Heparan sulfate regulates ephrin-A3/EphA receptor signaling

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Increasing evidence indicates that many signaling pathways involve not only ligands and receptors but also various types of coreceptors and matrix components as additional layers of regulation. Signaling by Eph receptors and their ephrin ligands plays a key role in a variety of biological processes, such as axon guidance and topographic map formation, synaptic plasticity, angiogenesis, and cancer. Little is known about whether the ephrin-Eph receptor signaling system is subject to such additional layers of regulation. Here, we show that ephrin-A3 binds to heparan sulfate, and that the presence of cell surface heparan sulfate is required for the full biological activity of ephrin-A3. Among the ephrins tested, including ephrin-A1, -A2, -A5, -B1, and -B2, only ephrin-A3 binds heparin or heparan sulfate. Ephrin-A3-dependent EphA receptor activation is reduced in mutant cells that are defective in heparan sulfate synthesis, in wild-type cells from which cell surface heparan sulfate has been removed, and in the hippocampus of conditional knockout mice defective in heparan sulfate synthesis. Ephrin-A3-dependent cell rounding is impaired in CHO cells lacking heparan sulfate, and cortical neurons lacking heparan sulfate exhibit impaired growth cone collapse. In contrast, cell rounding and growth cone collapse in response to ephrin-A5, which does not bind heparan sulfate, are not affected by the absence of heparan sulfate. These results show that heparan sulfate modulates ephrin/Eph signaling and suggest a physiological role for heparan sulfate proteoglycans in the regulation of ephrin-A3-dependent biological processes.

The function of many important morphogens and guidance molecules has been shown to involve accessory molecules as coreceptors or as a mechanism to control the topological distribution of the guidance molecules (7, 8, 12–14). Curiously, there is little information on whether ephrin-Eph signaling is influenced by heparan sulfate or other accessory regulatory molecules. In this study, we examined whether ephrin-Eph signaling is modulated by heparan sulfate. Our results surprisingly reveal that ephrin-A3 uniquely binds heparan sulfate. Ephrin-A3-mediated EphA receptor activation and biological activities are attenuated in cells lacking heparan sulfate. Cortical neurons deficient in heparan sulfate synthesis, isolated from conditional Ext1 knockout mice (7), exhibit greatly impaired growth cone collapse in response to ephrin-A3, as do wild-type cortical neurons from which cell surface heparan sulfate has been removed by heparitinase digestion. These results provide evidence for an involvement of heparan sulfate in ephrin/Eph receptor signaling and suggest that heparan sulfate modulates ephrin-A3-dependent biological processes.

Results

Binding of Ephrin-A3 to Heparin and Heparan Sulfate. To examine the binding of various ephrins to heparan sulfate, we generated a library of the recombinant ectodomains of most A and B ephrins. These recombinant ephrins all included the entire extracellular domain, up to the last amino acid before the stretch of hydrophobic amino acids that serve as either the signal for GPI-anchor attachment (in the case of the A ephrins) or the transmembrane domain (in the case of the B ephrins), followed by the FLAG epitope. Culture supernatants from transfected 293T cells containing the secreted ephrin ectodomains were applied to heparin-Sepharose and eluted by a stepwise increase in NaCl concentration. Only ephrin-A3 bound to heparin (Fig. L4). None of the other ephrins examined (ephrin-A1, -A2, -A5, -B1, -B1, or -B2) nor the ectodomains of several Eph receptors (EphA2, -A3, -A4, or -B2) exhibited detectable binding to heparin. Elution of ephrin-A3 from heparin-Sepharose required 0.4–0.8 M NaCl. This suggests that ephrin-A3 binds with an affinity similar to those of several known heparin-binding factors, such as Sonic hedgehog, Wnt1, the Ig-like domain-containing isoform of neuroregulin 1, and various chemokines (15–18).

To further confirm the significance of the above results, we performed two additional binding experiments. First, we examined binding of full-length untagged ephrins to heparin. Cell lysates from 293T cells that had been transfected with full-length untagged ephrin-A3 or -A5 were applied to heparin-Sepharose, and eluted ephrin-A3 and -A5 were detected with the respective antibodies. Consistent with the results obtained with FLAG-
tagged ectodomains, the native form of ephrin-A3 bound to heparin, whereas that of ephrin-A5 did not (Fig. 1B). Second, we examined whether ephrin-A3 binds not only to heparin but also to heparan sulfate. Heparin is synthesized exclusively in connective tissue mast cells (19) and, therefore, binding to heparin does not necessarily imply the biological significance of the interaction in other cell type. Binding of soluble forms of ephrins and Eph receptors to heparan sulfate was examined in an ELISA-type assay. As shown in Fig. 2, ephrin-A3 displayed binding to heparan sulfate.

Fig. 1. Ephrin-A3 binds heparin. (A) Binding of soluble ephrin and Eph proteins to heparin-Sepharose. FLAG-tagged ectodomains of the indicated ephrins and Eph receptors were expressed in 293T cells and the culture supernatants (Sample) were incubated with heparin-Sepharose. Unbound materials were collected (Pass) and bound materials were eluted with indicated NaCl concentrations. Each fraction was analyzed by immunoblotting with anti-FLAG antibody. (B) Binding of full-length, native ephrin-A3 and ephrin-A5 to heparin-Sepharose. Lysates of 293T cells transfected with full-length ephrin-A3 or ephrin-A5 were incubated with heparin-Sepharose. After washing with 0.2 M NaCl, bound materials were eluted with 1.2 M NaCl (Bound). Each fraction was analyzed by immunoblotting with anti-ephrin-A3 or anti-ephrin-A5 antibody.

Fig. 2. Ephrin-A3 binds heparan sulfate. Interactions of ephrins and Eph receptors with heparan sulfate were examined by an ELISA-type assay. Puriﬁed recombinant ectodomains of ephrins and Eph receptors fused to Fc and a hexahistidine tag (R&D Systems) were coated on copper-treated wells. Biotinylated heparan sulfate was incubated in these wells, and binding was measured by colorimetric enzyme reaction. Error bars represent standard deviation from quadruplicate wells.

Fig. 3. Ephrin-A3-dependent EphA signaling is impaired in cells defective in heparan sulfate synthesis and in cells treated with heparitinase. Levels of EphA receptor autophosphorylation were analyzed in cells lacking heparan sulfate expression. (A) Experiments using CHO cells. (Left) Comparison between wild-type (WT) and pgsD-677 mutant (pgsD-677) cells. pgsD-677 cells show reduced EphA2 phosphorylation in response to ephrin-A3 (compare lane 3 and lane 4). (Right) Removal of cell surface heparan sulfate by heparitinase treatment reduces EphA2 autophosphorylation in wild-type CHO cells (compare lane 9 and lane 10). (H’ase) heparitinase treated cells; (Cont) mock treated cells. Note that phosphorylation in response to ephrin-A5 was not affected in either pgsD-677 cells or wild-type cells treated with heparitinase (lanes 5, 6, 11, and 12). (B) Experiments using cortical neurons. (Left) Comparison between wild-type (WT) and Ext1 null (Ext1-KO) cortical neurons. Ext1 null neurons show reduced EphA4 phosphorylation in response to ephrin-A3 (compare lanes 3 and 4). (Right) Removal of cell surface heparan sulfate by heparitinase treatment also reduces EphA4 autophosphorylation in wild-type cortical neurons (compare lane 9 and lane 10). (H’ase) heparitinase treated cells; (Cont) mock treated cells. Note that the response to ephrin-A5 was not affected in either Ext1 null neurons or wild-type neurons treated with heparitinase (lanes 5, 6, 11, and 12). Bar graphs below each blot represent fold-changes in the intensity of immunoreactive bands. Error bars represent standard deviation of the relative intensity from three independent experiments. (PY-EphA2) tyrosine phosphorylated EphA2; (PY-EphA4) tyrosine phosphorylated EphA4. (C) Effect of the absence of heparan sulfate on EphA4 tyrosine phosphorylation in vivo. Phosphorylation of endogenous EphA4 in the hippocampus is compared between CaMKII-Cre;Ext1flox/flox (lane 2) and control mice (lane 1). Bar graphs below each blot represent fold-changes in the intensity of immunoreactive bands. Error bars represent standard deviation of the relative intensity from three control and three conditional knockout mice. * P < 0.005.
specific binding to heparan sulfate, whereas all other A and B ephrins and EphA2 and -B2 did not show detectable binding.

Abrogation of Cellular Heparan Sulfate Expression Impairs Ephrin-A3-Induced Epha Signaling. Engagement with ephrin-A ligands causes autophosphorylation of Eph receptors on tyrosine residues, which is the first step leading to activation of downstream signaling pathways (11). We therefore examined whether lack of heparan sulfate affects ephrin-A3-dependent EphA receptor tyrosine phosphorylation using two cell types that endogenously express different EphA receptors. First, we analyzed EphA2 phosphorylation in wild-type CHO cells and their heparan sulfate-deficient mutant pgsD-677 (Fig. 3A, lanes 1–6), pgsD-677 cells (20), a subline of CHO cells, lack the ability to synthesize heparan sulfate due to the disruption of Ext1, which encodes a glycosyltransferase essential for heparan sulfate synthesis (5, 6). Treatment with ephrin-A3–Fc induced substantial phosphorylation of EphA2 in wild-type CHO cells (lane 3) but much weaker phosphorylation in pgsD-677 cells (lane 4). In contrast, ephrin-A5 induced similar levels of EphA2 phosphorylation in wild-type and pgsD-677 cells (lanes 5 and 6). We further examined whether enzymatic elimination of cell surface heparan sulfate by heparitinase treatment affects EphA2 autophosphorylation (Fig. 3A, lanes 7–12). This treatment also reduced ephrin-A3-dependent EphA2 phosphorylation (lane 10) but had no effect on ephrin-A5-dependent phosphorylation (lanes 11 and 12).

Second, we examined signaling in cortical neurons, which express EphA4 and undergo growth cone collapse in response to ephrin-A3 and -A5. Ext1-null and wild-type cortical neurons were prepared from embryonic day (E)15.5 Nestin-Cre;Ext1lox/lox (7) and control embryos. We have previously demonstrated that heparan sulfate expression is abrogated in these neurons. In wild-type neurons, treatment with ephrin-A3 induced strong EphA4 phosphorylation (Fig. 3B, lane 3). In Ext1-null neurons, EphA4 phosphorylation in response to ephrin-A3 was significantly reduced (lane 4). Heparitinase treatment of wild-type neurons also reduced EphA4 phosphorylation (compare lanes 9 and 10). However, loss of heparan sulfate, either by genetic ablation of Ext1 or heparitinase treatment, did not affect ephrin-A5-induced EphA4 phosphorylation (compare lanes 5 and 6 and lanes 11 and 12).

Finally, we examined the role of heparan sulfate in ephrin-A3 signaling in vivo. Ephrin-A3 is the predominant ephrin-A ligand expressed in the adult hippocampus and has been proposed to play a major role in the activation of endogenous EphA4 in this brain structure (21). If heparan sulfate is physiologically involved in ephrin-A3 signaling, the phosphorylation level of EphA4 should be reduced in mice lacking heparan sulfate in the hippocampus. To test this, we generated conditional Ext1 knockout mice using the CaMKII-Cre2834 transgene (22). [Conditional Ext1 knockout mice driven by Nestin-Cre could not be used because they die at birth (7).] The CaMKII-Cre2834 transgene drives forebrain-specific recombination only after birth and is particularly efficient in the hippocampus [ref. 22; see also supporting information (SI) Fig. S1]. Consistent with a physiological role of heparan sulfate, tyrosine phosphorylation of EphA4 was significantly reduced in Ext1-Cre;Ext1flox/flox mice compared with wild-type littermates (Fig. 3C).

Fig. 4. Cells lacking heparan sulfate exhibit impaired cell rounding in response to ephrin-A3. (A) Morphological changes in response to ephrin-A3 and -A5 were examined in wild-type CHO cells (a–c), heparitinase-treated wild-type CHO cells (d–f), and pgsD-677 mutant CHO cells (g–i). Cells were treated with control Fc (a, d, and g), ephrin-A3–Fc (b, e, and h), or ephrin-A5–Fc (c, f, and i), and then stained with rhodamine-phalloidin. Ephrin-A3-treated wild-type CHO cells show almost complete cell rounding (b). In contrast, both heparitinase-treated wild-type cells and pgsD-677 mutants maintain a certain level of spreading and stress fibers, although they undergo partial cell retraction (e and h). The effects of ephrin-A5 were not influenced by the absence of heparan sulfate (c, f, and i). (Scale bar, 10μm.) (B) Expression of Ext1 restores the ephrin-A3-mediated cell rounding response in pgsD-677 cells. Myc-tagged Ext1 cDNA was transfected in pgsD-677 cells and cells were stimulated with ephrin-A3–Fc (b, e, and h), ephrin-A5–Fc (c, f, and i), or control Fc (a, d, and g). Cells were double-labeled with rhodamine-phalloidin (a–c) and anti-Myc antibody (d–f) to visualize cell morphology and to identify cells expressing transfected Ext1, respectively. Lower (g–i) represent merged views.

Note that ephrin-A3 induces cell rounding in pgsD-677 cells expressing transfected Ext1 (indicated by arrowheads in b, e, and h), whereas nontransfected cells in the same culture does not exhibit cell rounding (indicated by an arrow in b, e, and h). Expression of Ext1 does not affect cell rounding induced by ephrin-A5 (compare cells indicated by an arrow and arrowhead in c and f). (Scale bar, 10μm.) (C) Quantitative analysis of cell rounding. See SI Text for details on the quantification and statistical procedures. (Open bars) control Fc (black bars) ephrin-A3; (shaded bars) ephrin-A5. Data represent mean ± SD (n = 4). * P < 0.02; ** P < 0.01; *** P < 0.001.
of EphA4 was significantly reduced in mutant hippocampal tissue compared with wild-type tissue (Fig. 3C).

Ephrin-A3-Induced Cell Rounding Is Impaired in CHO Cells Lacking Heparan Sulfate. Activation of ephrin-A/EphA signaling induces a variety of cellular responses, one of which is retraction of the cell periphery and cell rounding (23, 24). Wild-type CHO cells underwent robust cell rounding in response to ephrin-A3 (Fig. 4Ab). In comparison, the cell-rounding response of pgsD-677 mutant cells was significantly impaired. Although ephrin-A3-treated pgsD-677 cells exhibited certain levels of retraction of the cell periphery, they remained substantially more spread than ephrin-A3-treated wild-type CHO cells (Fig. 4Ah). Similarly, heparitinase-treated wild-type CHO cells remained more spread than untreated cells after ephrin-A3 stimulation (Fig. 4Ac). However, cell retraction and rounding in response to ephrin-A5 were similar in heparan sulfate-deficient cells (both heparitinase-treated wild-type cells and pgsD-677 cells) and wild-type cells (compare Fig. 4c, f, and i; Fig. 4C).

To confirm that the impaired response of pgsD-677 cells to ephrin-A3 was due to the lack of heparan sulfate, rather than other nonspecific changes in these cells, we examined whether transfection of Ext1 restores normal cell rounding in response to ephrin-A3 (Fig. 4B). pgsD-677 cells transfected with Ext1 underwent robust cell rounding (indicated by arrowheads in Fig. 4B b, e, and h), whereas nontransfected cells in the same culture did not (indicated by arrows in Fig. 4B b, e, and h). Thus, the impaired cell rounding response of pgsD-677 cells is a direct consequence of the lack of heparan sulfate. Expression of Ext1 did not make any difference in the cell rounding response to ephrin-A5 (Fig. 4Bi; Fig. 4C).

Ephrin-A3-Induced Growth Cone Collapse Is Impaired in Cortical Neurons Lacking Heparan Sulfate. To gain insight into the role of heparan sulfate in ephrin-A3-mediated axon pathfinding, we examined growth cone collapse in Ext1-null and heparitinase-treated cortical neurons. Ephrin-A3 potently induced growth cone collapse in E15.5 wild-type neurons (Fig. 5Aa), causing the complete collapse of >70% of the growth cones (Fig. 5B, WT, black bar). In contrast, growth cone collapse was significantly impaired in Ext1-null neurons (Fig. 5Ac). Although some shrinkage occurred in many growth cones, complete collapse was observed much less frequently in Ext1-null neurons (Fig. 5B, Ext1-KO, black bar). The decrease in the number of collapsed growth cones in Ext1-null neurons is not due to faster recovery of previously collapsed growth cones, because a time course study demonstrated that Ext1-null neurons exhibit impaired growth cone collapse at any time points between 5 and 30 min of ephrin-A3 stimulation (data not shown). Impairment of growth cone collapse was also observed in heparitinase-treated wild-type neurons (Fig. 5Ab; Fig. 5B, WT + H’ase, black bar), whereas the absence of HS did not affect growth cone collapse in response to ephrin-A5 (Fig. 5B, shaded bars). Finally, transfection of Ext1 restored ephrin-A3-dependent growth cone collapse in Ext1-null neurons (Fig. 5Ad; Fig. 5B, Ext1-KO + Ext1-myc, black bar). Together with the results of the cell retraction and rounding assay, these results demonstrate that cell surface heparan sulfate plays a functional role in potentiating the biological activity of ephrin-A3.

Discussion

In this study, we demonstrate the interaction of ephrin-A3 with heparin and heparan sulfate and its biological significance. Ephrin-A3 binds not only to heparin, which is expressed only by connective tissue mast cells, but also to heparan sulfate, which is expressed broadly in developing tissues. The affinity of the interaction is as strong as that of several growth factors and morphogens for which unambiguous genetic evidence has demonstrated the physiological relevance of the interaction with heparan sulfate (25–27). The biological significance of the interaction is supported by functional assays with cultured CHO cells and cortical neurons and by the phosphorylation analysis of endogenous EphA4 in the adult hippocampus of conditional Ext1 knockout mice. Although it was somewhat surprising to find that binding to heparan sulfate is unique for ephrin-A3 among the ephrin-A and -B ligands, the
specificity observed in our biological experiments is consistent with the specificity of the binding.

Our results differ from those obtained in a carbohydrate microarray-based screening of heparin-binding proteins recently reported by Shi and Heish-Wilson (28). In those experiments, ephrin-A1-Fc and -A5-Fc were found to bind to heparin coated on microarrays. Ephrin-A3 was not included in their study, and the interaction of ephrin-A1-Fc and -A5-Fc with heparan sulfate was not examined. We do not know the reason for this discrepancy, although it might be due to the difference in assay systems. In any event, our data demonstrate that ephrin-A1-Fc and -A5-Fc do not bind to heparan sulfate (see Fig. 2), and that the biological effects of ephrin-A5 are not affected by the ablation of cellular heparan sulfate (see Figs. 4 and 5).

The specific interaction of ephrin-A3 with heparan sulfate suggests that ephrin-A3 binds heparan sulfate via a region unique to this ephrin. The ectodomain of A-type ephrins consists of an N-terminal receptor-binding domain and a C-terminal juxtamembrane linker region. The N-terminal domain is highly conserved (50–60% amino acid identity among different A-type ephrins; >90% between human and mouse within ephrin-A orthologs). In contrast, the sequence of the C-terminal region is different in different A-type ephrins but highly conserved across species (100% amino acid identity between human and mouse ephrin-A3 or -A5). Suggesting that this juxtamembrane linker region may be responsible for heparan sulfate binding. Consistent with this, truncated ephrin-A3 lacking the entire juxtamembrane region does not bind to heparin (unpublished data). However, this region does not contain any recognizable sequence motifs that may serve as heparin-binding sites. Moreover, mutation of some of the basic residues within the juxtamembrane region has not led to unambiguous identification of specific residues that are essential for heparin-binding (unpublished data). Identification of the location of the heparin-binding site may require detailed analyses of the 3D structure of this region of ephrin-A3 in complex with heparin or heparan sulfate.

The features of the ephrin-A3–heparan sulfate interaction bear some interesting similarities to the interaction of neuregulin isoforms containing an Ig-like domain with heparan sulfate (29). Like the ephrins, neuregulins function mainly as membrane-anchored ligands for receptor tyrosine kinases, but they can also function as proteolytically shed, soluble ligands (30). ADAM family metalloproteinases have been implicated in the shedding of neuregulins (31). Interestingly, A-type ephrins are also cleaved by ADAM family metalloproteinases (32). Although regulated ephrin-A cleavage upon EphA receptor binding has recently attracted much attention, other studies have demonstrated constitutive release of A-type ephrins from cells (33–35) and suggest possible long-range activities of released A-type ephrins (36). Although the physiological significance of these shedding events remains to be determined, these observations suggest the possibility that heparan sulfate serves to capture released ephrin-A3 on the cell surface. Such a mechanism may act to increase the local concentration of ephrin-A3 on the cell surface and/or to cluster the secreted monomeric ephrin-A3. Thus, when bound to cell surface heparan sulfate, secreted ephrin-A3 may still activate EphA receptors through a novel cell contact-independent mechanism.

In conclusion, we demonstrate the specific interaction between ephrin-A3 and heparan sulfate and its effect on ephrin-A3/EphA signaling in vitro and in vivo. Ephrin-A3 has been shown to function in the guidance of various types of axons in the developing nervous system (37–39) and in the control of dendritic spine morphology (21). Ephrin-A3 has also been proposed to play a role in promoting the growth of pancreatic cancer cells (40). Heparan sulfate is expressed in cell types and locations relevant to these events (41–43). Our results suggest that ephrin-A3/EphA signaling in these diverse biological settings may be controlled not only by the ligand-receptor interaction but also by an additional layer of regulation involving heparan sulfate.

Methods

Binding and Phosphorylation Assays. Binding of ephrins to heparin and heparan sulfate was examined by heparin-Sepharose chromatography and an ELISA-type assay with biotinylated heparan sulfate, respectively. Ephrin-A-induced EphA receptor phosphorylation was examined by an immunoprecipitation/immunoblotting assay in wild-type and the psg-D677 heparan sulfate-deficient CHO cells (20) and in primary cortical neurons from wild-type and Nestin-Cre Ext1lox/lox conditional knockout (7) embryos.

Assays for Cell Rounding and Growth Cone Collapse. Cell rounding in response to ephrin-A treatment was examined by using wild-type and psg-D677 CHO cells as described in ref. 23. Growth cone collapse in response to ephrin-A treatment was examined by using wild-type and Ext1 null cortical neurons. Morphological changes were scored by an observer blinded to experimental conditions.

For detailed description of experimental procedures, see SI Text.

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