Contactin-1 Is a Functional Receptor for Neuroregulatory Chondroitin Sulfate-Ε

Chondroitin sulfate (CS) plays critical roles in central nervous system development and regeneration, and individual modifications of CS form a “sulfation code” that regulates growth factor signaling or neuronal growth. Although we have shown that CS-E polysaccharide, but not CS-A or -C polysaccharide, has an inherent ability to promote neurite outgrowth toward primary neurons, its molecular mechanism remains elusive. Here, we show the involvement of a plasma membrane-tethered cell adhesion molecule, contactin-1 (CNTN-1), in CS-E-mediated neurite extension in a mouse neuroblastoma cell line and primary hippocampal neurons. CS-E, but not CS-A, -C, or heparan sulfate, engaged CNTN-1 with significant affinity and induced intracellular signaling downstream of CNTN-1, indicating that CS-E is a selective ligand for a potential CS receptor, CNTN-1, leading to neurite outgrowth. Our data provide the first evidence that biological functions of CS are exerted through the CS receptor-mediated signaling pathway(s).

The inherent potential of CS-E is of special interest for therapeutic application to central nervous system injury. CS-E binds to several humeral factors, such as midkine (MK) and brain-derived neurotrophic factor (BDNF) (10, 11), and stimulates neurite outgrowth through the activation of MK and BDNF signaling inputs to primary neurons (9), suggesting the possible roles of CS-E as a coreceptor and/or reservoir for neurotrophic factors. In contrast, although it has been postulated that functional receptor molecules receiving a sulfation code of CS reside on the neuronal membrane surface (12), identification of such potential molecules remains challenging.

Focusing on neuroregulatory roles of CS, we show here the involvement of a cell adhesion molecule, contactin-1 (CNTN-1), in CS-E-mediated neuroregeneration in a neuroblastoma cell line and primary hippocampal neurons. CS-E engaged CNTN-1 and induced intracellular signaling downstream of CNTN-1, indicating that CS-E is a ligand for a potential CS receptor, CNTN-1. Our data provide the first evidence for functional expression of CS through the CS receptor-mediated signaling pathway(s).

EXPERIMENTAL PROCEDURES

Cell Culture, Real-time RT-PCR, and Immunocytochemistry—Neuro2a (N2a) cells (American Type Culture Collection) were...
grown in Dulbecco’s modified Eagle’s medium (Sigma) containing 10% fetal bovine serum. The cDNA encoding mouse CNTN-1 (GenBank™ accession no. NM_007727) was subcloned into a pCMV expression vector (Stratagene). The fidelity of the plasmid construct (pCMV/CNTN-1) was confirmed by DNA sequencing. N2a cells were transfected with pCMV/CNTN-1 using FuGENETM 6 transfection reagent (Roche Applied Science) according to the manufacturer’s instructions. Clonal cell lines (N2a/CNTN-1 cells) were selected and grown in the presence of 800 μg/ml G418 (Invitrogen). To avoid any side effects of G418 on neuronal differentiation, N2a/CNTN-1 cells were passaged and maintained in G418-free medium for at least 7 days before the following experiments. Single-cell suspensions of hippocampal cells were prepared from E16 mouse brains as described previously (13). For the culture of crudely purified neurons, hippocampal cells were precultured on Petri dishes in Neurobasal™ medium (Invitrogen) containing 10% fetal bovine serum for 3 h at 37 °C to remove more adherent non-neuronal cells. After 3 h, non-adherent hippocampal cells were plated on poly-l-lysine-precoated plates and maintained in Neurobasal™ medium supplemented with B27 supplement (Invitrogen) for 3 days. Total RNA was extracted from parent N2a, N2a/CNTN-1, and crudely purified hippocampal cells using an RNeasy® Mini Kit (Qiagen). Amplification of target cDNAs, including CNTN-1 for comparative expression analysis) and glyceraldehyde-3-phosphate dehydrogenase (as an internal control), was initially conducted by RT-PCR. Quantitative real-time RT-PCR was performed using a FastStart DNA Master plus SYBR Green I in a LightCycler ST300 (Roche Applied Science). Primer sequences were as follows: CNTN-1, 5'-AAGCCTATACCTGGATATT-3' (forward) and 5'-CTGACGTAGTTATCTCGG-3' (reverse); glyceraldehyde-3-phosphate dehydrogenase, 5'-CATCTGAGGGCCCACGTG-3' (forward) and 5'-GAAGCCATGAGCCCATGA-3' (reverse). To evaluate the cell-surface distribution of CNTN-1, the cultured cells were labeled with an anti-CNTN-1 polyclonal antibody (goat IgG, final concentration of 5 μg/ml) was added to the medium 2 h after cell seeding. The cultured cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and labeled with anti-MAP2 (1:250) plus anti-neurofilament (Sigma, mouse IgG, 1:250) antibodies followed by development using an M.O.M™ Immuno detection kit (Vector) with 3,3’-diaminobenzidine as a chromogen. Microscopic images of immunostained cells were analyzed using morphological analysis software (Mac SCOPE, Mitani Corp). In each condition, clearly isolated cells with at least one process longer than the cell body diameter were counted as a “neurite-bearing cell.” The length of the longest neurite was measured by tracing the corresponding neurite of ~100 neurite-bearing cells. At least three independent experiments per condition were carried out.

Interaction Analysis—The binding of various GAGs to CNTN-1 was examined using a BIAcore J system (GE Healthcare) as described previously (14), with slight modifications. Briefly, recombinant human CNTN-1 (R & D Systems) was immobilized on a CM5 sensor chip (GE Healthcare) according to the manufacturer’s instructions. GAGs in a series of concentrations ranging from 10 to 100 μg/ml in running buffer were applied to flow cells, and changes in resonance units were recorded. Data were analyzed using BIAevaluation 3.0 software (GE Healthcare) using a 1:1 Langmuir binding model.

In Situ Binding of CS-E to the Cell Surface of N2a/CNTN-1 Cells—CS-E was biotinylated as described previously (11). Biotinylated CS-E (15 μg) was incubated with Avidin-Fluorescein (R & D, 0.1 μg) for 30 min. The fluorescein-labeled CS-E (final concentration, 100 μg/ml) was added to serum-free culture medium (Dulbecco’s modified Eagle’s medium) of parent N2a or N2a/CNTN-1 cells and incubated for 2 h. After washing with PBS, fluorescence on the cell surface was visualized on an all-in-one type fluorescence microscope BZ-8000 (Keyence, Osaka, Japan).

Kinase Assay—N2a/CNTN-1 cells grown in 100-mm dishes were incubated in 8 ml of serum-free Dulbecco’s modified Eagle’s medium for 24 h and further treated with CS-A, CS-C, or CS-E (final concentration, 50 or 100 μg/ml) for 2 h. The cells were harvested and lysed in 500 μl of M-PER® Mammalian Protein Extraction Reagent (Pierce) containing 1 mM Na4VO4 and protease inhibitor mixture (Nacalai Tesque). For immunoprecipitation, the cell lysate (300 μg of protein) was incubated with anti-p59fn (FYN3, Santa Cruz Biotechnology) polyclonal antibody for 60 min at 4 °C, then protein A-Sepharose (Pierce) beads were added, and the incubation continued overnight at 4 °C. An aliquot of the immunoprecipitate was assayed for kinase activity as described previously (15), with slight modifications. Briefly, the kinase assay was performed in a 20-μl reaction containing 10 mM Pipes (pH 7.0), 5 mM MnCl2, 0.5 mM dithiothreitol, 0.25 mM Na4VO4, and 1 nmol of ATP at 37 °C for 10 min. The reaction mixture was mixed with sample-loading buffer and heated at 100 °C. The reaction sample and the untreated aliquot of the immunoprecipitate were analyzed by Western blotting as described previously (16). For immunode-
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For simplicity, we attempted to uncover the mechanisms underlying CS-E-mediated neurite extension using a neuroblastoma cell line, Neuro2a (N2a) cells, instead of primary neurons. Because they differentiate and extend neurites in response to serum deprivation, the possible contributions of neuritogenic humoral factors, being derived from non-neuronal cells and/or sera, are largely excluded from this neuronal differentiation system. Surprisingly, when N2a cells were cultured on CS-E-precoated substrate under serum-free conditions in the presence of a serum replacement, neuritogenesis was not observed, even after 48-h culture (Fig. 1a). Furthermore, neither MK nor BDNF treatment improved the neuritogenesis of N2a cells on CS-E substrate (supplemental Fig. S1). These initial observations raised the possibility that the insensitivity of N2a cells to CS-E is due to defects (or relatively low expression) of particular cell surface molecules potentially sensing CS-E. In the search for candidate molecules by comparative gene expression analysis using cDNA libraries prepared from N2a and cultured hippocampal cells, we found that contactin-1 (CNTN-1), a glycosyl-phosphatidylinositol-anchored cell adhesion molecule of the immunoglobulin superfamily, was not expressed in N2a cells (Fig. 1b). The widely accepted features of CNTN-1 that modulate neurite outgrowth and interact with multiple macromolecules, including a CSPG RPTP/phosphacan (17–21), prompted us to validate the function of CNTN-1 as a neuronal cell surface receptor for CS-E.

To assess the involvement of CNTN-1 in CS-E-mediated neurite outgrowth, N2a cells were stably transfected with an expression vector carrying CNTN-1 cDNA. The significant expression of CNTN-1 and its cell surface distribution in established N2a clones (N2a/CNTN-1) were confirmed by quantitative RT-PCR and immunocytochemistry, respectively (Fig. 1c and supplemental Fig. S2). Unlike parent N2a cells, vigorous neurite sprouting was observed in N2a/CNTN-1 cells when plated on CS-E substrate, as judged by the increased fraction of neurite-bearing cells (Fig. 1, a and d). In contrast, no such appreciable promotion was seen even in N2a/CNTN-1 cells when cultured on substrate precoated with CS-A, CS-C, or CS-E.
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N2a/CNTN-1 cells, the neurite outgrowth-promoting effect of pal neurons cultured on CS-E substrate (Fig. 2). Notably, combined treatments with the CNTN-1-neutralizing antibody and either MK- or BDNF-neutralizing antibodies (9) completely abolished the CS-E-mediated neurite outgrowth promoting effects, when the poly-DL-ornithine was deemed as a basal control substrate (Fig. 2c). These results suggested that not only humoral factors, MK and BDNF, but also CNTN-1 is important in CS-E-mediated neuritogenesis in primary hippocampal neurons and also indicated that MK and BDNF might be largely dispensable for neurite outgrowth via CNTN-1.

To further clarify the receptor function of CNTN-1 specific for CS-E, the molecular interaction of CNTN-1 with various GAG preparations was examined using a surface plasmon resonance biosensor, BIAcore. Recombinant CNTN-1 was immobilized on the carboxymethyl-dextran sensor chip surface, and aqueous solutions of GAG preparations (80 μg/ml) were individually injected over the sensor surface to detect direct binding. An overlaid sensorgram disclosed the clear binding of CS-E (Fig. 3a); in contrast, CS-A, CS-C, and HS showed less binding than did CS-E. This binding preference of CNTN-1 was positively correlated with the structure-dependent neuritogenic activity of CS preparations toward both N2a/CNTN-1 cells and primary hippocampal neurons. Evaluation of kinetic parameters revealed that CS-E interacted with CNTN-1 with significant affinity (apparent equilibrium dissociation constant, $K_d = 1.4 \pm 0.1 \mu M$) (supplemental Fig. S4 and Table 1), which was comparable to measurement of carbohydrate-recognizing proteins, including animal lectins and their target glycoconjugates (23, 24). In contrast, all the other GAGs examined had 2 or 3 orders of magnitude lower apparent affinity for CNTN-1 compared with CS-E (Table 1). Based on the kinetic data, the binding ability of CS-E to cell-surface CNTN-1 was examined by addition of a fluorescein-labeled CS-E to the culture medium. As shown in Fig. 3b, the fluorescent signal was detected on the cell surface of N2a/CNTN-1 cells, whereas no detectable signal was observed on parent N2a cells. Thus, the direct interaction of CS-E, but not CS-A, CS-C, and HS, with cell-surface CNTN-1 seemed to be a prerequisite for subsequent actions leading to neurite outgrowth on CS substrata.

Several lines of evidence suggest that CNTN-1-mediated signaling is expressed as the activation of cytoplasmic, non-

**FIGURE 2. Functional involvement of CNTN-1 in CS-E-mediated neurite outgrowth in primary hippocampal neurons.** a, most MAP2-positive hippocampal neurons are CNTN-1-immunoreactive. Scale bar, 100 μm. b, representative morphology of primary hippocampal neurons cultured on CS-E substrate in the presence (+) or absence (−) of a neutralizing antibody against CNTN-1. Scale bar, 100 μm. c, a CNTN-1-neutralizing antibody inhibits the neurite elongation of primary hippocampal neurons grown on CS-E substrate ($n = 250$, $p < 0.00001$, antibody (+) versus (−), Mann-Whitney’s U test). Combined treatments with the CNTN-1-neutralizing antibody and either MK- or BDNF-neutralizing antibodies completely abrogated the neurite outgrowth ($n = 100$, $p < 0.000001$, antibodies (+) versus (−), Mann-Whitney’s U test).

HS from bovine kidney, another class of representative sulfated GAGs (supplemental Fig. S3). Notably, application of a functionally blocking anti-CNTN-1 polyclonal antibody in culture medium effectively abrogated neuritogenesis in N2a/CNTN-1 cells on CS-E substrate (Fig. 1d and supplemental Fig. S3). Additional quantitative analysis measuring the length of the longest neurite of individual N2a/CNTN-1 cells also revealed that CS-E displayed marked neurite outgrowth-promoting effects compared with the poly-DL-ornithine control, CS-A, CS-C, and HS (Fig. 1e). These findings clearly indicate that the functional expression of CNTN-1 is required for neuritogenesis in N2a cells grown on CS-E substrate.

We next investigated whether CNTN-1 is also required for CS-E-induced neurite outgrowth of primary hippocampal neurons. In support of the mRNA expression in cultured hippocampal cells (Fig. 1a), immunoreactivity for CNTN-1 was detected in the majority of MAP2-positive neurons in the culture (Fig. 2a), enabling us to test the above-mentioned functional blocking assay. As reported previously (22), elongation of prominent long neurites was frequently observed in hippocampal neurons cultured on CS-E substrate (Fig. 2b). As observed in N2a/CNTN-1 cells, the neurite outgrowth-promoting effect of CS-E was also effectively inhibited by treatment with the CNTN-1-neutralizing antibody (Fig. 2, b and c). Notably, combined treatments with the CNTN-1-neutralizing antibody and either MK- or BDNF-neutralizing antibodies (9) completely abolished the CS-E-mediated neurite outgrowth promoting effects, when the poly-DL-ornithine was deemed as a basal control substrate.

**Length of the longest neurites (μm)**

| Condition                        | Length (μm) |
|----------------------------------|-------------|
| CS-E/anti-CNTN-1 Ab (+)          | 500         |
| CS-E/anti-CNTN-1 Ab (-)          | 300         |
| CS-E/anti-CNTN-1 Ab (+) and anti-MK Ab (+) | 200        |
| CS-E/anti-CNTN-1 Ab (+) and anti-BDNF Ab (+) | 100        |
| P-ornithine/anti-CNTN-1 Ab (-)   | 0           |

**Scale bar**

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FIGURE 3. CS-E binds to CNTN-1 and stimulates intracellular signaling in N2a/CNTN-1 cells. a, GAG preparations (80 μg/ml) were individually injected over the recombinant CNTN-1-immobilized sensor chip. The beginning of the association and dissociation phases is marked by arrows A and B, respectively. RU, resonance units. b, exogenously added fluorescein-labeled CS-E accumulated on the cell surface of N2a/CNTN-1 cells, whereas no detectable fluorescent signal was observed on parent N2a cells. c and d, phosphorylation activity of Fyn kinase in N2a/CNTN-1 cells treated with CS-E. Fyn kinase was immunoprecipitated from lysates of N2a/CNTN-1 cells treated with CS-A, CS-C, or CS-E. Phosphorylated Fyn after a kinase assay reaction (Auto + endo) of each aliquot of the immunoprecipitate was detected by immunoblot using an anti-phosphotyrosine antibody (c, top), whereas untreated aliquots were probed for endogenously phosphorylated (Endo) and total Fyn kinase (c, middle and bottom, respectively). d, relative Fyn kinase activity (autophosphorylation per unit of Fyn protein, [(Auto + endo) − (Endo)]/total) was calculated by densitometric quantification of the Western blots. Values were obtained from an average of two independent experiments and normalized to that of control cells untreated with CS preparations. Error bars show the range of values obtained.

receptor-type tyrosine kinase Fyn, which regulates neurite outgrowth (25–27); therefore, to evaluate the functional aspects of CS-E as a signaling molecule, we examined in vitro autophosphorylation activity of Fyn immunoprecipitated from lysates of N2a/CNTN-1 cells cultured in the presence or absence of CS preparations. Treatment with CS-E (50 or 100 μg/ml) brought about a significant increase of Fyn kinase activity in a concentration-dependent manner, which neuronal cell soma (7, 9), resulting in additive or synergistic acceleration of neurite extension (see Fig. 2c).

It has been reported that neuronal CNTN-1-mediated neurite outgrowth is induced via trans-heterophilic interaction with a glial CSPG, RPTPβ/phosphacan (18–21). Although the contribution of CS moieties of RPTPβ/phosphacan to such neuron-glia interaction remains to be investigated, functional similarities between CS-E and RPTPβ/phosphacan in neurite was 168% or 245% of the autophosphorylation activity of non-treated cells, respectively (Fig. 3, c and d). By contrast, treatment with CS-A or CS-C did not augment the basal kinase activity observed in non-treated cells (Fig. 3, c and d). In addition, no significant increment in kinase activity occurred in parent N2a cells, even when treated with the higher concentration (100 μg/ml) of CS-E (supplemental Fig. S5). These results indicate that CS-E can act as an effective ligand for neuronal cell-surface receptor CNTN-1 that stimulates the intracellular signaling pathways involved in neuritogenesis.

Taking advantage of the observation that N2a cells were less responsive to the neuritogenic CS-E substrate, we described here how a glycosylphosphatidylinositol-linked cell adhesion molecule CNTN-1 can function as a neuronal cell-surface receptor for CS-E. This is the first report showing that CS sugar chains indeed behave as extracellular signaling molecules that can induce intracellular signaling in a sulfation pattern-dependent manner. Based on these findings, in conjunction with the capability of CS-E to bind humoral factors (10, 11, 28), we propose bidirectional regulatory modes of action for CS-E-mediated neurite outgrowth in cultured primary neurons as follows. CS-E substrate stimulates intracellular signaling cascades leading to neurite outgrowth by interacting directly with plasma membrane-residing CNTN-1. In addition, CS-E substrate concentrates soluble neuritogenic factors, derived from cultured cells and/or medium, onto its own surface and effectively presents them to their respective receptors on the extending neurites and/or
outgrowth suggest that neuritogenic sulfation domains composed of CS-E-like structures may be embedded in CS moieties of RPTPβ/phosphacan expressed by particular glial cells. In fact, significant proportions of CS-E-like structures are found in the mammalian brain (2). Given that the sulfation modification of CS chains is a cell/tissue-specific and core protein-dependent process, the emergence of neuritogenic CSPGs in the central nervous system may be also spatiotemporally regulated; therefore, further identification of CSPGs carrying the CS-E-like structures, but not typical CS-A- and CS-C-like structures, is essential to elucidate the functional aspect of CS as a neuritogenic molecule. In addition, future studies focusing on the sulfotransferases responsible for synthesis of the CS-E-like structures will advance our understanding of the functional importance of the sulfation code in the central nervous system development and regeneration and will aid in the development of novel, additional therapeutic approaches for central nervous system injuries and neurodegenerative diseases.

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