Characterization of Slit Protein Interactions with Glypican-1*

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We have demonstrated previously that the Slit proteins, which are involved in axonal guidance and related developmental processes in nervous tissue, are ligands of the glycosylphosphatidylinositol-anchored heparan sulfate proteoglycan glypican-1 in brain (Liang, Y., Annan, R. S., Carr, S. A., Popp, S., Mevissen, M., Margolis, R. K., and Margolis, R. U. (1999) J. Biol. Chem. 274, 17885–17892). To characterize these interactions in more detail, recombinant human Slit-2 protein and the N- and C-terminal portions generated by in vivo proteolytic processing were used in an enzyme-linked immunosorbent assay to measure the binding of a glyptic-Fc fusion protein. Saturable and reversible high affinity binding to the full-length protein and to the C-terminal portion that is released from the cell membrane was seen, with dissociation constants in the 80–110 nM range, whereas only a relatively low level of binding to the larger N-terminal segment was detected. Co-transfection of 293 cells with Slit and glypican-1 cDNAs followed by immunoprecipitation demonstrated that these interactions also occur in vivo, and immunocytochemical studies showed colocalization in the embryonic and adult central nervous system. The binding affinity of the glypican core protein to Slit is an order of magnitude lower than that of the glycanated proteoglycan. Glypican binding to Slit was also decreased 80–90% by heparin (2 μg/ml), enzymatic removal of the heparan sulfate chains, and by chlorate inhibition of glypican sulfation. The differential effects of N- or O-desulfated heparin on glypican binding also indicate that O-sulfate groups on the heparan sulfate chains play a critical role in heparin interactions with Slit. Our data suggest that glypican binding to the releasable C-terminal portion of Slit may serve as a mechanism for regulating the biological activity of Slit and/or the proteoglycan.

In previous studies we biochemically characterized a major heparan sulfate proteoglycan of nervous tissue (1, 2) that we later cloned and identified as the rat homologue of glypican-1 (3), the initial member of a family of glycosylphosphatidylinositol-anchored heparan sulfate proteoglycans that is currently composed of six vertebrate proteins. High levels of glypican-1 mRNA are present in brain and skeletal muscle, and in situ hybridization histochemistry demonstrated that glypican-1 mRNA is especially prominent in cerebellar granule cells, large motor neurons in the brain stem, and CA3 pyramidal cells of the hippocampus (4). These results and parallel immunocytochemical studies indicate that glypican-1 is predominantly a neuronal product in the late embryonic and postnatal rat nervous system. Based on genetic studies, it would appear that the glypicans play a significant role in cell growth and development. Daily, the Drosophila homologue of glypican-1, is required for the control of cell division in the developing visual system and for morphogenesis of other tissues (5), and the human homologue of glypican-3/OCI-5 (GPC3) is found to be mutated in patients with the Simpson-Golabi-Behmel overgrowth syndrome (6). Our demonstration of a novel nuclear localization of glypican-1 in nerve tissue suggests that it may also be involved in the regulation of cell division and survival by direct participation in nuclear processes (7).

To better understand the functions of glypican-1 in nervous tissue we began studies aimed at identifying endogenous ligands on the premise that knowledge of such proteins might aid in elucidating the role(s) of this proteoglycan in developmental and other neurobiological processes. Using an affinity matrix in which a recombinant glypican-Fc fusion protein expressed in 293 cells was coupled to protein A-Sepharose, we isolated from rat brain two proteins that were detected by SDS-polyacrylamide gel electrophoresis as a single 200-kDa silver-stained band from which 16 partial peptide sequences were obtained by nano-electrospray tandem mass spectrometry (8). Mouse expressed sequence tags containing two of these peptides allowed the synthesis of probes that enabled us to isolate from a rat brain cDNA library a 4.1-kilobase clone that encoded two of our peptide sequences and represented a member of the Slit protein family. All of the five known rat and human Slit proteins, which share a number of structural features such as N-terminal leucine-rich repeats and C-terminal epidermal growth factor-like motifs, contain 1523–1534 amino acids, and our peptide sequences corresponded best to those present in human Slit-1 and Slit-2. In situ hybridization histochemistry showed that these glypican-1 ligands are synthesized by neurons, such as hippocampal pyramidal cells and cerebellar granule cells, in which we also previously demonstrated glypican-1 mRNA and immunoreactivity (4).

Slit proteins are conserved proteins of ~200 kDa that are expressed by neurons and glial cells, where they are associated with the cell membrane and have been shown to play an important role in axon guidance, axon branching, and cell migration (for a review, see Ref. 9). By binding to the Robo receptor of developing neurons, Slit functions as a chemorepellent signal to prevent axons or neuronal cell bodies from entering the septum area or the floor plate in the spinal cord. Slit proteins also undergo proteolytic processing in vivo, and the resulting membrane-attached N-terminal fragment has been shown to promote branching of spinal sensory axons. The function of the released C-terminal domain has not yet been elucidated, but it may have a biological activity distinct from that of the N-terminal fragment.

In the present investigation we have demonstrated that

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glypican-1 co-immunoprecipitates with the human Slit-2 protein and its C- and N-terminal fragments. More detailed studies of this interaction by ELISA1 showed that glypican-1 has a much higher affinity for full-length Slit and its C-terminal domain than for the N-terminal fragment. Scatchard analysis of the glypican-Slit interactions yielded dissociation constants in the nanomolar range that are comparable with the values reported for the binding of Slit to the Robo receptor (10) and for the cleavage site were deleted in the construct FL(UC) hSlit2, producing an uncleavable full-length protein. B, in the rat glypican-1 construct, the glycosylphosphatidylinositol anchor was replaced with the human IgG Fc sequence. The five potential heparan sulfate attachment sites were maintained in the fusion protein.

AP, alkaline phosphatase; MYC, c-Myc sequence EQLISEEDL; HIS, hexahistidine tag.

### EXPERIMENTAL PROCEDURES

#### Preparation of Glypican-1-Fc Fusion Proteins—Human embryonic kidney 293 cells were transfected with a glypican-1-Fc fusion protein construct (7) using LipofectAMINE 2000 and grown in serum-free Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (12). Conditioned medium and 1 M NaCl extracts of 293 cells transfected with the glypican-1-Fc construct was verified by DNA sequencing and N-terminal peptide gel electrophoresis. ImmunobLOTS were probed using a monoclonal anti-His antibody (Qiagen) to detect the human full-length Slit-2, the uncleavable full-length Slit-2, and the C-terminal human Slit-2, and a polyclonal anti-Slit-2 antibody was used to detect the N-terminal human Slit-2.

**ELISA—96-well plates (Corning Costar, catalog no. 9018) were coated overnight with the human full-length Slit-2 or its deletion mutants at a saturating concentration of 4 µg/well in phosphate-buffered saline. After removing the unbound protein by washing with TBST (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20), the wells were blocked with 10% fetal bovine serum in TBST for 2 h and then incubated for 18 h at room temperature with varying concentrations of glypican-1-Fc bound to the Slit-2 proteins. The colorimetric reaction product from the o-phenylenediamine substrate was measured at 450 nm using a Dynatech MRX ELISA plate reader. Nonspecific binding was calculated as the binding of glypican-1-Fc to wells coated with 100 µg of bovine serum albumin. From serial dilutions of a known concentration of glypican-1-Fc directly coated on the wells and the corresponding immunoreactivity absorbance, a standard curve was created and used to quantitate the amount of glypican-1-Fc bound to the Slit-2 proteins.**

For the various tagged Slit constructs used in the ELISAs, wells were coated with the following proteins and peptides (5 µg/well): alkaline phosphatase (Type XXIV from human placenta, Sigma), the c-Myc peptide EQLISEEDL-OH (BIOSOURCE International, Hopkinton, MA), and the C-terminally His-tagged ADP-ribosylation factor-like protein 2 (15) that was a generous gift of Nicholas Cowan (New York University Medical Center). N-Desulfated and N,O-desulfated heparins that were used in inhibition studies were kindly provided by Robert Linhardt (University of Iowa).

**Immunocytochemistry—A polymerase chain reaction product encoding amino acids 1260–1472 of rat Slit-2 was cloned into pGEX-2T (Amersham Pharmacia Biotech) and the glutathione S-transferase fusion protein was expressed in Escherichia coli. The correctness of the construct was verified by DNA sequencing and N-terminal peptide sequences.**

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1 The abbreviation used is: ELISA, enzyme-linked immunosorbent assay.
2 Ba-Charvet, K. T. N., Brose, K., Ma, L., Wang, K. H., Marillat, V., Sotelo, C., Tessier-Lavigne, M., and Chédotal, A. (2001) J. Neurosci. 21, 4281–4289.
sequencing of the expressed fusion protein after thrombin cleavage. The fusion protein was isolated by a preliminary adsorption to glutathione-agarose beads, eluted with glutathione, and then further purified by SDS-polyacrylamide gel electrophoresis. For the initial immunization, rabbits were injected with crushed gel slices containing the recombinant protein band, as well as with protein transferred to a nitrocellulose membrane after electrophoresis and untransferred protein electroeluted from gel slices and emulsified in Freund’s complete adjuvant (–100 μg total of protein/rabbit). The first and second boosts (at 2-week intervals, 40–50 μg of protein/rabbit) used gel slices and nitrocellulose membrane, and only gel slices (~25 μg of protein/rabbit) were used for subsequent boosts at 1-month intervals. Antibody titers were followed by a dot binding assay (16).

Slit was localized in embryos by immunofluorescence microscopy of cryostat sections (17) using fluorescein-conjugated anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA) as secondary antibody and in Vibratome sections of perfusion-fixed postnatal rat brain using peroxidase-conjugated secondary antibody as described previously (16).

RESULTS

Glypican-1 Interacts in Vivo with Slit Proteins—The Slit protein constructs used in our studies are shown in Fig. 1. To determine whether glypican interacts with Slit in vivo, 293 cells were transiently co-transfected with Fc-tagged glypican-1 and different Slit-2 expression plasmids, followed by the immunoprecipitation of glypican-1-Fc with protein A-Sepharose. Using an anti-His monoclonal antibody, immunoblot analysis revealed that FL(C) hSlit2, FL(UC) hSlit2, and C-terminal hSlit2 proteins were immunoprecipitated together with the glypican fusion protein, and similar results were obtained with the N-terminal hSlit2 protein that was detected by an anti-Slit polyclonal antibody (Fig. 2). In a control experiment, no signal was detected when the cells were transfected only with FL(C) hSlit2 (Control).

Immunochemical studies of embryonic and adult rats also showed a colocalization of glypican-1 and Slit proteins in the spinal cord, cerebral cortex, hippocampus, and retina and optic nerve (Fig. 3), as well as in other areas of the central nervous system such as the thalamus, internal capsule, optic chiasm, and olfactory tract (data not shown). The finding that glypican and Slit occur in the same locations during the various stages of central nervous system development strongly supports the biological significance of their high affinity interactions demonstrated in solid-phase binding assays as described below.

Interactions of Glypican-1 with Slit in Binding Assays—Solid-phase binding assays showed that the interactions of glypican with Slit proteins reached equilibrium by ~12 h and were reversible. The addition of 2 mM CaCl$_2$, MgCl$_2$, or MnCl$_2$ alone or in combination did not increase binding, which was decreased by only 10–20% in the presence of 20 mM EDTA or EGTA, indicating that glypican binding to Slit does not require the presence of divalent cations. The binding of glypican to Slit was saturable, and Scatchard plots indicated a single class of high affinity binding sites with dissociation constants of 80–100 nM for the N-terminal construct (Figs. 4 and 5).

As a control for the possibly nonspecific binding of glypican to the alkaline phosphatase, c-Myc, and His tags in the Slit constructs, we also measured the binding of glypican to wells coated with these tags either alone or (in the case of the hexahistidine tag) in the form of an irrelevant His-tagged protein. Although the concentration of these tags in the control wells would be expected to be much greater than that of those in the wells coated with the various Slit constructs, glypican binding to the c-Myc, alkaline phosphatase, and His controls was only 0.7, 6, and 7.4%, respectively, of that to the full-length Slit protein.

Role of Heparan Sulfate in the Binding of Glypican-1 to Slit—Heparin inhibited the binding of glypican to Slit in a concentration-dependent manner, with 80% inhibition of binding at a heparin concentration of 2 μg/ml (~0.4 μM) and a maximum inhibition of ~90% at 5–10 μg/ml (Fig. 6). Binding was also inhibited to the extent of 80–90% by heparitin-sulfate lyase treatment of the glypican-Fc fusion protein (data not

**Fig. 2.** Glypican-1 interacts in vivo with full-length hSlit-2 and its variants. 293 cells were transiently transfected with Fc-tagged glypican-1 and the indicated Slit-2 expression plasmids. Glypican-1-Fc was immunoprecipitated with protein A-Sepharose. Co-immunoprecipitating FL(C) hSlit2, FL(UC) hSlit2, and C-terminal hSlit2 proteins were detected by immunoblot analysis using the anti-His monoclonal antibody. N-terminal hSlit2 protein was detected by an anti-Slit polyclonal antibody. No signal was detected when the cells were transfected only with FL(C) hSlit2 (Control).

**Fig. 3.** Colocalization of the C-terminal portion of Slit and glypican-1 in the rat central nervous system. A and B, immunofluorescence staining of embryonic day 13 (E13) spinal cord. Asterisk, dorsal root entry zone; DRG, dorsal root ganglion; vr, ventral root; vf, ventral funiculus; ml, mantle layer. C and D, E16 retina and optic nerve. on, optic nerve; inl, inner nuclear layer of the retina. E and F, E19 cerebral cortex. mz, marginal zone; cp, cortical plate; sp, subplate; vz, ventricular zone. G and H, immunoperoxidase staining of CA1 and CA3 pyramidal cells and the dentate gyrus (DG) in adult hippocampus. Bars, A/B and G/H, 300 μm; C/D and E/F, 150 μm.
shown) and by the inhibition of sulfation of the heparan sulfate chains by chlorate treatment of the 293 cells during expression of recombinant glypican (Fig. 7). Completely N-desulfated (and N-reacetylated) heparin at a concentration of 2 μM inhibits glypican binding to Slit by 73%, which is on the same order as the binding of glypican produced by chlorate-treated cells, whereas there is no inhibition by completely N- and O-desulfated (and N-reacetylated) heparin (Fig. 7). These findings indicate that O-sulfation of heparan sulfate is the critical structural feature for glypican binding to Slit and that N-sulfation, which is largely unaffected by chlorate treatment (11, 18), has a much lesser role in this process. In Madin-Darby canine kidney cells, 20 mM sodium chlorate selectively inhibited 6-O-sulfation of heparan sulfate whereas 50 mM chlorate inhibited both 2- and 6-O-sulfation (11). In our studies we found that no additional inhibition of binding was achieved by increasing the chlorate concentration from 20 to 50 mM (Fig. 7).

The results of our inhibition studies are consistent with the minimal binding of the glypican core protein that was isolated from a conditioned medium by ion-exchange chromatography and migrated on SDS-polyacrylamide gel electrophoresis as a compact band of the expected molecular size. For each of the four Slit constructs, glypican binding was found to be only 10–11% of that seen with the glycanated proteoglycan (data not shown), and the dissociation constant is an order of magnitude lower than that of the glycanated protein (Fig. 8A). The binding of the core protein was not affected by treatment with heparitin-sulfate lyase or chondroitinase ABC (data not shown), demonstrating that there is a low but significant binding of the glypican core protein to Slit and that this residual binding is not because of the presence of a small amount of glycosaminoglycan. This conclusion is supported by experiments demonstrating that in the presence of heparin, glycanated glypican binds to Slit with the same low affinity as that of the core protein (Fig. 8B), and the affinity of the core protein is unchanged when the saturation study is performed in the presence of 2 μM heparin (data not shown).
between the glypicancore protein and Slit, binding is largely mediated by the glycosaminoglycan chains, and O-sulfate residues of the heparan sulfate are a critical factor in this interaction.

Slit was first identified as a secreted protein expressed by midline glia in Drosophila. An analysis of the null mutations of Slit indicated that dSlit is necessary for the proper formation of commissural and longitudinal axon tracts in the fly (19). Genetic and biochemical studies provided strong evidence that Slit is a ligand for the repulsive guidance transmembrane receptor Roundabout (Robo) and that it functions as a short-range chemorepellent for axons crossing the midline and as a long-range chemorepellent for the migration of axons away from the ventral midline (20, 21). Three Robo receptors (Robo-1, Robo-2, and Rig-1) and three Slit proteins (Slit-1, Slit-2, and Slit-3) have been cloned as the mammalian homologues of the Drosophila counterparts and found to be expressed, with complementary patterns, in various parts of the developing and adult brain and spinal cord (for a review, see Ref. 9). The mechanism of midline guidance appears to have been conserved in vertebrates because the chemorepellent activity of Slit has been demonstrated on various types of nervous tissue explants. Slit proteins repel spinal motor neurons and hippocampal and olfactory bulb axons (10, 22, 23) and can participate in the repulsive activity of the septum on the neurons that migrate toward the olfactory bulb from the subventricular zone (24, 25). The Robo/Slit receptor-ligand couple therefore appears to be involved in regulating the growth of axons projecting to the appropriate regions of the brain, functioning as a repellent and preventing axons from crossing non-target areas.

The function of Slit-2 has been extensively studied in the developing rodent visual system. Slit-2 inhibits in vitro the outgrowth of the retinal ganglion cell axons that extend from the eye toward the diencephalon and then ascend dorsolaterally to reach the midbrain and forebrain (26–28). However, Slit-2 does not have any differential effect on the path of those axons that grow ipsilaterally or cross contralaterally at the level of the optic chiasm, a ventral midline structure (26). These findings raise the possibility that additional axon guidance molecules synergize with Slit-2 and render the projecting axons responsive to divergence cues. Indeed, Slit-2 protein was reported to bind in vitro with high affinity to netrin-1, a bifunctional molecule that can act as a chemotactant as well as a repellent on different populations of axons (10, 29). In a search for other molecules that might modulate axon guidance across midline structures, Zou et al. (30) have recently shown that Slit-2 and two semaphorin proteins (Sema 3B and Sema 3F) contribute to repelling commissural axons that have crossed the midline floor plate, directing them away from the ventral gray matter and into the surrounding fiber tracts.

We have previously reported that at least two members of the Slit family, Slit-1 and Slit-2, could be isolated by interaction with a glypicanc-1 affinity matrix and that both glypicanc and the Slit proteins are expressed in overlapping patterns in various regions of the brain (8). As a continuation of that study we have now demonstrated, using a solid-phase binding assay, that glypicanc-1 binds to full-length Slit-2 and to its C-terminal region with dissociation constants in the nanomolar range that are comparable with those of other neural receptor-ligand pairs. However, the affinity of glypicanc, a heparan sulfate proteoglycan, for Slit is an order of magnitude lower than that of neural chondroitin sulfate proteoglycans such as neurocan and phosphacan for cell surface and extracellular matrix proteins of the brain (31). Co-immunoprecipitation from mamma-

**DISCUSSION**

In this report we have described the specific, high affinity binding of glypicanc-1, a major heparan sulfate proteoglycan of nervous tissue, to Slit-2, an extracellular matrix protein that has been shown to have repulsive activity in axonal guidance and pathfinding. Our study has shown that a C-terminal fragment of Slit that is released from the cell membrane by in vivo proteolytic processing can account for glypicanc binding. We also have determined the affinities of glypicanc interactions with various forms of Slit and have shown that this interaction occurs in vivo and that there is a co-localization of glypicanc and Slit at the various stages of central nervous system development. Although there is a definite but low affinity interaction

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**FIG. 6.** Effects of varying concentrations of heparin on the binding of glypican-1-Fc to full-length human Slit-2. Values represent the percent of maximum binding as defined by the binding obtained with untreated glypican and are averages of triplicate determinations ± S.E.

**FIG. 7.** Inhibitory effects of chlorate treatment and modified heparins on the binding of glypican to Slit. Glypican-1-Fc was isolated from the medium of 293 cells cultured in the presence of 20 mM or 50 mM sodium chlorate to inhibit O-sulfation of the heparan sulfate chains. Binding of the undersulfated glypicans to full-length Slit-2 was determined as described under "Experimental Procedures" and compared with the binding of normally sulfated glypican in the presence of 2 μM N-desulfated (N-dsHep) or N,O-desulfated (N,O-dsHep) heparin. Values represent the percent of maximum binding as defined by that obtained using glypican produced by untreated cells in the absence of heparin (Control) and are averages of triplicate determinations ± S.E.
their interactions with other molecules remains to be defined, can and Slit in the regulation of their biological activities and Therefore, although the full significance of the binding of glypic-
ancy and Slit-2 and glypican-1 also interact with
complex mechanism for the regulation of axonal guidance.
other cell-surface and matrix molecules to constitute a more
system, it is likely that Slit-2 and glypican-1 also interact with
it plays a critical role. Because the sulfation pattern of chon-
promotes secondary axon branching has been attributed, glypi-
can was found to bind to the C-terminal portion of Slit-2 to the
same extent as to the full-length protein. It is therefore possible
that the release of the C-terminal fragment from the cell mem-
brane and its binding to components of the extracellular matrix
might represent a mechanism for the formation of ligand gra-
dients and for signaling to distant targets.
It is also noteworthy that >80% of glypican-1 binding to
Slit-2 is mediated by the glycosaminoglycan chains of the pro-
teoglycan and that O-sulfation of the heparan sulfate chains
plays a critical role. Because the sulfation pattern of chon-
droitin sulfate and heparan sulfate has been shown to change
during brain development (16, 32, 33), the heparan sulfate
plays an important regulatory function of glypican during nervous tis-
ue histogenesis.

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