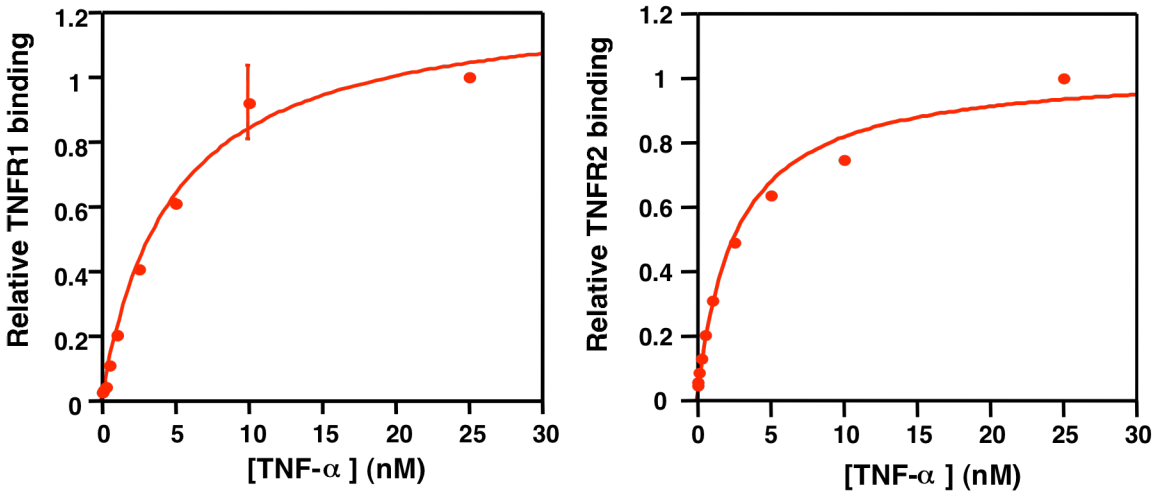


Figure 7.8: Optimization of the biotinylated TNF-α concentration for TNFR1 and TNFR2 ELISA studies.

Caspase assay

U937 cells (ATCC) sensitive to TNF-α-induced apoptosis[36,38] were cultured in RPMI 1640 medium containing 10% fetal bovine serum at 37 °C and 5% CO_2/O_2. Cells were grown in every other well of a 384-well NUNC sterile, clear plate (25,000 cells/well, 25 μL/well) for 18 h. Solutions of the carbohydrates at the indicated concentrations (0.01 – 4000 μM in sterile PBS) were pre-incubated with TNF-α (1 μL/well of a 125 ng/mL solution in sterile PBS) at rt. After 2h, the solutions were added to the cells and incubated for 18 h. At this time, caspase 3/7 activity was analyzed as described by Shi *et al.* using the Apo-One homogeneous caspase 3/7 assay kit (Promega) according to the manufacturer's protocol.[39] Readings were taken every 30 min for 18 h at rt on a Victor plate reader. By this time, caspase 3/7 activity had reached a plateau, and the endpoints of the various treatments were used for data analysis. Endpoint fluorescence values for controls containing only cells were subtracted from the experimental endpoint values, and the resulting values were then normalized with respect to that of the lowest carbohydrate concentration. Each carbohydrate concentration was

repeated in triplicate. The CS-E tetrasaccharide and polysaccharide had no effect on the extent of apoptosis in the absence of TNF-α. Figure 7.9 shows full caspase assay curves with varying cell and TNF-α amounts to demonstrate how the assay was optimized and the plateau of the caspase activity during the assay.

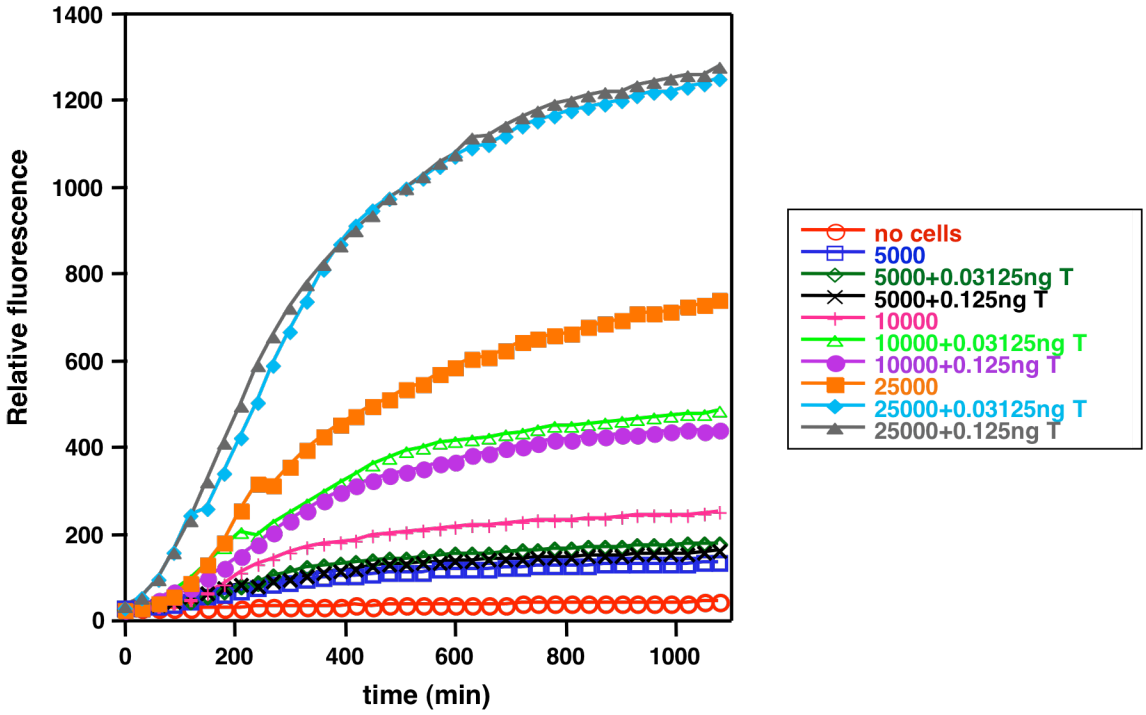


Figure 7.9: Optimization of the caspase assay. Varying amounts of cells (5000, 10,000 and 25,000 per well of a 384-well plate) were treated with either 0.03125 ng or 0.125 ng of TNF-α (T). 25,000 cells per well with 0.125 ng of TNF-α added gave the highest response over the cell control, and these conditions were used in subsequent assays.

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