Structural and Functional Analysis of Slit and Heparin Binding to Immunoglobulin-like Domains 1 and 2 of Drosophila Robo*

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Recognition of the secreted protein Slit by transmembrane receptors of the Robo family provides important signals in the development of the nervous system and other organs, as well as in tumor metastasis and angiogenesis. Heparan sulfate (HS) proteoglycans serve as essential co-receptors in Slit-Robo signaling. Previous studies have shown that the second leucine-rich repeat domain of Slit, D2, binds to the N-terminal immunoglobulin-like domains of Robo, IG1-2. Here we present two crystal structures of Drosophila Robo IG1-2, one of which contains a bound heparin-derived oligosaccharide. Using structure-based mutagenesis of a Robo IG1-5 construct we identified key Slit binding residues (Thr-74, Phe-114, Arg-117) forming a conserved patch on the surface of IG1; mutation of similarly conserved residues in IG2 had no effect on Slit binding. Mutation of conserved basic residues in IG1 (Lys-69, Arg-117, Lys-122, Lys-123), but not in IG2, reduced binding of Robo IG1-5 to heparin, in full agreement with the Robo-heparin co-crystal structure. Our collective results, together with a recent crystal structure of a minimal human Slit-Robo complex (Morlot, C., Thielens, N. M., Ravelli, R. B., Hemrika, W., Romijn, R. A., Gros, P., Cusack, S., and McCarthy, A. A. (2007) Proc. Natl. Acad. Sci. U.S.A. 104, 14923–14928), reveal a contiguous HS/heparin binding surface extending across the Slit-Robo interface. Based on the size of this composite binding site, we predict that at least five HS disaccharide units are required to support Slit-Robo signaling.

During development, growing axons navigate through the embryo by responding to a number of attractive and repulsive signals. The key ligand-receptor systems involved in axon guidance have been highly conserved during metazoan evolution (1, 2). Although initially discovered in the central nervous system (CNS), these molecules have additional, equally fundamental functions outside of the CNS. One of the best studied systems involves the interaction of Slit and Robo at the CNS midline of invertebrates and vertebrates (3). Slit is a protein secreted by midline glia cells, while Robo is a transmembrane receptor expressed on the axon growth cone; Slit-Robo signaling results in axon repulsion away from the midline. In mammals, Slit-Robo signaling is required for the proper development of the CNS (3), lung (4), kidney (5), and mammary gland (6), as well as for leukocyte chemotaxis (7) and endothelial cell migration (8). Mutations in ROBO genes have been linked to two human disorders, horizontal gaze palsy with progressive scoliosis (9) and congenital abnormalities of the kidney and urinary tract (10). Aberrant Slit-Robo signaling also contributes to human cancers, by promoting tumor formation, angiogenesis, and metastasis (8, 11). A molecular understanding of the Slit-Robo interactions is thus of great fundamental and biomedical interest.

Slit proteins are characterized by an N-terminal tandem of leucine-rich repeat (LRR) domains, D1–4. Their C-terminal regions consist of multiple epidermal growth factor-like domains, a laminin G-like domain, and a cystine knot domain. Robos have an extracellular domain resembling cell adhesion molecules (five immunoglobulin-like (IG) domains followed by three fibronectin type 3-like domains), a single transmembrane helix, and a large cytosolic domain predicted to be natively unstructured. Various cytosolic binding partners have been identified that link Robo activation to the cytoskeletal rearrangements underlying growth cone repulsion (3). Previous structure-function studies by us and others (12, 13) have shown that the Slit-Robo interaction is mediated by D2 of Slit and IG1-2 of Robo. These findings were very recently confirmed and much extended by a crystal structure of a Slit D2–Robo IG1 complex (14).

Biochemical and genetic experiments have shown that heparan sulfate (HS) is absolutely required for Slit-Robo signaling (15–18). HS consists of repeating disaccharide units that are modified by variable epimerization and sulfation; the HS chains are attached to core proteins to form HS proteoglycans. Heparin is a highly sulfated form of HS (19, 20). We previously identified Slit residues involved in heparin binding and demonstrated that one important function of HS/heparin is to promote the formation of a ternary Slit-Robo-HS signaling

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The atomic coordinates and structure factors (codes 2vr9 and 2vra) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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3 The abbreviations used are: CNS, central nervous system; LRR, leucine-rich repeat; IG, immunoglobulin-like; HS, heparan sulfate; FGF, fibroblast growth factor; FGFR, FGF receptor; r.m.s.d., root mean-square deviation.
complex (21). Here, we report two crystal structures of Droso-
phila Robo IG1-2, one of which contains a bound heparin-
derived oligosaccharide. Using structure-based mutagenesis, we have identified Robo residues involved in Slit and heparin binding. Combined with the recent structure of a human Slit2 D2-Robo1 IG1 complex (14), our results provide new insight into how HS/heparin strengthens the Slit-Robo interaction and thereby contributes to Slit-Robo signaling.

**EXPERIMENTAL PROCEDURES**

**Expression Vectors**—Initial constructs were made by PCR amplification from complete cDNA clones of Drosophila Slit and Robo, kindly provided by Guy Tear (King’s College, Lon-
don, UK). The C-terminally His-tagged Robo IG1-2 construct has been described (21). Untagged Robo IG1-2 was constructed by PCR amplification from the tagged construct and ligation into the original pCEP-Pu vector (22), using the NheI and XhoI restriction sites. The forward and reverse primers were, respectively, 5′-CGGAAATTCGCTAGCCAGGCCATCGACCCACG-
tc and 5′-AGGATCTCGAGTATTTGACCT-GGACAAATCGCTTGGCAGTAC. The expressed protein after cleavage of the BM-40 signal peptide has the sequence APLAGQYQS...IVQVK, with the first four residues derived from the vector. The Robo IG1-5 Fc construct has been described (21). Robo IG1-5 Fc mutants were made as follows. A fragment of the wild-type construct encompassing most of the IG1-2 region was mutated by strand overlap extension PCR and cloned back into the Robo IG1-5 Fc construct, using the NheI and BstBI restriction sites. The forward primer was as for Robo IG1-2, and the reverse primer was 5′-CGGAGATCCCTTGAGACCGGGGAGCTGGCGCCAAACG. Specific mutagenic primers were used to introduce the desired mutations (primer sequence available on request). For the R57A/I59A/E60A mutants, the strand overlap extension method could not be used, because the sequence to be mutated is too close to the 5′ restriction site. Therefore, the mutagenic sequence was included in the forward primer 5′-CGGAAATTCGCTAGCCAG-
GCCATACCCACGTCCTGAGCGCAATCCACGGTAC. The expressed protein after cleavage of the BM-40 signal peptide has the sequence APLAGQYQS...IVQVK, with the first four residues derived from the vector.

**Protein Production**—All proteins were purified from the conditioned medium of episomally transfected 293-EBNA cells as described (12). Briefly, cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum (Invitrogen), transfected using FuGENE reagent (Roche Applied Science), and selected with 1 μg/ml puromycin (Sigma). His-tagged Robo IG1-2 was purified from serum-free conditioned medium using a 5-ml HisTrap column (GE Healthcare). Untagged Robo IG1-2 and His-tagged Slit D1–4 were purified using 5-ml HiTrap Heparin HP affinity columns (GE Healthcare). The Robo IG1-5 Fc proteins (wild-type and mutants) were purified using 1-ml HiTrap rProtein-A FF columns (GE Healthcare). SlitD1–4 and Robo IG1-5 Fc proteins were dialyzed into PBS for binding experiments. The Robo IG1-2 proteins used for crystallization were further purified on a 24-ml Superdex75 column (GE Healthcare) in TBS (His-
tagged protein) or 0.02 M Na-HEPES pH 7.5, 0.15 M NaCl (untagged protein). All proteins were analyzed by SDS-PAGE and quantified by measuring their absorption at 280 nm.

**Crystallography**—His-tagged Robo IG1-2 in TBS buffer was concentrated to 19 mg/ml. Crystals were obtained by the hanging drop method at room temperature using 0.15 M KH₂PO₄, 12.5% PEG8000 as precipitant. Crystals were frozen in liquid nitrogen in 0.15 M KH₂PO₄, 12.5% PEG8000, 25% glycerol. Diffraction data to 3.2 Å resolution were collected at 100 K on beamline 9.6 at the SRS Daresbury (λ = 0.87 Å). The crystals belong to space group P4₁₂₁, a = b = 115.40 Å, c = 144.71 Å. There are three Robo IG1-2 molecules in the asymmetric unit, resulting in a solvent content of 63%.

A Robo-heparin complex was prepared by mixing untagged Robo IG1-2 with a 1.4-molar excess of a heparin octasaccharide (Iduron, Manchester, UK). The complex was purified on a Superdex75 gel filtration column (GE Healthcare) in 0.02 M Na-HEPES pH 7.5, 0.15 M NaCl, and concentrated to 15 mg/ml. Crystals were obtained at room temperature in nano-crystalization drops, using a Mosquito pipetting robot (TTP LabTech, Melbourne, UK). The drops consisted of 100 nl of protein solution and 100 nl of reservoir solution (10% PEG6000, 0.1 M Na-HEPES, pH 7.5 and 5% MPD) as precipitant. Crystals were harvested directly from the nanodrops and frozen in liquid nitrogen in 10% PEG6000, 0.1 M Na-HEPES pH 7.5, 20% glycerol. Diffraction data to 3.2 Å resolution were collected at 100 K on beamline 10.1 at the SRS Daresbury (λ = 1.12 Å). The crystals belong to space group P2₁, a = 64.93 Å, b = 84.43 Å, c = 107.13 Å, β = 98.54°. There are four Robo IG1-2 molecules in the asymmetric unit, resulting in a solvent content of 59%.

The diffraction data were processed with MOSFLM and pro-
grams of the CCP4 suite (23). The tetragonal structure of His-
tagged Robo IG1-2 was solved by molecular replacement with PHASER (24, 25) using domain IG2 of MuSK (26) as a search model. The monoclinic Robo IG1-2 structure was solved by molecular replacement using the partially refined structure of the tetragonal form as a search model; the IG domains had to be located individually to obtain a solution. Both structures were built with O (27) and COOT (28), and refined with CNS (29). Non-
ystallographic symmetry restraints were applied only during the early stages of refinement. Data collection and refinement statistics are summarized in Table 1. The figures were made with PYMOL. The electrostatic surfaces were calculated with APBS (30). The coordinates of the tetragonal and monoclinic crystal forms of Drosophila Robo IG1-2 have been deposited in the Protein Data Bank (entries 2v9r and 2vra, respectively).

**Solid-phase Protein Binding Assay**—The solid-phase binding assay was carried out as described (12). Briefly, Slit D1–4 (100 μg/ml) was coated onto 96-well microtiter plates (NUNC Maxisorp). Wells were blocked with TBS/casein/Tween 20 and washed with TBS. Robo IG1-5 Fc protein was added for 1 h. After four washes, bound protein was detected by alkaline phosphatase-conjugated goat anti-human Fc antibody (Sigma).

**Heparin Affinity Chromatography**—300 μg of each Robo IG1-5 Fc protein (wild-type and mutants) were injected onto a 5-ml HiTrap Heparin HP column (GE Healthcare) equilibrated in 0.05 M Tris-HCl, pH 7.5. Bound proteins were eluted with a
linear NaCl gradient of 0–1 M NaCl over 5 column volumes at 2 ml/min.

Docking Calculations—To obtain an atomic model of the Slit D2-Robo IG1-2 complex, Robo IG1 in the crystal structure of the human Slit2 D2-Robo1 IG1 complex (14) was replaced with the human Robo1 IG1-2 pair (crystal form 1) (14). An atomic model of a heparin decasaccharide was obtained from the crystal structure of a ternary fibroblast growth factor (FGF)-fibroblast growth factor receptor (FGFR)-heparin complex (31). AutoDock 4 (32) was used to add polar hydrogens and charges to the heparin decamer and the Slit D2-Robo IG1-2 complex. The complex was covered by a 1 Å grid, and a total of 10 independent docking runs were carried out with AutoDock 4, using a Lamarckian genetic algorithm and the following set of parameters: elitism rate, 1; mutation rate, 0.02; crossover rate, 0.8. The protocol was validated by performing similar docking runs on two independent structures of the tetragonal form containing the untagged protein in the presence of a heparin ligand, and a monoclinic form containing the protein with a C-terminal His tag, and a monoclinic form containing multiple copies of the tetragonal structure can be superimposed with r.m.s. deviations of 0.9–1.3 Å (180 Ca atoms), and the four copies of the monoclinic structure can be superimposed with r.m.s. deviations of 0.6–1.0 Å (180 Ca atoms). Molecule A of the monoclinic structure could be traced completely and will serve as the reference model in the following description.

The IG1 and IG2 domains of Drosophila Robo belong to the I-set of the IG domain superfamily, a subclass found in many cell adhesion molecules, receptor protein tyrosine kinases, and other signaling receptors (34). These domains adopt a typical β-sandwich fold with two antiparallel sheets packing against each other (Fig. 1A). The opposing sheets are made up of strands A/A’-B/B’-E-D and C-F/G/G’, respectively, and are connected by a disulfide bond between strands B and F. In both IG1 and IG2, there is a cis-proline in the reverse turn following strand B’ (Pro-83 and Pro-183, respectively), a feature also seen in the related IG domains of MuSK (26) and axonin (35). Strands C and D of both IG1 and IG2 are connected by long loops, which are partially disordered in all but one of the crystallographically independent Robo IG1-2 molecules. The single exception is molecule A of the monoclinic form, where the C-D loop of IG2 is folded into an α-helix. Finally, compared with other I-set IG domains, Robo IG1 also has a relatively long loop connecting strands E and F.

There is essentially no linker between the two IG domains in the Robo IG1-2 tandem. In the monoclinic form, the last β-strand of IG1 (G’) extends into domain IG2 and hydrogen bonds with a short extended segment in the A-B loop of IG2 (strand B’), resulting in an almost linear arrangement of the two IG domains. In the tetragonal form, a substantially different conformation is observed: the β-interaction in the linker is lost, resulting in a rotation by 35° of IG2 relative to IG1 (Fig. 1B). The
hinge point of this rotation is at Ile-150, which in the monoclinic form is still part of the extended G' strand.

Mapping of Slit Binding Site—To identify residues involved in Slit binding, we designed a series of Robo point mutants and tested them in a Slit binding assay. Because the Slit-Robo signaling mechanism is conserved in higher eukaryotes (3) we looked for conserved residues exposed on the surface of the Robo molecule. Because the Robo binding site on Slit contains Tyr, His, and Leu residues (12), we expected the Slit binding site to contain a mixture of hydrophobic and polar residues. Taking into account these characteristics, and using a combination of sequence alignment (Fig. 2), surface conservation, and electrostatics (Fig. 3), we identified three potential Slit binding patches in the Robo IG1-2 tandem: (1) Arg-57/Ile-59/Glu-60 residing in strand A of IG1; (2) Thr-74/Phe-114/Arg-117 residing on the solvent-exposed face of strands B and E of IG1; (3) Arg-154/Asp-155/Asp-156/Arg-158/Lys-180 residing in the bulge preceding strand A of IG2, and in the adjacent B-B' loop (Lys-180).

We introduced mutations in the context of the Robo IG1-5 Fc construct, which binds strongly to immobilized Slit D1–4 in a solid phase assay (21). Altogether, we made five mutants: R57A/I59A/E60A, T74E, F114E, R117E (all in IG1), and R154A/D155A/D156A/R158A in IG2. All but the first mutant protein could be purified from the conditioned medium of episomally transfected 293 cells; the R57A/I59A/E60A mutant was not secreted by the cells (Fig. 4A and data not shown). When we tested the mutants for Slit binding, we found that mutation of Thr-74, Phe-114, or Arg-117 (all in IG1) essentially abolished binding, whereas the simultaneous mutation of four conserved residues in IG2 had only a marginal effect (Fig. 4B). Thus, the major Slit binding site of Drosophila Robo is located in IG1, near the center of the A/A'-B/B'-E-D sheet of the IG domain β-sandwich.

Mapping of Heparin Binding Site—In a previous study, we showed that HS/heparin is absolutely required for Slit-Robo signaling and reconstituted a minimal ternary complex consisting of Slit D2, Robo IG1-2 and heparin; we also identified heparin binding residues in Slit D2 by site-directed mutagenesis (21). To better understand the formation of this ternary signaling complex, we now wanted to identify Robo residues involved in heparin binding. Visual inspection of the electrostatic potential on the Robo surface revealed a very basic region at the domain interface (Fig. 3). This basic patch is created by the side chains of Lys-69, Arg-117, Lys-122, and Lys-123 in IG1 and Arg-154, Arg-158, and Lys-180 in IG2, all of which are conserved between invertebrate and vertebrate Robos (Fig. 2). Arg-117 is involved in Slit binding (see above), but its location at the border between the Slit binding site and the pronounced basic patch prompted us to also test its contribution to heparin binding.

We made three additional mutants, again in the context of Robo IG1-5 Fc: K69E and K122A/K123A in IG1, and K180E in IG2. We tested our entire panel of Robo mutants for heparin binding using affinity chromatography, taking the NaCl concentration required for elution from a heparin column as a measure of relative heparin affinity. Mutation of four residues
in IG1 (Lys-69, Arg-117, Lys-122, Lys-123) substantially reduced binding to the heparin column, whereas all other mutants showed only a marginal reduction compared with wild-type Robo IG1-5 Fc (Fig. 4C and data not shown). The Fc tag does not interact with the heparin matrix, because a human Robo4 Fc protein did not bind to the column at all (data not shown). The collective results of our analysis demonstrate that Robo IG1 contains not only the Slit binding site, but also the HS/heparin binding site of Robo.

Structure of a Robo-Heparin Complex—After we had completed building all four Robo IG1-2 molecules in the monoclinic crystal form, strong residual electron density was observed near the interdomain regions of molecules A and B, which are related by a non-crystallographic dyad (Fig. 5). Similar, but weaker and disconnected, density is also present in the analogous contact between molecules C and D (data not shown). We interpreted these electron density features as bound heparin octasaccharide molecules, which had been
included in our crystallization trials. At the contact between Robo IG1-2 molecules A and B, four sugar moieties could be modeled with confidence. We observed strong globular density for the sulfate groups (one and two sulfates per iduronic acid and glucosamine, respectively), as well as continuous density for the sugar backbone (Fig. 5C). After refinement of a heparin tetrasaccharide, there remained patchy residual density consistent with the presence of further sugar moieties, but these could not be modeled. Comparison with other protein-heparin co-crystal structures (31, 33, 36) reveals that the heparin tetrasaccharide included in our crystallographic model of Robo IG1-2 adopts a canonical conformation (Fig. 5D).

Consistent with our mutational analysis, the heparin oligosaccharide is bound at the pronounced basic patch in Robo IG1. The heparin binding residues Lys-69, Arg-117, Lys-122, and Lys-123 are all close to the oligosaccharide, but only the side chains of Lys-69 and Arg-117 are seen to make specific interactions with the acidic groups of the ligand (Fig. 5B). The side chains of Lys-122 and Lys-123 are disordered, most likely due to multiple modes of electrostatic interactions with the ligand. The presence of a heparin octasaccharide bridging two Robo IG1-2 molecules is almost certainly the result of fortuitous crystal packing, but the crystallographic observation of a bound heparin oligosaccharide nevertheless lends strong support to our mutational analysis of heparin binding. It is worth noting that Robo IG1-2 in solution remains monomeric in the presence of heparin octasaccharide (data not shown).

**DISCUSSION**

We have carried out a comprehensive structure-function analysis of IG domains 1 and 2 of *Drosophila* Robo. First, using structure-based mutagenesis, we found that the major determinants for both Slit and HS/heparin binding are fully contained in IG1. Second, we present a crystal structure of Robo IG1-2 with a bound heparin oligosaccharide, confirming and extending the mutational data. Finally, we provide crystallographic evidence for substantial conformational flexibility at the domain junction, which may be functionally relevant.

While we were completing our study, Morlot et al. (14) reported the crystal structure of a human Slit2 D2-Robo1 IG1 complex. This important structure revealed that IG1 of hRobo1 is bound by the concave face of hSlit2 D2, confirming our earlier prediction based on mutagenesis of *Drosophila* Slit D2 (12). hRobo1 residues involved in Slit2 binding are contributed by two distinct regions of IG1: polar and charged residues in strands A and B (Glu-72, Ser-75, Asn-88, and Lys-90) recognize the N-terminal LRRs of hSlit2 D2, and apolar residues in strands B, D, and E (Pro-84, Thr-86, Met-120, Leu-122, Phe-128, and Leu-132) recognize the central and C-terminal LRRs.

Our present mutagenesis, in the context of *Drosophila* Robo IG1-5, identified residues Thr-74, Phe-114, and Arg-117 (hRobo1 residues Thr-86, Phe-128, and Arg-131, respectively) as being important for Slit binding (Fig. 4B). The strongest effect on Slit binding was seen with our F114E mutant, consistent with the location of this residue (Phe-128 in hRobo1) at the very heart of the Slit-Robo interface (14). Arg-117 (Arg-131 in hRobo1) is located at the periphery of the interface, but makes a very close contact with a tyrosine...
residue on Slit D2, which we have previously shown to be important for Robo binding (12). Similarly, Thr-74 (Thr-86 in hRobo1) interacts with another tyrosine residues previously implicated in Robo binding (12). Finally, our inspection of the Drosophila Robo IG1-2 structure led us to suspect an involvement of residues 57–60 in Slit binding. We could not test this hypothesis, as the relevant mutant protein was degraded by the 293 cells, but the structure of Morlot et al. (14) shows that these residues are indeed involved in Slit binding.

Neither our present mutagenesis nor the structure of Morlot et al. (14) suggest a prominent role of Robo IG2 in Slit binding. This contradicts a previous study by domain deletion mutagenesis of hRobo1, which reported that deletion of either IG1 or IG2 abolished Slit binding (13). One possible explanation is that deletion of IG2 destabilized the adjacent IG1 domain and thus affected Slit binding indirectly. However, we do not rule out the alternative possibility that IG2 indeed provides an, as yet unspecified, contribution to Robo function. In this regard, we were unable to observe a stable binary complex between Drosophila Robo IG1-2 and Slit D2 in solution (data not shown), whereas the longer Robo IG1-2 construct readily forms such a complex with Slit D2 (21).

An intriguing parallel between our present structural analysis of Drosophila Robo IG1-2 and the study of Morlot et al. (14) is the existence of two distinct Robo conformations. Morlot et al. (14) determined two structures of hRobo1 IG1-2, which differed dramatically in the interdomain angle, in almost exactly the same manner as our two structures (Fig. 1B). One conformation (form 1 of hRobo1 IG1-2, monoclinic form of Drosophila Robo IG1-2; r.m.s. deviation 1.7 Å for 190 Cα atoms) is fully extended and features continuous β-strand interactions across the domain interface. The other conformation (form 2 of hRobo1 IG1-2, tetragonal form of Drosophila Robo IG1-2; r.m.s. deviation 1.7 Å for 187 Cα atoms) is kinked by 35–40° with respect to the extended conformation. Whether or how this mode of flexing is related to Robo function is unclear at present, but its conservation in two orthologous proteins is certainly intriguing.

An important aim of our present investigation was to gain further insight into the essential role of HS/heparin in Slit-Robo signaling (15–18,21), an aspect that was not addressed by Morlot et al. (14). We previously identified conserved basic residues in Slit D2, which mediate heparin binding and are required for Slit function in a growth cone collapse assay (21). We now complement these findings with the identification of the corresponding heparin binding site of Robo, both by mutagenesis (Fig. 4C) and direct crystallographic observation of a bound heparin oligosaccharide (Fig. 5). Because the role of HS/heparin is conserved across species (15–18), our results can be combined with the structure of human Robo1 IG1 bound to human Slit2 D2 (14) to gain...
insight into the Slit-Robo-HS signaling complex (Fig. 6). An electrostatic surface representation of the Slit D2-Robo IG1-2 complex reveals a contiguous basic patch that extends across the Slit-Robo interface and, crucially, encompasses all Slit and Robo residues shown to be important for heparin binding (Ref. 21 and this study).

How does HS/heparin bind to the extended basic surface patch in the Slit-Robo complex? We do not think that the heparin oligosaccharide bound to isolated Robo IG1-2 (Fig. 5) faithfully recapitulates HS/heparin in the ternary Slit-Robo-HS complex, as it is located in a crevice near the Robo IG1-2 boundary (Fig. 6B). Rather, we think that longer HS/heparin chains (five or more disaccharide units) might span the Slit-Robo interface like a bridge without fully entering into the crevice. We attempted to use computational docking (see “Experimental Procedures”) to gain more detailed insight into the Slit-Robo-heparin complex; a similar approach has recently been used to analyze heparin binding to the thrombospondin-1 N-terminal domain (37). While our docking calculations invariably positioned the heparin oligosaccharide on the composite basic surface of the Slit-Robo complex, repeated runs did not produce a unique mode of binding (data not shown). We think that this may reflect the actual situation in vivo, as it is quite plausible that an extended HS chain could straddle the Slit-Robo interface in more than one way and still foster a stable ternary signaling complex. In this regard, we note an interesting analogy to the association of Hedgehog and its receptor, Ihog, which is strictly heparin-dependent in solution. Crystallization of the ternary complex failed to reveal the position of the heparin oligosaccharide, but mutagenesis confirmed that a composite basic surface spanning the Hedgehog-Ihog interface is the site of heparin binding (38). Even in the FGF-FGFR system, where the role of heparin is comparatively well understood, inconsistent modes of heparin binding have been observed in two independent studies (31, 33).

How does Slit D2 binding to Robo IG1 lead to Robo transmembrane signaling? One possibility is that the conformational switch at the IG1-2 junction is somehow involved in transmitting a signal, but additional structures will be required to test this hypothesis. Another, perhaps more plausible, possibility is that Robo signaling is the result of a change in the oligomeric state of Robo, as has been proposed for the related netrin receptor, DCC (39). The minimal Slit-Robo complex in solution has 1:1 stoichiometry (14, 21), but this may not be the case when the complex is assembled on long, native HS chains at the cell membrane. To answer these important questions, biophysical studies of full-length transmembrane Robos will be required.

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