Specific Structural Features of Heparan Sulfate Proteoglycans Potentiate Neuregulin-1 Signaling*

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Neuregulins are a family of growth and differentiation factors that act through activation of cell-surface erbB receptor tyrosine kinases and have essential functions both during development and on the growth of cancer cells. One alternatively spliced neuregulin-1 form has a distinct heparin-binding immunoglobulin-like domain that enables it to adhere to heparan sulfate proteoglycans at key locations during development and substantially potentiates its activity. We examined the structural specificity needed for neuregulin-1-heparin interactions using a gel mobility shift assay together with an assay that measures the ability of specific oligosaccharides to block erbB receptor phosphorylation in L6 muscle cells. Whereas the N-sulfate group of heparin was most important, the 2-O-sulfate and 6-O-sulfate groups also contributed to neuregulin-1 binding in these two assays. Optimal binding to neuregulin-1 required eight or more heparin disaccharides; however, as few as two disaccharides were still able to bind neuregulin-1 to a lesser extent. The physiological importance of this specificity was shown both by chemical and siRNA treatment of cultured muscle cells. Pretreatment of muscle cells with chlorate that blocks all sulfation or with an siRNA that selectively blocks N-sulfation significantly reduced erbB receptor activation by neuregulin-1 but had no effect on the activity of neuregulin-1 that lacks the heparin-binding domain. These results suggest that the regulation of glycosaminoglycan sulfation is an important biological mechanism that can modulate both the localization and potentiation of neuregulin-1 signaling.

Heparan sulfate proteoglycans (HSPGs)1 are ubiquitous components of the cell surface and extracellular matrix that are critical for normal development and regulation of cell growth (1–3). HSPGs have been implicated in a wide range of biological processes including cell adhesion, motility, proliferation, differentiation, tissue morphogenesis, and carcinogenesis (4, 5). Many of these biological processes are mediated through numerous heparin-binding growth and differentiation factors that include fibroblast growth factors (FGFs) (6, 7), platelet-derived growth factor (8), bone morphogenic proteins (9), heparin-binding growth-associated molecule (10), and neuregulins (NRGs) (11).

The NRGs have many functions in the nervous system and cardiac development and in the regulation of cell growth and carcinogenesis (12). Four NRG genes have been discovered thus far of which NRG1 is the best characterized. The NRG1 gene produces multiple alternatively spliced membrane-bound and soluble isoforms. Soluble isoforms of NRG1 are composed of an epidermal growth factor (EGF)-like domain, which is sufficient for receptor binding and activation of homodimers and heterodimers of erbB2, erbB3, and erbB4, and an immunoglobulin-like domain, NH2-terminal to the EGF-like domain, that is responsible for binding heparin (13–15). Interactions with HSPGs appear to direct the distribution of NRG1 in the developing nervous system. For example, HSPGs including agrin become restricted to the basal lamina of neuromuscular synapses coincident with NRG1 during embryonic development in the chick (16). Here agrin and NRG1 can work synergistically by forming localized complexes that optimally concentrate acetylcholine receptors at the synapses (17). This synergy results by both potentiation of NRG1-induced erbB receptor phosphorylation and by producing a sustained signal critical for up-regulating acetylcholine receptor genes (13). NRG1 accumulation along early motor and sensory axons, radial glia, spinal axonal tracts, and neuroepithelial cells through associations with HSPGs raises other potentially important roles for NRG1 in nervous system development (16).

Because HSPGs are expressed on the surface of virtually every cell, a key question is how heparin-binding growth factors preferentially associate with HSPGs on some cells but not others. Recent evidence suggests that this specificity may be encoded by the diversity generated by differences in sugar composition and sulfation pattern of the glycosaminoglycan (GAG) chains (2, 18). HSPGs are composed of repeating disaccharides of glucuronic acid or iduronic acid and N-acetyl or O-sulfated pentasaccharide components for optimal binding but differ in the requirements for O-sulfation (20). Hepatocyte growth factor, platelet-derived growth factor, and lipoprotein lipase all depend on the presence of one or more N-acetylgalactosamine 6-O-sulfate groups (21–23), and antithrombin III requires a 3-O-sulfated N-sulfoglucosamine unit (24). Further specificity in the FGF signaling system may also result from either simultaneous or sequential

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1 The abbreviations used are: HSPG, heparan sulfate proteoglycan; FGF, fibroblast growth factor; NRG, neuregulin; EGF, epidermal growth factor; Ig, immunoglobulin; IgEGF, form of NRG with both Ig and EGF-like domains; GAG, glycosaminoglycan; p185, tyrosine-phosphorylated erbB receptors; GMSA, gel mobility shift assay; NDST, N-deacetylase/N-sulfotransferase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; siRNA, small interference RNA.
interactions between heparan sulfate and FGF and its receptors (25, 26).

Here we have defined specific structural features required for NRG1-heparin interactions and shown the physiological importance of these features in vitro. We demonstrate a clear hierarchy of importance for specific sulfate groups with the N-sulfate group being most important followed by 2-O- and 6-O-sulfate groups for binding to NRG1 and blocking erbB receptor phosphorylation. Consistently, the removal of all of the endogenous sulfate groups with chlorate or selective blockade of N-sulfation with an siRNA directed against the enzyme that promotes N-sulfation resulted in decreased NRG1-induced erbB receptor activation. This specificity provides a means by which tissues can localize and potentiate NRG1 signaling through modifications in GAG composition.

EXPERIMENTAL PROCEDURES

Reagents—Recombinant human NRG1 β1-polypeptides were from R&D (Minneapolis, MN). The isolated EGF-like domain corresponded to amino acids 177–246, and the intact IgEGF form of NRG1 corresponded to amino acids 14–246. Sodium chlorate was purchased from Sigma. The heparan sulfate antibody, 10e4, was from Seikagaku. De-N-sulfated, De-2-O-sulfated, and De-6-O-sulfated heparins were prepared as described previously (27). Different sized heparin fragments from 12 to 2 disaccharides in length were prepared by high resolution gel chromatography of low molecular weight heparin as described previously (28). For all of the experiments, the heparins were used at equal weight/volume (μg/μl) concentrations.

Cell Culture and Western Blots for p185 Phosphorylation—L6 cells were used to quantify erbB receptor phosphotyrosine (p185) as described previously (13). Cells were plated at 50,000 cells/well and grown for 8 days. Chlorate was filter-sterilized and incubated with L6 cells for 2 days prior to the assay. Inhibition assays of p185 were performed by preincubating 75 μM IgEGF NRG1 and various concentrations of the heparins at room temperature for 20 min. 150 μl of this mixture was then applied to 8-day-old L6 cells for 45 min at 37 °C in 10% CO2. The medium was aspirated, and the cells were solubilized in 50 μl of sample buffer and boiled for 5 min as described previously (13). 15 μl of this buffer was resolved on a 5% denaturing polyacrylamide gel. The phosphorylated forms of the erbB receptors (p185) were detected by Western blot analysis using the phosphotyrosine monoclonal antibody 4G10 (Upstate Biotechnology) and then verified and quantified by reprobing with a mixture of antibodies against erbB2 (Neomarkers Ab-17) and erbB3 (Neomarkers Ab-6). Quantitation was performed by determining the ratio of p185/erbB2 + erbB3) using Metamorph imaging system (Universal Imaging Corp.) on non-saturated images as described previously (29).

Gel Mobility Shift Assay (GMSA)—NRG1 alone or NRG1 plus various heparins at various concentrations were incubated with binding buffer (2.7 mM KCl, 4.3 mM Na2HPO4, 10 mM MgCl2, 1.4 mM KH2PO4, 12% glycerol, 1 mM dithiothreitol) for 20 min and run on a 5% (29:1 acrylamide:bis) non-denaturing gel (10 mM Tris, pH 7.4, 1 mM EDTA) for 20 min at 200 V as described previously (30, 31). Although the electrophoresis buffer consisted of 40 mM Tris, pH 8.0, 40 mM acetic acid, 1 mM EDTA, changing the pH to 9.0 above the isoelectric point of NRG1 (pI = 8.8) did not appreciably change the mobility of NRG1 with or without added heparin. However, the band resolution was sharper at pH 8.0 and was therefore used for our studies here. Protein was transferred to a polyvinylidene fluoride membrane (Millipore Corp.) for 1 h at 100 V. NRG1 bands were detected by Western blot analysis using a polyclonal rabbit antiserum (AD03) developed against the IgEGF polypeptide (Assay Designs Inc.). The filters were then probed with a goat anti-rabbit antibody coupled to peroxidase (Chemicon) and exposed to X-blue film (Eastman Kodak Co.) after treatment with chemiluminescence reagents, and the bands were quantified as above.

siRNA Silencing—siRNA molecules were designed with on-line software from Qiagen using the entire rat NDST-1 cDNA sequence (GenBank accession number M92042). The target sequence of 1406–1427 was chosen because of a 19/21-base pair identity to NDST-2. 24 h after plating, L6 cells were incubated with either 2 μg of NDST siRNA or random non-silencing siRNA (Qiagen) in 12 μl of suspension buffer diluted to 200 μl of L6 media. The cells were then incubated at 37 °C for 3 days and either treated with NRG1 for 45 min followed by phosphotyrosine Western blot as above. Parallel siRNA-treated cultures were used to document reduced NDST mRNA levels by quantitative real-time RT-PCR and sulfation activity by immunostaining with an N-sulfate-specific antibody. Total RNA was isolated using the RNeasy kit.
Fig. 3. N-Sulfation is more important for inhibiting NRG1-induced erbB phosphorylation than 2-O- and 6-O-sulfation. A, 75 pm NRG1 was incubated in duplicate with 0, 1, 10, and 100 μg/ml of heparin for 20 min and applied to L6 cells. erbB receptor phosphorylation (p185) was progressively inhibited as heparin concentration increased. Western blots were reprobed for erbB receptors (erbB2/3) to normalize the data. B, the same assay was performed with desulfated, De-N-sulfated, De-2-O-sulfated, and De-6-O-sulfated heparins. Completely desulfated and De-N-sulfated heparins were least effective at inhibiting erbB receptor phosphorylation followed by the De-2-O- and De-6-O-sulfated heparins and quantified as the percent of control (C). Schematic representations of each of the modified heparins are included next to each gel with sulfate groups marked with an S.

from Qiagen according to the manufacturer’s instructions. To reduce genomic DNA contamination, the column was treated with DNase (137.5 μg/ml) for 15 min. The concentration of mRNA eluted from the column was measured using the Ribogreen kit (Molecular Probes), and 1 μg of RNA from each sample was reverse-transcribed using SuperScript II (Invitrogen). The following primers were designed using Primer Express software against NDST-1: primer set 1 (forward 5′-GAG GAC AAA CGC CAC AAA GAC-3′, and reverse, 5′-GGG CTG TGG TGC CTG TTT T-3′); primer set 2 (forward, 5′-CCA GCT CCG AGA CCT TTG AG-3′, and reverse, 5′-GTG TTG GAG GGA ATA GGG AAG A-3′); primer set 3 (forward, 5′-AAA GTG ATG GAC ACA GTG CAG AA-3′, and reverse, 5′-GCT GGC ACC AAA ATC CTT TC-3′); and GAPDH (forward, 5′-AGT ATG ACT CTA CCC ACG GCA AGT-3′, and reverse, 5′-TCT CGC TCC TGG AAG ATG GT-3′). Each of these primer sets produced a single band of the correct size of ~100 bp. We used AmpliTaq Gold polymerase (Promega) with the following cycling parameters: 95°C for 10 min followed by 40 cycles of 95°C for 15 s, 60°C for 10 s, and 72°C for 50 s. For real-time quantitative PCR, we used the SybrGreen system (Molecular Probes) on an MJ Research Opticon machine and calculated the change in gene expression relative to GAPDH for each sample as described previously (29). Immunostaining was performed on L6 cells plated at 50,000 cells/well on an 8-well Flexiperm chamber and fixed with 4% paraformaldehyde for 15 min at room temperature. After washing three times with phosphate-buffered saline, cells were blocked for 40 min with 0.1% Triton X-100, 2% goat serum in phosphate-buffered saline and then incubated with the 1064 antibody at a 1:30 dilution in the block buffer overnight at 4°C, washed 3 × 5 min in phosphate-buffered saline, and then visualized with the Alexa 546 goat-anti-mouse IgM (Molecular Probes) at 1:300 for 2 h at room temperature using a Nikon Eclipse 600 epifluorescent microscope with a Princeton Micromax cooled CCD camera. Quantification of fluorescence was performed using Metamorph software (Universal Imaging) in a similar manner as described previously (17). The entire fields of three separate pairs of NDST-1 siRNA and control siRNA-treated cells from 8-well Flexiperm chambers were compared after subtracting the background of nearby regions without cells. siRNA-treated cells were reduced to 39, 43, and 60% of the values from control-treated cells, which gave an average of a 47 (± 11)% reduction relative to the control. The mean ± S.E. represents one standard deviation.

RESULTS

Specific Heparin Sulfate Groups Are Required for NRG1 Binding—We prepared a set of chemically modified heparins lacking either all of the sulfate groups (completely desulfated), the N-sulfate groups (De-N-sulfated), the 2-O-sulfate groups (De-2-O-sulfated), or the 6-O-sulfate groups (De-6-O-sulfated) to assess their importance in heparin-NRG1 interactions using a GMSA modeled after that described by Rosenberg and colleagues (30). Heparin oligosaccharides were chosen, because they were highly and uniformly sulfated, but had a high degree of structural similarity to the endogenous cell-surface heparan sulfates (32). This GMSA utilized a non-denaturing polyacrylamide gel, which separated protein/oligosaccharide complexes based on their charge to mass ratio and conformation. The buffer conditions were optimized so that NRG1 (pI = 8.8) was close to a net neutral charge, resulting in minimal mobility in the charged field (Fig. 1A). However, when complexed with heparin, NRG1-heparin complexes migrated further and demonstrated a “shift” in the mobility of NRG1 by Western blot. Fig. 1A shows that
increasing concentrations of heparin shift NRG1 in a dose-dependent manner that is quantified in Fig. 1B.

Using this gel mobility shift assay, we compared fully sulfated heparin to completely desulfated and De-N-sulfated heparin. The completely desulfated heparin was unable to shift NRG1 mobility (Fig. 2A). This is not surprising because the relatively neutrally charged desulfated heparin should have very low mobility even if complexed with NRG1. However, the De-N-sulfated sugars, which should still migrate in the electric field, also did not shift NRG1, suggesting a critical importance of N-sulfation for NRG1 binding. We next examined the importance of 2-O- and 6-O-sulfation on NRG1 binding using heparins lacking each of these groups. Both of these chemically modified heparins were equally effective in shifting NRG1, although not as effective as fully sulfated heparin, and more effective than the De-N-sulfated heparin. This finding suggests an intermediate but equal importance of these two sulfate groups for NRG1 binding (Fig. 2B).

The Same Rank Order of Desulfated Heparins Block NRG1-induced Receptor Phosphorylation in L6 Muscle Cells—A limitation of our gel shift assays using Western blots for NRG1 rather than radiolabeled oligosaccharides, as has been done for other heparin-binding proteins (30, 31), was that unbound NRG1 barely entered the gel, even at a higher pH. This produced variability in the intensity of the NRG1 band representing unbound NRG1 at the top of the gel. Because of this problem and as a second independent means to confirm the relative importance of specific heparin sulfation patterns on heparin-NRG1 interactions, we examined the ability of these same desulfated heparins to block erbB receptor phosphorylation in L6 muscle cells. Although the exact mechanism by which soluble heparins block erbB receptor activation is not known, it is a useful assay to compare the affinity of NRG1-heparin interactions in a biologically relevant system and has been used previously to define the relative affinities of different sulfated oligosaccharides (14). Fig. 3A shows that NRG1-induced erbB receptor phosphorylation is inhibited by fully sulfated heparin in a dose-dependent manner. Fig. 3B shows a reduced efficacy for each of the modified heparins to block NRG1-induced erbB receptor phosphorylation. Similar to the gel mobility shift assay, completely desulfated and De-N-sulfated heparins had little inhibition even at 100 μg/ml. Similarly, the 2-O- and 6-O-desulfated heparins again showed similar intermediate effects compared with fully sulfated heparin. The results were quantified in Fig. 3C and were presented as a percentage of control. Curiously, low concentrations (1 μg/ml) of the De-N-sulfated heparin had a modest stimulatory effect on receptor phosphorylation. This reproducible observation raises the possibility that low concentrations of a low affinity heparin can potentiate erbB receptor activation by releasing NRG1 from endogenous HSPG-binding sites on the cell surface.

Heparin Chain Length Effects on NRG1 Binding and erbB Receptor Blockade—The results thus far suggest an importance of the specific sulfation pattern to promote NRG1-heparin interactions and demonstrate an hierarchy of importance for the sulfate groups. We next investigated the importance of chain length on the NRG1-heparin interactions using a set of fully sulfated heparin fragments decreasing in size from 12 to 2 disaccharides on both GMSA (Fig. 4A) and receptor phosphorylation assays (Fig. 4B). As the heparin chain length was decreased, equal weight/volume concentrations of these oligosaccharides produced an upward shift in the predominant NRG1-heparin complex. The relative amount of NRG1 shifted also decreased with shorter heparin chain length, suggesting that longer heparins bind NRG1 more efficiently. If we had used equimolar amounts of each oligosaccharide, we would have expected an even greater difference between the larger and smaller oligosaccharides. The appearance of multiple bands for a given oligosaccharide length raises the possibility that either the multiple NRG1 monomers bind a single heparin chain or multiple oligosaccharide monomers bind a single NRG1. Heparins as small as two disaccharides in length were able to shift small amounts of NRG1 seen on the right side of the gel (and clearly seen on longer film exposures, data not shown). When we compared the ability of various sized heparin fragments to block NRG1-induced receptor phosphorylation parallel to the effects of NRG1 binding in Fig. 4A, we found that, as heparin chain length decreased, its ability to inhibit NRG1-induced erbB receptor phosphorylation was progressively impaired (Fig. 4B). Once the chain length was reduced to approximately four disaccharides or smaller, there was no significant inhibition by heparin.

Endogenous Heparan Sulfate Groups Potentiate NRG1-induced erbB Receptor Activation—We have shown previously that endogenous HSPGs significantly enhanced erbB receptor activation by heparin-binding forms of NRG1 (IgEGF) but not by forms of NRG1 that lack the heparin-binding Ig-like domain (EGF) (13). Therefore, we examined the role of endogenous sulfation by treating L6 muscle cells for 48 h with chlorate, which competitively inhibits the formation of 3’-phosphoadenosine 5’-phosphosulfate, the sulfate donor for sulfotransferase reactions (33–35). Using the full-length heparin-binding form of NRG1 (IgEGF), chlorate treatment reduced receptor phosphorylation by 53%, whereas no reduction was seen with
the EGF form that lacks the heparin-binding domain (Fig. 5, A and B). This documents the importance of endogenous HSPG sulfate groups to potentiate cellular interactions with NRG1.

Based on the specificity observed in the GMSA and erbB phosphorylation inhibition assays, we examined the importance of the endogenous \( N \)-sulfate group for HSPG-NRG1 interactions in muscle cells. We did this by reducing endogenous \( N \)-sulfation with an siRNA against \( N \)-acetylglucosamine \( N \)-deacetylase/\( N \)-sulfotransferase-1 (NDST-1), which is the most ubiquitously expressed of the four \( N \)-sulfotransferase isoforms and shares a close homology with NDST-2 (4, 36). The treatment of L6 cells with this siRNA resulted in a 2–3-fold reduction in NDST-1 mRNA using three different primer pairs (primer set 1, \( H_1 \)1002 2.3-fold; primer set 2, \( H_1 \)1002 3.2-fold; and primer set 3, \( H_1 \)1002 1.4-fold). The reduction in mRNA levels translated into a 47 (\( H_1 \)1006 11)% reduction in the staining of the \( N \)-sulfate groups using 10e4 antibodies that have been shown to require \( N \)-sulfate groups for their recognition (Fig. 6 A) (37). Compared with the control siRNA, NDST-1 siRNA produced a 58% reduction in NRG1-induced (IgEGF) receptor phosphorylation (\( p < 0.004 \)). An insignificant 20% reduction in NRG1-induced erbB phosphorylation was observed with NDST-1 siRNA treatment using the isolated EGF domain of NRG1 (\( p < 0.289 \)) (Fig. 6, B and C). These results suggest an importance of endogenous \( N \)-sulfation on NRG1-HSPG interactions that parallels our results with the chemically modified heparins.

**DISCUSSION**

Given that virtually all of the cells express HSPGs and that the list of heparin-binding factors continues to grow, mechanisms are needed to discriminate which factors bind to and concentrate along a given cell surface in response to dynamic requirements during development and throughout life. Variations in the amount of HSPGs and their specific sulfation patterns are ways that this could be accomplished. If a certain growth factor is needed, the cell could regulate the expression of enzymes that modify the GAG chains accordingly. In fact, regional specificity in HSPG structure has been observed with anti-heparan sulfate monoclonal antibodies, each of which...
stains a unique region of the skeletal muscle during development (38). The localized expression of two different 6-O-sulfotransferase enzymes in the anterior and posterior proximal chick limb bud, respectively, and a more uniform expression of 2-O-sulfotransferase throughout the entire limb bud suggest that this diversity is not random but highly coordinated during development through the expression of synthetic enzymes (39). Post-synthetic modifications of heparan sulfate chains have also been described that may regulate the sulfation state of HSPGs through the activation of specific sulfatases. This includes the avian sulfatase Q-sulf1, which targets the S-domains of heparan sulfate and promotes Wnt signaling (40), and the mammalian homologue, Hsfult-1, that has been shown to be required to up-regulate growth factor signaling in cancer (41).

Here we have used a set of chemically modified heparins to identify the specific sulfation requirements for optimal NGF1 binding. Using two independent assays, GMSA and erbB receptor phosphorylation, we found a reproducible hierarchy of importance with the N-sulfate groups most important for NGF1-heparin interactions followed by the 2-O- and 6-O-sulfate groups. The similar affinities seen with the De-2-O- and the De-6-O-sulfated heparins suggest that each contributes to NGF1 binding to a similar extent. In addition to the sulfation state, the disaccharide chain length was important for optimal NGF1-NRG1 interactions. We showed that, whereas 8–12 disaccharides in length were necessary for maximal binding to NGF1, heparin fragments as small as two disaccharides could still bind but to a limited extent. This raises further questions as to the exact stoichiometry and potential functions of NGF1-heparin interactions with heparins of various sizes. Because the erbB receptors exist as both homodimers and heterodimers, binding multiple NGF1 polypeptides to a given oligosaccharide chain may potentiate erbB receptor signaling.

The specific sulfuration requirements for NGF1-heparin interactions described here may also help explain the precise localization and potentiation of biological functions of NGF1 that associates with endogenous HSPGs at distinct tissue regions during development (11). We found that blocking all of the endogenous NGF1 interaction with chloride significantly reduced the ability of NGF1 to activate erbB receptors in L6 muscle cells. The effect of chloride treatment on reducing NGF1 activity in muscle cells was not expected as pronounced as that seen previously after removing all of the HSPG GAGs with heparitinase (13) or by β-oxylosidase treatment that removes and releases GAGs into the extracellular milieu (42). This is perhaps not too surprising, because the desulfuration produced by chloride is often not complete and can be selective for specific sulfate groups (33).

The physiological importance of N-sulfation in mediating NGF1-heparin interactions was substantiated by reducing endogenous N-sulfation in muscle cells using an siRNA against NDST-1. These cells showed a 2-fold reduction in NGF1-induced receptor phosphorylation comparable to the effects seen with chloride treatment that was not seen with an isolated NRG1 EGF domain that lacks the heparin-binding domain. The effectiveness of the siRNA treatment was documented by a 50% decrease in staining intensity with an antibody that associates with endogenous HSPGs at distinct tissue regions of heparan sulfate and promotes Wnt signaling (40), and the mammalian homologue, Hsfult-1, that has been shown to be required to up-regulate growth factor signaling in cancer (41).

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