A sulfated carbohydrate epitope inhibits axon regeneration after injury

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Edited by* Peter G. Schultz, The Scripps Research Institute, La Jolla, CA, and approved January 23, 2012 (received for review December 27, 2011)

Chondroitin sulfate proteoglycans (CSPGs) represent a major barrier to regenerating axons in the central nervous system (CNS), but the structural diversity of their polysaccharides has hampered efforts to dissect the structure-activity relationships underlying their physiological activity. By taking advantage of our ability to chemically synthesize specific oligosaccharides, we demonstrate that a sugar epitope on CSPGs, chondroitin sulfate-E (CS-E), potently inhibits axon growth. Removal of the CS-E motif significantly attenuates the inhibitory activity of CSPGs on axon growth. Furthermore, CS-E functions as a protein recognition element to engage receptors including the transmembrane protein tyrosine phosphatase PTPσ, thereby triggering downstream pathways that inhibit axon growth. Finally, masking the CS-E motif using a CS-E-specific antibody reversed the inhibitory activity of CSPGs and stimulated axon regeneration in vivo. These results demonstrate that a specific sugar epitope within chondroitin sulfate polysaccharides can direct important physiological processes and provide new therapeutic strategies to regenerate axons after CNS injury.

A major obstacle to functional recovery after CNS injury is the inhibitory environment encountered by regenerating axons. Chondroitin sulfate (CS) polysaccharides and their associated proteoglycans (CSPGs) are the principal inhibitory components of the glial scar, which forms after neuronal damage and acts as a barrier to axon regeneration (1–3). It is well established that the inhibitory activity of CSPGs is derived from their CS chains, as chondroitinase ABC (ChABC) treatment promotes axon regeneration, sprouting, and functional recovery after injury in vivo (4–6). However, the mechanisms by which CS polysaccharides inhibit axon growth are poorly understood. Dissection of the structural determinants and mechanisms underlying CS activity is essential for understanding the barriers to axon regeneration and for developing new treatments to promote regeneration and functional recovery after spinal cord and other CNS injuries.

CS polysaccharides are composed of repeating disaccharide units, which undergo regiochemical sulfation during development and after injury (7–10). The CS-A (GlcA-4SGalNAc), CS-C (GlcA-6SGalNAc), and CS-E (GlcA-4S,6SGalNAc) disaccharides represent major sulfation motifs in the mammalian CNS (Fig. S1). Although the diverse sulfation patterns of CS polysaccharides lie at the heart of their biological activity, these complex patterns have also hampered efforts to understand the biological functions of CS. For example, genetic approaches are challenged by the presence of multiple sulfotransferase isoforms with overlapping specificities, and deletion of a single sulfotransferase gene can propagate global changes throughout the carbohydrate chain. The structural complexity of CS has also thwarted biochemical efforts to isolate well-defined, sulfated molecules. As such, only heterogeneous mixtures or purified samples biased toward abundant, readily isolable sequences have been available for biological investigations. Although studies have suggested that the CS-A, CS-E, and CS-C motifs are upregulated after neuronal injury and may play roles in axon regeneration (7, 9, 11), only heterogeneous polysaccharides were utilized for those studies, and there have been conflicting data, confounding the question of whether specific sulfation sequences are important. Indeed, because of the lack of structure-activity relationships, relatively nonspecific mechanisms have also been proposed, such as those brought about by steric blockage of the extracellular space (12), arrays of negatively charged sulfate (7), or obstruction of substrate adhesion molecules (13).

Here, we exploited chemically synthesized CS oligosaccharides and glycopolymers to examine systematically the role of specific sulfation sequences in axon regeneration. Our studies demonstrate that the CS-E sulfation motif is a key structural determinant responsible for the inhibitory activity of CSPGs. Moreover, we provide mechanistic insights into how CS-E enables CSPGs to inhibit axon growth through the identification of a specific neuronal receptor for CS-E. Finally, we show that blocking the inhibitory CS-E sugar motif can reverse CSPG-mediated inhibition and promote axon regeneration in vivo, providing a unique therapeutic approach to neural regeneration.

Results and Discussion

CS-E-Enriched Polysaccharides Inhibit Neurite Outgrowth and Repel Axons. To understand the role of specific sulfation motifs, we used CS polysaccharides enriched in particular motifs and exploited our ability to chemically synthesize defined CS-A, CS-C, and CS-E oligosaccharides. First, we compared the inhibitory effects of CSPGs and CS polysaccharides enriched in CS-A, CS-C, or CS-E disaccharide units on neurite outgrowth. Neurite outgrowth of dissociated dorsal root ganglion (DRG) neurons was inhibited by 58% of untreated control levels when grown on CSPGs (Fig. 1*A*). ChABC digestion largely abolished the effects, confirming the importance of the CS chains. CS polysaccharides enriched in the CS-E motif potently inhibited neurite outgrowth to approximately 50% of untreated control levels as suggested previously (7) and in a dose-dependent manner (Fig. 1 *A* and *B*). In contrast, polysaccharides enriched in the CS-A or CS-C motif had no appreciable effects on neurite outgrowth at the same glucuronic acid concentrations. The lack of inhibition observed for CS-A and

Author contributions: J.M.B., J.X., B.Z., and L.C.H.-W. designed research; J.M.B., J.X., B.Z., K.-S.C., C.J.R., C.I.G., S.E.T., and D.E.M. performed research; M.R., S.E.T., N.U., M.L.T., and O.H. contributed new reagents/analytic tools; J.M.B., J.X., B.Z., K.-S.C., C.J.R., C.I.G., D.E.M., E.C.P., D.F.C., and L.C.H.-W. analyzed data; and J.M.B., B.Z., D.F.C., and L.C.H.-W. wrote the paper.

The authors declare no conflict of interest.

*This Direct Submission article had a prearranged editor.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1121318109/-/DCSupplemental.

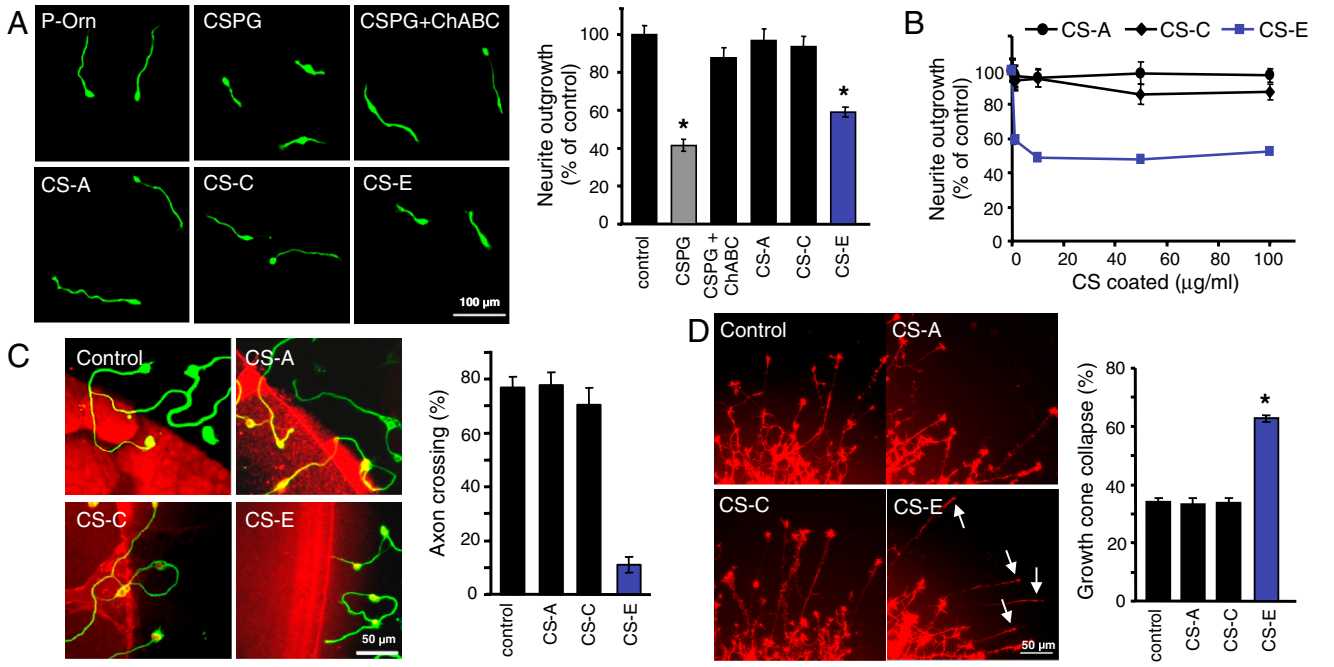

Fig. 1. CS-E-enriched polysaccharides inhibit DRG neurite outgrowth and induce growth cone collapse. (*A*) Dissociated chick E7 DRGs were cultured on a substratum of poly-DL-ornithine (P-Orn control), CSPGs, chondroitinase ABC-treated CSPGs, or CS polysaccharides enriched in the CS-A, CS-C, or CS-E sulfation motifs. Representative images and quantitation of average neurite length (±SEM, error bars) from three experiments ($n = 50–200$ cells per experiment). (*B*) Polysaccharides enriched in the CS-E sulfation motif, but not the CS-A or CS-C motifs, inhibit DRG neurite outgrowth in a dose-dependent manner. (*C*) CS-E-enriched polysaccharides repel axon crossing in a boundary assay. Polysaccharides (1 mg/mL) or PBS control were mixed with Texas Red and spotted on P-Orn coated coverslips. Dissociated rat P5-9 CGN neurons were immunostained with an anti-βIII-tubulin antibody. Representative images and quantitation of percentage of axon crossing (±SEM, error bars) from two experiments ($n = 30–50$ axons per experiment). (*D*) CS-E-enriched polysaccharides induce growth cone collapse. DRG explants from chick E7-9 embryos were grown on a P-Orn/laminin substratum, treated with medium (control) or the indicated polysaccharides, and stained with rhodamine-phalloidin. Representative images and quantitation of growth cone collapse (±SEM, error bars) from five experiments ($n = 50–100$ growth cones per experiment). Arrows indicate collapsed growth cones. All statistical analyses were performed using the one-way ANOVA test (*$P < 0.0001$, relative to control).

CS-C, even when used at 100-fold higher concentrations than CS-E (Fig. 1*B*), suggests that the inhibitory activity of CS-E polysaccharides is not simply due to their high overall negative charge. Similar results were obtained with cerebellar granule neurons (CGNs; Fig. S2), whose neurite growth is inhibited by CSPGs (14).

As CSPGs in the glial scar form an inhibitory boundary to growing axons, we examined whether polysaccharides enriched in the CS-E sulfation motif could repel axons in a boundary assay. Like CSPGs (15), CS-E-enriched polysaccharides formed an inhibitory zone that was strongly repellent to CGN axons (Fig. 1*C*). In contrast, axons freely crossed into boundaries enriched in the CS-A or CS-C motifs. CS-A-enriched polysaccharides also exhibited repulsive behavior as reported (8), but much higher concentrations of sugar were required (Fig. S3*A*).

It is known that CSPGs can acutely collapse growth cones to form dystrophic axonal structures that no longer extend, thus leading to long-term inhibition of regrowth (16). To examine whether CS-E is involved in the acute phase of the inhibitory response, we performed growth cone collapse assays. Application of CS-E-enriched polysaccharides to DRG or CGN explants significantly increased the number of collapsed growth cones within minutes (Fig. 1*D* and Fig. S3*B*), whereas CS-A- and CS-C-enriched polysaccharides had no effect. Taken together, these results indicate that CS polysaccharides are sufficient to recapitulate the inhibitory effects of CSPGs on neurons, and this activity depends critically on the CS sulfation pattern.

Pure CS-E Potently Inhibits Neurite Outgrowth and Collapses Growth Cones. Although natural polysaccharides enriched in CS-E shed light on how specific sulfation motifs function in CSPG-mediated axon inhibition, these data should be interpreted cautiously because polysaccharides containing a single, pure sulfation sequence have not traditionally been isolated from natural sources, and thus the possibility that the inhibitory activity is due to minor, contaminating motifs cannot be eliminated. Indeed, about 40% of the CS-E-enriched polysaccharide contains other sulfation motifs, and rare sulfation sequences are likely to be biologically important, as in the case of heparan sulfate glycosaminoglycans (17). As such, the intrinsic structural complexity and heterogeneity of CS pose a major obstacle to understanding structure-activity relationships.

To overcome this problem, we synthesized homogeneously sulfated glycopolymers displaying only the CS-A, CS-C, or CS-E sulfation motifs (Fig. 2*A*). Norbornene-linked CS-A, CS-C, or CS-E disaccharides were polymerized using ruthenium-catalyzed ring-opening metathesis polymerization (ROMP) chemistry. This approach generates glycopolymers of pure, defined sulfation sequence with molecular weights and biological activities comparable to natural CS polysaccharides (18). Previously, we showed that these molecules were powerful tools to study the roles of specific CS motifs in promoting neurite outgrowth of developing hippocampal neurons (19). In the context of DRG neurons, glycopolymers containing pure CS-E inhibited neurite outgrowth, whereas those containing pure CS-A or CS-C had minimal activity (Fig. 2*B* and Fig. S4). Moreover, the monovalent CS-E disaccharide at the same uronic acid concentration did not inhibit neurite outgrowth, confirming that the multivalent presentation of CS-E is critical for biological activity. Similarly, we found that glycopolymers containing pure CS-E potently induced growth cone collapse in DRG explants (Fig. 2*C*), whereas CS-A or CS-C glycopolymers had no effect. As CS polysaccharides are found as a complex mixture of different sulfation patterns in vivo, we also examined the activity of a glycopolymer mixture. A 1:1 mixture of CS-A and CS-E glycopolymers had no further effects on neurite outgrowth compared to the pure CS-E glycopolymer alone, confirming that sulfated mixtures do not confer additional inhibitory properties (Fig. S5).

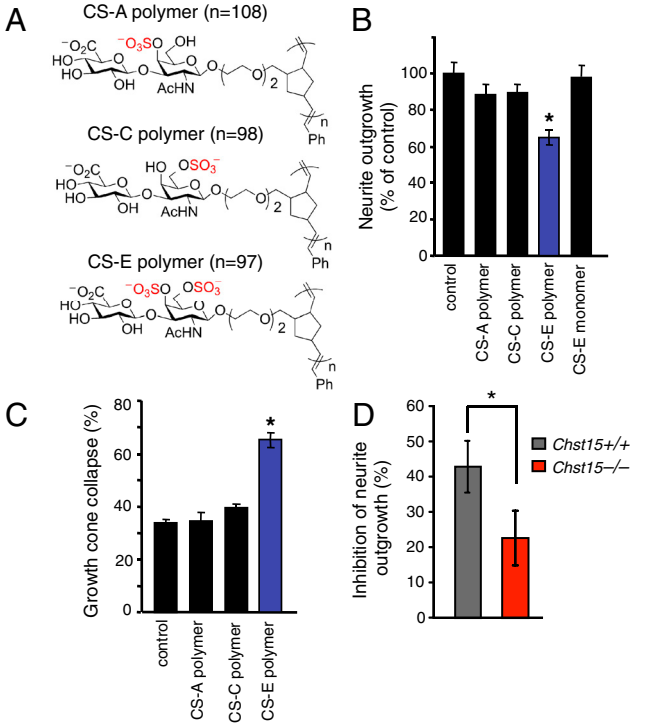

Fig. 2. The CS-E motif is a potent inhibitor of axon growth. (*A*) Structures of synthetic glycopolymers displaying pure CS-A, CS-C, and CS-E disaccharides. (*B*) The synthetic CS-E glycopolymer inhibits neurite outgrowth of chick E7 DRGs, whereas the CS-A glycopolymer, CS-C glycopolymer, and monovalent CS-E disaccharide have little effect. (*C*) The synthetic CS-E glycopolymer induces DRG growth cone collapse. (*D*) CSPGs from CS-E-deficient mice show significant loss of inhibitory activity on DRG neurite outgrowth. Mouse P8 DRGs were cultured on CSPGs purified from *Chst15* knockout or wild-type mice. Statistical analyses were performed using the one-way ANOVA test (*$P < 0.0001$, relative to control).

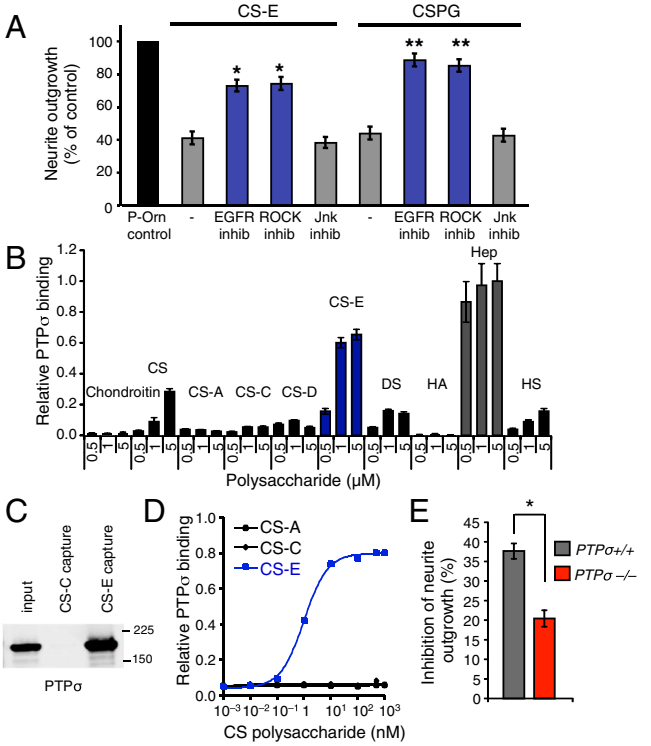

Fig. 3. The CS-E sulfation motif inhibits axon growth via PTPσ. (*A*) Inhibitors against EGFR (AG1478, 15 nM) and ROCK (Y27632, 5 μM) rescued CS-E- and CSPG-mediated inhibition of neurite outgrowth in dissociated rat P5-9 CGN cultures, whereas JNK inhibitor II (10 μM) had no effect. Quantitation of neurite outgrowth from three experiments is reported. (One-way ANOVA, *$P < 0.0001$, relative to CS-E control without inhibitors, **$P < 0.0001$, relative to CSPG control without inhibitors; $n = 50$–200 cells per experiment). (*B*) PTPσ binds selectively to CS-E-enriched polysaccharides on glycosaminoglycan microarrays. Microarrays were incubated with PTPσ-Fc, followed by a Cy3-conjugated antihuman IgG secondary antibody, and analyzed using a GenePix 5000a scanner. Graphs show quantification from three experiments ($n = 10$ per condition). (*C*) Coprecipitation of CS-E and PTPσ. Full-length PTPσ-mycHis was expressed in COS-7 cells and incubated with biotinylated CS-E or CS-C polysaccharides bound to streptavidin beads. PTPσ binding was detected by immunoblotting with an anti-myc antibody. (*D*) Specific, high affinity binding of CS-E polysaccharides to PTPσ. (*E*) *PTPσ* -/- neurons show significantly less inhibition by CS-E than wild-type control neurons. For each genotype, the percentage inhibition of neurite outgrowth is plotted relative to neurons treated with only P-Orn. Quantification from three experiments is shown. (One-way ANOVA, *$P < 0.005$, relative to control; n => 200 cells per experiment).

To complement our chemical approaches, we also investigated the contribution of the CS-E motif using genetic methods. We isolated CSPGs from mice containing a targeted gene disruption of *N*-acetylgalactosamine 4-sulfate 6-*O* sulfotransferase 15 (Chst15), the enzyme that generates CS-E via addition of a sulfate group to the 6-*O* position of GalNAc on CS-A (20). Consistent with potent inhibitory activity for CS-E, removal of CS-E from CSPGs resulted in significant loss of inhibitory activity on DRG neurite outgrowth (Fig. 2*D*). The remaining inhibitory effect of CSPGs from *Chst15*$^{-/-}$ mice is likely due to the proteoglycan core protein or other proteins in the mixture, as treatment with ChABC to remove CS chains did not reduce the inhibitory effects any further. Taken together, our chemical and genetic studies demonstrate conclusively that the CS-E motif is a potent inhibitor of axon growth and a critical inhibitory structure on CSPGs.

The CS-E Motif Activates Inhibitory Signaling Pathways. To investigate the molecular mechanisms by which CS-E inhibits axon growth, we examined the ability of CS-E to activate signaling pathways associated with inhibition of axon regeneration. CSPGs and myelin inhibitors have been shown to activate Rho/Rho-kinase (ROCK) and epidermal growth factor receptor (EGFR) pathways (8, 14, 15). Pharmacological inhibition of these signaling pathways effectively reversed the inhibitory effects of CSPGs on CGNs (Fig. 3*A* and Fig. S6). Specifically, the EGFR competitive inhibitor AG1478 and the ROCK inhibitor Y27632 restored neurite outgrowth to within 79–88% of untreated control levels, in agreement with previous studies (14, 15). Importantly, we found that the EGFR and ROCK inhibitors also neutralized the inhibitory activity of CS-E polysaccharides and rescued neurite outgrowth to a similar extent. In contrast, inhibition of c-Jun N-terminal kinase (JNK) pathways using JNK inhibitor II showed no effect on either CS-E- or CSPG-mediated neurite inhibition, as expected (15). Moreover, treatment of COS-7 cells with CS-E or CSPGs led to activation of RhoA (Fig. S7). Thus, CS-E activates intracellular signaling pathways involved in CSPG-mediated inhibition of axon regeneration, further supporting the notion that this sugar epitope is a major inhibitory component of CSPGs.

The CS-E Motif Inhibits Neurite Outgrowth via PTPσ. The ability of CS-E to trigger downstream signaling pathways suggests that CS-E may directly engage protein receptors at the cell surface, thereby initiating intracellular signaling. Recently, CSPGs were shown to interact with protein tyrosine phosphatase PTPσ, a transmembrane receptor known to bind heparan sulfate proteoglycans (21, 22). *PTPσ* gene disruption reduced axon inhibition by CSPGs in culture (22) and enhanced regeneration in sciatic, facial, optic, and spinal cord nerves in vivo (22–25). However, it remains unknown whether (and which) specific sulfation motifs on CS mediate the interactions of CSPGs with PTPσ.

In light of our results showing that CS-E is a major inhibitory motif on CSPGs, we examined the potential interaction between CS-E and PTPσ using carbohydrate microarrays (26). A soluble PTPσ-Fc fusion protein, but not other receptors such as EphA2-Fc or Fc alone, bound efficiently to CS-E polysaccharides arrayed on poly-lysine-coated glass slides (Fig. 3*B* and Fig. S8). PTPσ showed strong binding to heparin and CS-E polysaccharides, with weaker binding to chondroitin sulfate and dermatan sulfate (both of which contain some CS-E) and heparan sulfate. Little or no binding to CS-A, CS-C, or CS-D polysaccharides was observed, highlighting the specificity of PTPσ for the CS-E sulfation motif.

To confirm further the PTPσ-CS-E interaction, biotinylated CS-E or CS-C polysaccharides were conjugated to streptavidin beads and incubated with COS-7 cell lysates expressing full-length PTPσ. We found that CS-E polysaccharides were capable of pulling down PTPσ, whereas CS-C polysaccharides showed no interaction (Fig. 3*C*). In addition to this heterologous cell system, we captured PTPσ from a rat brain membrane protein-enriched fraction and identified the protein by mass spectrometry analysis (Fig. S9). Lastly, we showed that biotinylated CS-E, but not CS-A or CS-C, polysaccharides bind immobilized PTPσ with high affinity according to a Langmuir binding model (Fig. 3*D*). The apparent dissociation constant ($K_{D,\mathrm{app}}$) of approximately 1 nM is similar to values reported for the association of PTPσ with the CSPGs neurocan and aggrecan (22).

Having demonstrated that CS-E interacts specifically with PTPσ, we next tested whether CS-E and PTPσ form a functional association. Deletion of PTPσ significantly attenuated CS-E-induced inhibition of neurite outgrowth in DRG neurons (Fig. 3*E*), indicating that PTPσ is required for CS-E to inhibit neurite outgrowth. Interestingly, residual inhibition by CS-E (approximately 22%) remained in PTPσ-deficient neurons, consistent with previous observations with CSPGs (22). These results suggest that CS-E may also engage other receptors, possibly leukocyte common antigen-related phosphatase (LAR) (27) and as-yet-undiscovered receptors, although we cannot rule out additional receptor-independent mechanisms, such as charge repulsion or reduced cell adhesion. Together, these studies demonstrate that the fine structure of CS chains mediates interactions with receptors involved in axon regeneration, and they identify PTPσ as a critical functional receptor for CS-E.

Generation of a Selective CS-E Blocking Antibody. An important implication of these results is that blocking CS-E interactions may prevent the inhibition caused by CSPGs and promote axon regeneration. To generate a CS-E blocking agent, we raised a monoclonal antibody against a pure synthetic CS-E tetrasaccharide (28). Although antibodies have been generated previously using CS polysaccharides as antigens (29, 30), their specificity has been limited by the structural heterogeneity of natural polysaccharides. Synthetic chemistry has the advantage of providing defined molecules of precise sulfation sequence, which can be used as antigens, for screening antibodies, and for characterizing binding specificities. An antibody generated in this manner was highly selective for the CS-E sulfation motif, as measured by dot blot, ELISA, carbohydrate microarrays, and surface plasmon resonance (Fig. 4*A* and Figs. S10 and S11). Strong binding to pure CS-E tetrasaccharides and natural CS-E polysaccharides was observed, with minimal binding to CS-A or CS-C tetrasaccharides and other glycosaminoglycan classes. Notably, this antibody also bound a mixture of CSPGs derived from chick brain (Fig. 4*B*), confirming the presence of the CS-E epitope on CSPGs, and blocked the interaction of CS-E polysaccharides with PTPσ (Fig. S12).

CS-E Blocking Antibody Promotes Axon Regeneration. To test whether blocking the CS-E epitope reverses the inhibitory effects of CSPGs, we added the CS-E antibody to DRG neurons grown on a substratum of CSPGs. Neurite inhibition by CSPGs was significantly decreased by addition of the CS-E antibody, with neurite outgrowth returning to 79% of control levels (Fig. 4*C*). In contrast, neither a CS-A monoclonal antibody nor an IgG control antibody had any effect on CSPG-mediated neurite outgrowth.

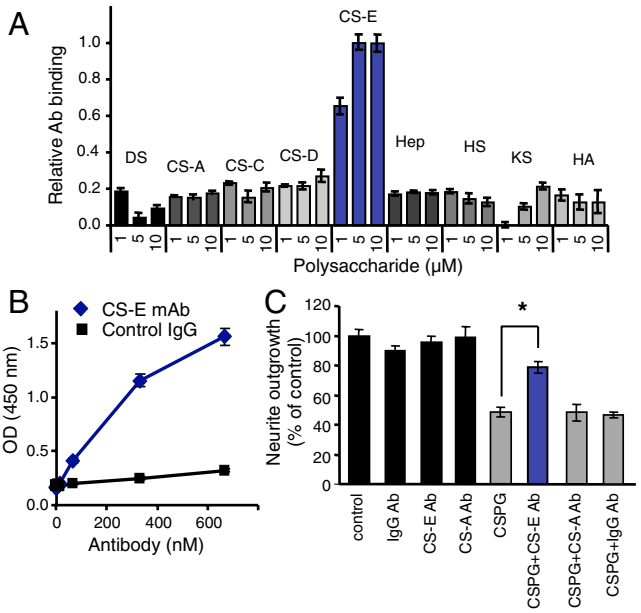

Fig. 4. A monoclonal antibody binds specifically to CS-E and blocks CSPG-mediated neurite inhibition. (*A*) Binding of the CS-E antibody to carbohydrate microarrays. Little binding to other sulfated CS polysaccharides or glycosaminoglycan classes was detected. Experiments were performed in triplicate ($n = 10$ per condition). (*B*) Dose-dependent binding of the anti-CS-E antibody to CSPGs, as shown by an enzyme-linked immunosorbent assay. The experiment was performed in triplicate, and average values (±SD, error bars) are shown for one representative experiment. (*C*) The CS-E antibody blocks CSPG-mediated inhibition of neurite outgrowth. Dissociated chick E7 DRGs were cultured on a substratum of P-Orn (control) or CSPGs (0.5 μg/mL) in the presence of the indicated antibodies (0.1 mg/mL) for 12 h. Quantitation from three experiments is shown (One-way ANOVA, $^*P < 0.0001$, relative to CSPG without antibody treatment control; $n = 50$–200 cells per experiment).

Having demonstrated specific blocking of CSPG activity in vitro, we next examined whether the CS-E antibody could promote axon regeneration in vivo. We performed an optic nerve crush injury in mice (31), which causes focal damage and glial scarring in the optic nerve and thus presents an ideal model for evaluating the effects of local application of the CS-E antibody on axon regeneration. Supporting the notion that CS-E is a prominent inhibitory component associated with CSPGs, pronounced upregulation of CS-E was rapidly observed around the lesion site within 1 d after the injury (Fig. 5*A*). To examine the effects of the CS-E antibody on axon regeneration, gelfoam soaked in a solution containing the CS-E or control IgG antibody was placed around the crush site of the nerve immediately after the injury and replaced twice at day three and six. The extent of axonal regrowth was assessed 2 weeks after injury by anterograde axon tracing with choleratoxin-B subunit (CTB), which was injected intravitreally 3 d before mice were killed. Little axon regeneration was observed in the control antibody-treated group. In contrast, the CS-E antibody treatment resulted in substantial axonal regrowth, with a sixfold increase in the number of regenerating axons when counted at 0.25 mm beyond the injury site, as compared with control antibody-treated mice (Fig. 5*B*). Notably, the extent of axon regeneration observed after CS-E antibody treatment was comparable to that seen in mice treated with ChABC alone (50 U/mL) or with ChABC and CS-E antibody applied simultaneously (50 U/mL and 1.7 mg/mL, respectively). Thus, blockade of CS-E activity induced a similar extent of axon regeneration as

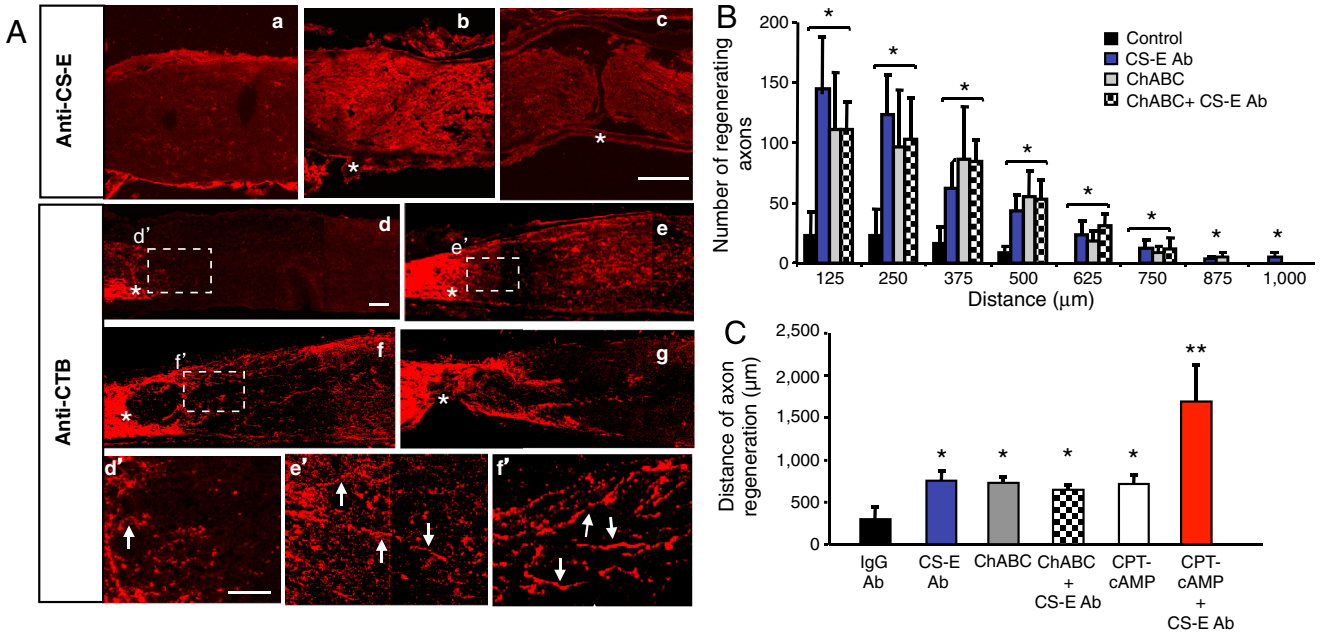

Fig. 5. CS-E neutralizing antibodies promote optic nerve regeneration. (*A*) a–c: Immunofluorescence labeling of CS-E expression in optic nerve sections at day 1 sham-operation (a), optic nerve crush injury (b), or optic nerve crush injury plus ChABC treatment (c). Note upregulation of CS-E around the injury site (b) that was removed by ChABC treatment (c). d–g: Representative epifluorescence photomicrographs of optic nerve sections taken from mice treated with control IgG (d), CS-E antibody (e), ChABC (f) or ChABC plus CS-E antibody (g). Asterisk indicates the crush site. Retinal ganglion cell axons (red) are labeled by an anterograde axon tracer, CTB, which was injected into the vitreous 3 d prior to scarify, followed by immunostaining with goat-anti-CTB antibody. In control antibody-treated mice (d), few regenerating axons are evident. In contrast, numerous regenerating axons were seen extending pass the crush site in CS-E antibody, ChABC or the combine-treated groups (e–g). Scale bars: 75 μm (a–g); 25 μm (d′–f′). Arrowheads indicate regenerating RGC axons. (*B*) Quantification of the numbers of regenerating axons at different distances from the injury site. Nerve fibers were counted at 125-μm intervals from the crush site from three nonconsecutive sections, and the number of fibers at a given distance was calculated (±SEM, error bars). Both the anti-CS-E-and ChABC-treated groups showed significantly more regenerating axons as compared with the control IgG antibody-treated group (ANOVA with Bonferroni posttests at each distance, $*P < 0.001$ as compared to controls; $n = 6$ for each group). (*C*) Quantification of the distances of axon regeneration. Longest distance of axon regeneration was measured from at least four nonconsecutive optic nerve sections from each mouse (±SEM, error bars). Combined treatment of CS-E mAb and CPT-cAMP more than tripled the distance of axon regeneration but did not affect the number of regenerating axons compared to the anti-CS-E or CPT-cAMP treatment alone (Fig. S13).

removal of CS chains from the CSPGs, underscoring the inhibitory potency of CS-E in vivo. To rule out the possibility that the observed axon regrowth after CS-E antibody treatment was simply due to improved cell survival, we stained retinal sections with an anti-βIII-tubulin antibody to image retinal ganglion cells and counted the number of surviving cells. No detectable increase in retinal ganglion cell survival was found in the CS-E antibody-treated mice, as compared with control antibody-treated mice (Fig. S13). Remarkably, these results indicate that the complex process of CSPG-mediated neuronal inhibition can be broken down into discrete, active components, which when blocked are sufficient to promote axonal regeneration in vivo.

Combining the CS-E Blocking Antibody with Other Treatments. The failure of axons to regenerate has been attributed to inhibitory molecules in the extrinsic environment and a reduced intrinsic regenerative capacity of mature CNS neurons (1, 2). We therefore examined the ability of the CS-E antibody to enhance axon regrowth in vivo when used in combination with 8-(4-chlorophenylthio)-cyclic AMP (CPT-cAMP), a cAMP analog known to penetrate the cell membrane and activate the intrinsic growth state of neurons (32). In agreement with stimulation of the growth potential of retinal ganglion cell axons, treatment of CPT-cAMP increased the number of regenerating axons by 12-fold compared to the control treated group (Fig. S13). Combined delivery of the CS-E antibody and CPT-cAMP stimulated longer axonal regrowth than either drug treatment alone, increasing the distance of regeneration by more than 3-fold (Fig. 5*C*). The number of regenerating axons compared to the CPT-cAMP treatment alone was not affected (Fig. S13), further supporting the notion that CS-E contributes to the environmental inhibition, but not the intrinsic growth status, of retinal ganglion cell axons. These results demonstrate the potential of combining the CS-E antibody to block inhibitory CSPGs in the extracellular matrix with growth-promoting treatments to enhance the regenerative outcome.

Conclusions

It has long been recognized that CSPGs are one of the major inhibitors of axon regeneration, but until recently, the structural determinants and mechanisms underlying their activity have been poorly understood. In particular, the precise role of the CS sugars and the importance of specific sulfation motifs have been unclear, limiting the development of molecular approaches to counteract CSPGs. Our studies identify a sugar epitope on CSPGs that is primarily responsible for the inhibitory effects of CSPGs. We show that the CS-E motif interacts directly with the PTPσ receptor and activates signaling pathways involved in inhibiting axon growth. These findings defy the conventional view that CSPGs function primarily as a mechanical barrier to axon regrowth and that chondroitin sulfate sugars play nonspecific, passive roles. The ability to upregulate particular sulfated epitopes on the sugar side chains may be essential for regulating CSPG activity by allowing for more precise control beyond mere expression of the core protein. Further, the concerted expression of diverse sulfated epitopes on different CSPGs could provide an elegant mechanism to coordinate the activities of various proteoglycan core proteins.

These studies also provide a potential strategy for promoting axon regeneration and neural plasticity after injury. We show that CS-E blocking strategies can increase axon regeneration in vivo and can be combined effectively with other treatments, such as stimulation of neuronal growth, to further improve the regenerative outcome. Previous studies have demonstrated that antibodies

delivered to the spinal cord can improve function after spinal cord injury (33, 34), and new techniques may even allow antibodies into the brain for the treatment of neurodegenerative diseases (35, 36). Additionally, the development of small-molecule antagonists of CS-E function should also be feasible by inhibiting the sulfotransferase Chst15. Targeting specific CS sugar epitopes using antibodies, small molecules, or other approaches may offer fewer undesirable side effects and a more stable, selective, and less immunogenic alternative to chondroitinase ABC, which is currently being evaluated as a therapeutic treatment for spinal cord injury. Given that CS-E appears to interact with multiple protein receptors and activate multiple signaling pathways, strategies that block the sulfated CS-E epitope may also prove more effective at neutralizing CSPGs than targeting individual CSPG receptors or pathways.

More broadly, our results demonstrate the importance of the fine structure of CS chains in modulating the activity of CSPGs in vivo. In contrast to heparan sulfate, where a handful of important sequences have been identified (17), much less is known about the roles of CS sulfation. We provide in vivo evidence that a specific CS-E sulfation motif within CS polysaccharides signals through protein receptors so as to direct important physiological responses. Our studies underscore the power of synthetic chemistry to deliver sulfated sequences with precise spacing and orientation to assess an underappreciated component of the mechanism. Given the importance of glycosaminoglycans in processes ranging from development to viral invasion and spinal cord injury, an expanded view of these sulfated sugars may provide new insights into many critical biological processes.

Methods

For a detailed description of the materials and methods used, see *SI Methods*.

Neurite Outgrowth Assays. E7 chick DRGs or P5-9 rat CGNs were grown on coverslips coated with poly-DL-ornithine, followed by CS-A, -C, -E polysaccharides or glycopolymers, CSPGs, ChABC-digested CSPGs, or PBS control. For the signaling pathway inhibitor studies, inhibitors against EGFR (AG1478, 15 nM), ROCK (Y27632, 5 μM), and JNK (JNK Inhibitor II, 10 μM) were added in solution at the start of culturing. For inhibition studies using neurons from $PTP\sigma^{-/-}$ mice, DRGs from adult KO mice or WT controls were grown on laminin-coated plates. Neurons were immunostained with an anti-βIII tubulin antibody, and neurite outgrowth was quantified using NIH software Image J or MetaMorph software. Statistical analysis was performed using the one-way ANOVA test; $n = 50$–500 cells per experiment, and results from at least three independent experiments were reported. Further details can be found in *SI Methods*.

Optic Nerve Regeneration Assay. Immediately after crush injury in the optic nerve of adult mice, gelfoam soaked in a solution containing control IgG, CS-E antibody, or ChABC plus CS-E antibody was placed around the crush site. Other groups of mice received an intravitreal injection of CPT-cAMP alone or CPT-cAMP plus CS-E antibody. To label retinal ganglion cell axons, a solution containing the anterograde axon tracer CTB was injected intravitreally 3 d before mice were killed. The extent of axonal regrowth was assessed 2 weeks after injury. For immunofluorescence labeling, sectioned optic nerve and retinal tissue was immunostained with anti-CS-E, anti-CTB, or anti-βIII-tubulin antibodies. To quantify the number of CTB-positive regenerating axons, the number of regenerating axons was counted at 125 μm stepwise from the crush site of the optic nerve. Further details can be found in *SI Methods*.

ACKNOWLEDGMENTS. We thank J. Vielmetter, Director of the Caltech Protein Expression Center, for assistance with the surface plasmon resonance analysis, I. Antoshechkin and the Caltech Millard and Muriel Jacobs Genetics and Genomics Laboratory for printing of the polysaccharide microarrays, and P. Clark for helpful comments on the manuscript. This research was supported by National Institutes of Health grants R01 GM093627-03 (L.H.W.) and 5T32 GM07616-30S1 (J.M.B.), the Roman Reed Spinal Cord Injury Research Fund of California (L.H.W.), and a Christopher & Dana Reeve Foundation Individual Research Grant (B.Z.).

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