Mammalian Homologues of the Drosophila Slit Protein Are Ligands of the Heparan Sulfate Proteoglycan Glypican-1 in Brain*

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Using an affinity matrix in which a recombinant glypican-Fc fusion protein expressed in 293 cells was coupled to protein A-Sepharose, we have isolated from rat brain at least 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. Mouse expressed sequence tags containing two of these peptides were employed for oligonucleotide design and synthesis of probes by polymerase chain reaction and enabled us to isolate from a rat brain cDNA library a 4.1-kilobase clone that encoded two of our peptide sequences and represented the N-terminal portion of a protein containing a signal peptide and three leucine-rich repeats. Comparisons with recently published sequences also showed that our peptides were derived from proteins that are members of the Slit/MEGF protein family, which share a number of structural features such as N-terminal leucine-rich repeats and C-terminal epidermal growth factor-like motifs, and in Drosophila Slit is necessary for the development of midline glia and commissural axon pathways. All of the five known rat and human Slit proteins contain 1523–1534 amino acids, and our peptide sequences correspond best to those present in human Slit-1 and Slit-2. Binding of these ligands to the glypican-Fc fusion protein requires the presence of the heparan sulfate chains, but the interaction appears to be relatively specific for glypican-1 insofar as no other identified heparin-binding proteins were isolated using our affinity matrix. Northern analysis demonstrated the presence of two mRNA species of 8.6 and 7.5 kilobase pairs using probes based on both N- and C-terminal sequences, and in situ hybridization histochemistry showed 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 (9). From this work and parallel immunocytochemical studies (9) we concluded that glypican-1 is predominantly a neuronal product in the late embryonic and postnatal rat nervous system. Glypican-1 was also found to be a dual modulator capable of enhancing the mitogenic response of fibroblast growth factor-1 but inhibiting the effects of fibroblast growth factor-7 in keratinocytes (10), and it can inhibit neurite outgrowth induced by amyloid precursor protein in vitro (11).

Genetic studies provide additional support for a role of glypicans 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 (12), and the human homologue of glypican-3/OCI-5 (GPC3) was found to be mutated in patients with the Simpson-Golabi-Behmel overgrowth syndrome (13). We have also recently demonstrated a novel nuclear localization of glypican-1 in nervous tissue (14), suggesting that it may be involved in the regulation of cell division and survival by direct participation in nuclear processes.

Because the functional roles of glypican-1 in nervous tissue remain unknown, we have begun studies aimed at identifying ligands that may aid in understanding how it is involved in developmental and other neurobiological processes. By affinity chromatography of brain extracts on a matrix in which a recombinant glypican-Fc fusion protein was coupled to protein A-Sepharose, we isolated and partially cloned proteins whose sequences allowed us to identify mammalian homologues of the Drosophila Slit protein as ligands of glypican-1. Slit, which was initially identified by cross-hybridization using the sequence coding for tandem epidermal growth factor repeats of Notch, a gene involved in Drosophila neurogenesis (15), is necessary for development of midline glia and commissural axon pathways in Drosophila (16). Although information has only very recently become available concerning the functions of mammalian Slit proteins, our results suggest that interactions of these presum-
ably extracellular proteins with cell surface glypican-1 may be important in axonal pathfinding and nervous tissue histogenesis.

**EXPERIMENTAL PROCEDURES**

**Preparation of Glypican-FC Fusion Protein Affinity Matrix—Human embryonic kidney 293 cells were transfected with a glypican-FC fusion protein construct (14) using LipofectAMINE (Life Technologies, Inc.) and grown in serum-free Dulbecco’s modified Eagle’s medium containing 1% ITS+ (Collaborative Biomedical Products, Bedford, MA). The conditioned medium was continuously collected and frozen after centrifugation for 30 min at 27,000 × g and addition of sodium azide to a concentration of 0.02%. The amount of the glypican-1 fusion protein in aliquots of the conditioned medium was estimated by Coomassie Blue staining following SDS-PAGE in comparison with bovine serum albumin standards, after binding to an excess of protein A-Sepharose beads (Zymed Laboratories Inc.) using a ratio of 0.5 ml of medium/20 μl of settled beads and elution twice by sample buffer.

To determine the proportion of the glypican-FC fusion protein that was synthesized in a glycanated form, 293 cells in a six-well plate were transfected with the fusion protein construct, and after 24 h cells were washed twice with a short term labeling medium (methionine/cysteine-free Dulbecco’s modified Eagle’s medium supplemented with 1% ITS+ followed by incubation for 1 h with the same medium. The medium was subsequently changed for 4 h to labeling medium containing 125 μCi of [35S]methionine/cysteine. The glypican-FC fusion protein was purified from the conditioned medium by adsorption to protein A-Sepharose beads, and an aliquot of the beads was digested with heparitin-sulfate lyase and released glypican-1 ligands, brains of 30- to 130-day-old Sprague-Dawley rats were homogenized in 4 volumes of 25 mM PBS, pH 7.2, containing 5 mM EDTA, 100 mM 6-aminohexanoic acid. The homogenate was centrifuged for 30 min at 45,000 × g and addition of sodium azide to a concentration of 0.02%. The amount of the glypican-1 fusion protein in aliquots of the conditioned medium was estimated by Coomassie Blue staining following SDS-PAGE in comparison with bovine serum albumin standards, after binding to an excess of protein A-Sepharose beads (Zymed Laboratories Inc.) using a ratio of 0.5 ml of medium/20 μl of settled beads and elution twice by sample buffer.

**AFFINITY CHROMATOGRAPHY OF RAT BRAIN EXTRACTS—** After reclarification by centrifugation (30 min, 27,000 × g), the glypican-1 fusion protein was bound to protein A-Sepharose beads with gentle mixing at 4 °C overnight, using a ratio of 1 mg of fusion protein (in 2–5 ml of concentrated medium)/ml of settled beads. The beads were thoroughly washed with cold PBS followed by 50 mM Tris-buffered saline, pH 8.0, containing 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS. After re-equilibration in 0.1 M phosphate buffer, pH 8.0, the glypican-1 fusion protein was cross-linked to the protein A (17) at room temperature using a 30-fold molar excess of dimethyl pimelimidate (Pierce) added four times at 10-min intervals. The coupling reaction was terminated by washing the beads with 1 M Tris-glycine buffer, pH 7.0, followed by PBS, and the beads were stored in PBS containing 0.02% sodium azide. Before each use of the affinity matrix, any free fusion protein was removed by washing with 1 M NaCl in 50 mM PBS followed by 0.1 M glycine, and the beads were then again equilibrated in 50 mM PBS.

**Affinity Chromatography of Rat Brain Extracts—** In an initial experiment to determine which subcellular fractions might be enriched in glypican-1 ligands, brains of 30- to 130-day-old Sprague-Dawley rats were homogenized using a Teflon-glass tissue grinder in 4 volumes of 25 mM PBS, pH 7.2, containing 5 mM EDTA, 100 mM 6-aminohexanoic acid. The homogenate was centrifuged for 10 min at 1,000 × g and washed once. The washed P1 pellet was then extracted by stirring overnight at 4 °C in PBS with protease inhibitors as described above but with the addition of 1% CHAPS, 0.2 M NaCl, 1 mM EDTA, 10 μM pepstatin A, and 10 μM leupeptin and extracted with stirring overnight at 4°C. After centrifugation for 1 h at 200,000 × g, the supernatants were saved as fractions S4N (a crude “nuclear” extract) and a membrane extract (S5M), respectively.

**Peptide Sequencing by Mass Spectrometry—** Proteins in the 1 M NaCl eluate from the glypican-1 affinity column were electrophoresed on several lanes of an 8% 1-mm minigel, silver-stained using a protocol for subsequent sequencing (19). A major band with an apparent molecular size of ~200 kDa was excised from two to three lanes of the gel and digested in situ with trypsin (19). The complete tryptic peptide mixture was desalted and concentrated (20) on an Eppendorf G-Spinner pipette tip (Brinkman) packed with Poros Rf resin (PE Biosystems, Framingham, MA) and eluted with 2 μl of 50% methanol in 5% formic acid into a nano-electrospray sample needle. The unfraccionated digest was analyzed by nano-electrospray (21) on a QTOF mass spectrometer (Micromass, Manchester, United Kingdom), and partial or complete sequences from 16 peptides were obtained by tandem mass spectrometry (22). Amino acid sequences of nine or more residues from 10 different peptides were used to search protein or EST data bases for matches.

**cDNA Library Screening—** The peptide sequences obtained by mass
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RESULTS

Affinity Chromatographic Isolation of Glyceran-1 Ligands—Initial experiments surveyed extracts prepared from several subcellular fractions of brain (see "Experimental Procedures") for the presence of glyceran-1 ligands, which were detected predominantly in the "crude nuclear fraction." This fraction was therefore used for all subsequent studies. Specific ligands were considered to be those proteins that bound only to the glyceran-Fc fusion protein affinity beads, but not to protein A-Sepharose beads that did not contain the fusion protein. SDS-PAGE analysis and silver staining revealed a major specific ligand with an apparent molecular size of ~200 kDa (Fig. 1A), and a second band with a slightly slower mobility could frequently also be resolved (data not shown).

Methionine/cysteine labeling of the glyceran-Fc fusion protein secreted by transfected 293 cells followed by SDS-PAGE before and after heparitinase treatment showed that approximately 40% of the fusion protein is glycated (data not shown). These results indicate that in comparison with endogenous glyceran-1 in rat brain and C6 glioma cells (14), the addition of heparan sulfate chains in transfected 293 cells lagged considerably behind the high level of expression of the glyceran-1 core protein. Interaction of the 200-kDa ligand with the glyceran-1 affinity matrix is at least partially mediated by the heparan sulfate chains, insofar as binding is abolished after treatment of the beads for 5 h with heparitinase (Fig. 1B). However, the 200-kDa band was not detected in eluates of brain proteins bound to heparin-agarose using identical conditions (data not shown).

Another specific ligand with an apparent molecular size of...
-22 kDa was also detected, and at least five major bands were seen in the 40–70 kDa range (Fig. 1A). However, the latter proteins bound equally well to protein A-Sepharose beads that did not contain the fusion protein they probably represent rat immunoglobulins or other nonspecific ligands. No sequence in the 40–70 kDa range (Fig. 1A) was obtained by interrogating the mass difference between the bands for N-terminal Edman sequencing or for protease treatment and high performance liquid chromatography fractionation and high performance liquid chromatography fractionation and mass spectrometry for 17 peptides were generated (Fig. 2A). When applied to an unfractionated trypsin digest of glycpcan-1 ligand for the peptide. The spectrum shows a nearly complete set of sequences obtained from the peptide P8 ion is shown in Fig. 2B. The difference of 227 Da can correspond to only three possible pairs of amino acids (Table I, b1). 

Two alignments of peptide sequences obtained from the 200-kDa glypican-1 ligand with those of Slit proteins. The numbering refers to amino acids in rat Slit-1/MEGF4. M8EST represents the sequence that was amplified by PCR from a mouse embryo cDNA library using primers designed from the mouse EST sequence. Peptides derived from the 200-kDa glypican-1 ligand are numbered as in Table I, and their sequences are underlined (except for peptide 9). Peptide 6 corresponds to the human keratin sequence (data not shown) and was presumably a result of contamination, whereas peptide 9 does not match perfectly with any reported protein and may represent either a new member of the Slit family or reflect amino acid polymorphism.

**Table I**

| Peptide Sequence Obtained by Nano-electrospray Tandem Mass Spectrometry |  |
|---|---|
| P1 | (200)NSVXYYGNK 200 = S,X; E,A; or V,T |
| P2 | (212)QXHENQXAVFR 212 = P,D or X,Y |
| P3 | (228)XSENQXAXPR 228 = D,X; N,N; P,M; or E,V |
| P4 | XDXISNQXAVPR |
| P5 | (278)QVXIDSXNKK 276 = F,E or X,Y |
| P6 | (200)DXDSXXAEVK 200 = S,X; E,A; or V,T |
| P7 | (227)XYNNEQXDFTK |
| P8 | (227)XYNSEXQDFR |
| P9 | (212)QXFCNQXQVER 212 = F,D or X,Y |
| P11 | (242)PXQXNPADDPR 242 = S,N; E,X; or K,N |
| P12 | QRXTQXTQTCG*SGPASXR |
| P13 | QNXQXPEXPXONQXAXSR |
| P14 | SXNXVVXYGNKTDXPR |
| P15 | XNNXESXSEATGFXK |
| P16 | RXDSSNNQNXAEPAPDAFQGLR |
| VSEXEDTFGEATSVSEXHTANQXESVR |

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**Fig. 2.** Alignments of peptide sequences obtained from the 200-kDa glypican-1 ligand with those of Slit proteins. The numbering refers to amino acids in rat Slit-1/MEGF4. M8EST represents the sequence that was amplified by PCR from a mouse embryo cDNA library using primers designed from the mouse EST sequence. Peptides derived from the 200-kDa glypican-1 ligand are numbered as in Table I, and their sequences are underlined (except for peptide 9). Peptide 6 corresponds to the human keratin sequence (data not shown) and was presumably a result of contamination, whereas peptide 9 does not match perfectly with any reported protein and may represent either a new member of the Slit family or reflect amino acid polymorphism.

Peptide sequences obtained by nano-electrospray tandem mass spectrometry for the peptide. The spectrum shows a nearly complete set of sequences obtained from the peptide P8 ion is shown in Fig. 2B. The difference of 227 Da can correspond to only three possible pairs of amino acids (Table I, b1). 

Two alignments of peptide sequences obtained from the 200-kDa glypican-1 ligand with those of Slit proteins. The numbering refers to amino acids in rat Slit-1/MEGF4. M8EST represents the sequence that was amplified by PCR from a mouse embryo cDNA library using primers designed from the mouse EST sequence. Peptides derived from the 200-kDa glypican-1 ligand are numbered as in Table I, and their sequences are underlined (except for peptide 9). Peptide 6 corresponds to the human keratin sequence (data not shown) and was presumably a result of contamination, whereas peptide 9 does not match perfectly with any reported protein and may represent either a new member of the Slit family or reflect amino acid polymorphism.

Using 3–4 residues of sequence data together with mass information (residual N- and C-terminal masses and peptide molecular weights), we generated short MS-derived peptide sequence tags (26) for the 17 peptides. These were searched against the current nonredundant protein data bases revealing that except for peptide 6, which is derived from keratin, there were no significant identity with previously identified proteins. Ten of the MS/MS spectra yielded sequences of nine or more amino acids (two of the peptide sequences differed by only a single amino acid), which we used to search EST data bases. This approach found ESTs from mouse embryo and myotube that matched peptides P3 and P8 (GenBank accession numbers AA396603 and AA645364, respectively).
Cloning of the 200-kDa Ligand (Rat Slit-2)—Using the EST sequences for primer design and rat and mouse brain cDNA as templates, we amplified by PCR the corresponding rat and mouse sequences (designated P3EST and P8EST, respectively) and used the P3EST as a probe to screen a rat brain cDNA library. The longest clone obtained from screening (designated P8EST) amplified from a mouse brain cDNA library show 91–93% amino acid identity to human Slit-2 (27), indicating that our partial sequences represent rat and mouse homologues of Slit-2 (Table II). Because some of our peptide sequences match better with MEGF4/Slit-1 than with Slit-2 (Fig. 3), it would appear that the peptide sequences in the 200-kDa gel band are derived from more than one Slit protein and that glypican-1 can bind to both Slit-1 and Slit-2 and possibly also to other related proteins.

Rothberg et al. (16) compared the LRRs and the conserved N- and C-terminal sequences surrounding the LRRs of the Drosophila Slit protein with other proteins containing LRRs. Similarity was found in several proteins involved in adhesive events such as the oligodendrocyte-myelin glycoprotein and the Toll protein of Drosophila, as well as with other proteins involved in extracellular protein-protein interactions such as the platelet glycoproteins IX, Ibβ, and Iba and small leucine-rich proteoglycans including decorin, biglycan, and fibromodulin. These conserved sequences flanking the LRRs can also be found in our partial sequence of rat Slit-2 (Fig. 4).

**Tissue Distribution and Cellular Sites of Synthesis of Slit-2**—P3EST and P8EST were used as templates to transcribe digoxigenin-labeled riboprobes for use in Northern analysis and in situ hybridization histochemistry. Northern analysis showed that both probes hybridized with 7.6- and 8.5-kb bands present in rat brain and C6 glioma cell mRNA (Fig. 5), confirming that both peptides (and ESTs) are derived from the same gene. Non-nervous tissues, including skeletal muscle, showed no message with the exception of lung, possibly due to the presence of bronchial smooth muscle.

In the hippocampus at 1 month postnatal, Slit-2 mRNA is present in CA1 and CA3 pyramidal neurons and in granule cells of the dentate gyrus (Fig. 6A), and consistent with the previously reported expression of glypican-1 in cerebellum (9), Slit-2 mRNA is seen in cerebellar granule cells (Fig. 6B). Although there is a weaker signal in white matter, which is probably in oligodendrocytes, glial cells do not appear to be a major source of Slit-2. In cerebrum at embryonic day 19 (E19), Slit-2 mRNA is present primarily in the cortical plate and subplate (but not in the intermediate cortical layer), as well as in the thalamic nuclei, hippocampal formation, caudate putamen, and cerebral cortex (Fig. 6B/C). In spinal cord at E13 and E16, Slit-2 mRNA is present predominantly in the ependymal and mantle layers, the floor plate, and dorsal and ventral root ganglia (Fig. 7A). In E16 brain Slit-2 mRNA is detected in the trigeminal ganglion and the ventricular zone, including the entire ganglionic eminence (Fig. 7B). Slit-2 expression can also be seen in the embryonic retina (Fig. 7C) and optic stalk (data not shown).
DISCUSSION

By affinity chromatography of rat brain extracts on a matrix containing a glypican-Fc fusion protein, we identified a 200-kDa ligand whose peptide sequences did not match any sequences then available in the data bases. During the course of our cloning of this ligand, other data became available indicating that the 200-kDa SDS-PAGE band contained peptide sequences derived from at least two proteins (rat Slit-1 and Slit-2) that are mammalian homologues of the Drosophila Slit protein. This overlapping of protein bands is not surprising insofar as all five of the human and rat Slit proteins that have been cloned up to now have very similar amino acid sequences and differ in size by only 11 or fewer (out of ~1,500) amino acids.

The Drosophila Slit protein is expressed by midline glia and is distributed along commissural axons. Reduction in slit expression results in a disruption of the developing midline cells and the commissural axon pathways. The presence of a putative signal peptide and the lack of a transmembrane domain together with other structural features indicate that the Slit proteins are secreted extracellular matrix proteins. Although Slit was not detected in a Tris-buffered saline extract of rat brain, like other extracellular matrix components it may be tightly bound to other cell surface or extracellular proteins and require detergents or dissociative conditions for extraction.

The amino acid sequence of the Slit proteins can be divided into four domains. These consist of four N-terminal LRRs, seven to nine epidermal growth factor-like repeats (seven in Drosophila and nine in vertebrates), a motif with a high degree of identity to agrin, laminin, and perlecan (designated the ALPS domain, Ref. 29), and a C-terminal cysteine-rich domain. LRRs are found in a number of intracellular and extracellular proteins and contribute to protein-protein interactions and cell adhesion (30, 31). The epidermal growth factor-like motif has been identified as an extracellular binding domain involved in cell adhesion and receptor-ligand interactions (32), the ALPS domain is responsible for protein-protein interactions and self-aggregation of agrin, laminin, and perlecan (for a review, see Ref. 29), and the cysteine-rich domain is considered to be essential for dimerization of proteins such as von Willebrand factor (33). It is likely that the Slit proteins function to link multiple ligands and thereby mediate cell interactions. In view of the complex domain structure of the Slit proteins and their heparitinase-sensitive interactions with glypican-1, it will be important to identify which protein domain(s) may also be involved in this binding.

During our MS-based sequencing experiments, we attempted CID-MS/MS on a 2,011-Da peptide from the 200-kDa protein tryptic digest. Although we were unable to generate any sequence-specific fragment ions, the peptide readily lost neutral fragments characteristic of tyrosine phosphorylation (34). These preliminary data therefore suggest that Slit proteins may be phosphorylated.

We demonstrated that two major rat Slit-2 mRNA species (8.6 and 7.5 kb) are expressed in adult brain and lung and in rat C6 glioma cells and that the localization of this mRNA in the postnatal hippocampal formation and developing cerebellum is similar to that of glypican-1. In contrast to these results, Northern analysis of human Slit-2 showed a single 8.5-kb mRNA expressed predominantly in adult spinal cord but also

FIG. 5. Expression of Slit-2 in rat tissues. Northern blots of mRNA from 7-day and adult brain, C6 glioma cells, and adult liver, heart, spleen, kidney, lung, and muscle were probed with digoxigenin-labeled P3EST and P8EST RNA transcripts.

FIG. 6. Localization of Slit-2 mRNA in rat postnatal hippocampus and cerebellum and embryonic cerebrum. In situ hybridization histochemistry of P28 rat hippocampal formation (A) shows mRNA in neurons of the dentate gyrus (DG) and the CA1–CA3 regions. In P8 cerebellum (B) the mRNA is primarily present in granule cells of both the external (arrow) and internal (arrowhead) granule cell layers. In E19 cerebrum (C) the mRNA is seen primarily in the cortical plate (CP) and subplate (arrows), as well as in the ventricular zone (VZ), hippocampal formation (H) and the caudate putamen (CA). Note that the intermediate cortical layer (ICL) does not show Slit-2 message. Bars, 300 μm.
nervous system.

In E13 spinal cord (A) Slit-2 mRNA is present predominantly in the ependymal layer (EL) and mantle layer (ML) and in the dorsal root ganglion (DRG). At E16 (B) Slit-2 mRNA is detected in the ventricular zone of the brain (arrows), including the entire ganglionic eminence (GE) and in the trigeminal ganglion (TG). Slit-2 expression can also be seen in the retina at E16 (C). Bars: A, 100 μm; B, 500 μm; C, 200 μm.

in fetal lung and kidney, and a 9-kb rat MEGF5/Slit-3 message was seen in brain, whereas a major 5.5-kb mRNA and a minor 9.5-kb species of human Slit-3 were found in adult endocrine tissues but were not detectable in brain (27). Although human Slit-1 has the same expression pattern as that of rat Slit-1, there may be species differences in the tissue distribution and alternative splicing of Slit-2 and Slit-3 in humans as compared with rodents. It is also noteworthy that whereas Drosophila Slit is expressed only by glia, rat MEGF4/Slit-1 and Slit-2 are predominantly neuronal products (Ref. 27 and Figs. 6 and 7).

In adult brain, rat MEGF4/Slit-1 is expressed in the hippocampus, cerebral cortex, and olfactory bulb but not in the cerebellum (27), where we detected Slit-2 mRNA. Because our various peptide sequences match both Slit-1 and Slit-2, it would appear that at least two Slit proteins were isolated by our affinity chromatographic procedure and that glypican-1 functions in nervous tissue may be mediated by its differential interactions with two or more members of the Slit protein family. Recent genetic and biochemical studies of Drosophila and mammals demonstrated that Slit proteins bind Robo, a repulsive guidance receptor on growth cones (35–37). At least one of the mammalian Slit proteins, Slit-2, is a repulsive molecule for olfactory bulb axons (37), embryonic spinal motor axons (35), and developing forebrain axons (38). Interestingly, a 140-kDa N-terminal fragment of Slit-2 was purified from a tissue extract that stimulates the elongation and branching of sensory axons and shown to be responsible for these effects, whereas full-length Slit-2 does not have this activity (39). Although it was also demonstrated that heparin can release Slit-2 from the cell surface (35), we found that Slit proteins could not be isolated from our brain extract when a heparin-agarose matrix was substituted for the glypiccan-Fc fusion protein affinity column. Because the 140-kDa N-terminal fragment of Slit-2 was not detected in our affinity column eluate, it is possible that glypiccan-1 binds full-length Slit proteins via both its core protein and the heparan sulfate chains and that this interaction regulates the proteolysis and thereby the generation of biologically active fragments of Slit proteins. The identification of interactions between glypiccan-1 and Slit proteins therefore not only provides the most direct evidence yet available for an involvement of glypiccan-1 in nervous tissue development, but also suggests a possible regulatory mechanism underlying the dual functionality of Slit proteins.

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