Slit is a large secreted leucine-rich repeat (LRR) protein with multiple roles in cell signaling and adhesion. They have well established and evolutionarily conserved functions as guidance cues in the developing nervous system (1, 2), but Slits are also important in the development of the vasculature (3) and other organs (4). The first class of Slit receptors to be identified were Robo family members, which are transmembrane proteins with an extracellular domain resembling cell adhesion molecules and a large cytosolic signaling domain (1, 2). Biochemical studies have defined the domains mediating the Slit-Robo interaction (5, 6), as well as some of the components of the signaling cascade downstream of Robo activation (7, 8), but how binding of Slit to Robo receptors conveys a signal across the cell membrane remains unknown.

The first indication that there might exist a second Slit receptor came from the observation that heparan sulfate (HS) was required for the repellent activity of Slit in vitro (9) and in vivo (10). The identity of this receptor was revealed by recent genetic studies in invertebrates, which showed that Slit signaling requires Robo to be co-expressed on the same cell with the HS proteoglycan syndecan (11–13). Syndecan is a membrane-spanning proteoglycan to which are covalently attached several HS chains, consisting of repeating sulfated disaccharide units (14). Heparin is a member of the HS family that is more highly and uniformly sulfated than other HS. Johnson et al. (12) showed that both Slit and Robo can be co-immunoprecipitated with syndecan, suggesting the presence of a ternary (or higher order) complex at the neuronal cell membrane. However, the composition and functional relevance of this putative ternary complex was not established. Because the Slit distribution was found to be altered in syndecan-deficient embryos, HS may also be required for Slit localization rather than signaling (12).

Here we provide direct biochemical and functional evidence for a ternary signaling complex composed of the second LRR domain of Slit, Robo, and heparin/HS. Other Slit domains are dispensable for signaling in an in vitro growth cone collapse assay, but heparin/HS is required absolutely. We conclude that heparin/HS is an integral component of the minimal Slit-Robo signaling complex.

**EXPERIMENTAL PROCEDURES**

**Expression Vectors**—All constructs were made by PCR amplification from complete cDNA clones, kindly provided by Dr. Guy Tear (King’s College, London, UK) and Dr. Lindsay Hinck (University of California, Santa Cruz). Primer sequences are available upon request. The PCR products were cloned into modified pCEP-Pu vectors, coding for proteins with a His tag either at the N terminus (Slit constructs) or at the C terminus (Robo constructs). A Robo IG1–5 Fc construct was made by replacing the C-terminal His tag with a human Fc sequence obtained by PCR amplification from the pFUSE-hFc1 vector.
(InvivoGen). Mutations in human Slit2 (hSlit2) D2 were introduced by strand overlap extension PCR. The insert sequences of all expression vectors were verified by DNA sequencing. The domain boundaries of the Slit LRR domains and the Robo D1–5 construct have been described previously (5). The new constructs have the following boundaries (sequence numbering includes the signal peptide): Slit EG1–5, NACFE...YPQTS (933–1137); Slit EG6-LG-EG7, QTSPC...TVTAA (1134–1430); Slit CT, QGEGS...TKKCY (1417–1504); Slit EG6-LG-EG7-CT, QTSPC...TKKCY (1134–1504); hSlit2 D2, SVLHC...FADLA (269–505); Robo D1-2, GQYQS...IVQVK (51–254); Robo D3-5, KPYFM...AADPS (254–546). The following vector-derived sequences are additionally present in the mature proteins: AADPS...KPYFM (254–268) and SVLHC...FADLA (269–505). The C terminus of the Robo IG1–5 Fc construct.

**RESULTS**

**Mapping of Heparin-binding Sites in Slit and Robo**—To locate the heparin-binding site(s) within the large Drosophila Slit protein, we used a panel of recombinant proteins spanning the entire molecule. In a previous study we had shown that the LRR region of Slit consists of four distinct domains, D1–4, each comprising an array of LRRs flanked by cysteine-rich caps, and we mapped the Robo-binding site to a highly conserved region on the concave face of Slit D2 (5). In addition to the LRR domain constructs available from that study, we prepared several new constructs spanning the C-terminal portion of Slit (Fig. 1). The EG1-5 construct contains the five epidermal growth factor-like domains following the LRR region and terminates at a natural proteolytic cleavage site of Slit (1). The EG6-LG-EG7-CT construct contains the remainder of the Slit C-terminal region; a truncated version of this construct, EG6-LG-EG7, was used to assess the contribution to heparin binding of the C-terminal (CT) cystine knot domain. It was not possible to study heparin binding by the CT domain directly, as the isolated CT domain was not secreted by 293 cells transfected with the relevant expression vector.

The recombinant Slit proteins were analyzed by heparin affinity chromatography. Several proteins did not bind to the heparin column; those that did eluted in single sharp peaks, and the NaCl concentration required for elution was taken as a measure of relative heparin affinity. We found that heparin-binding sites are located at either end of the Slit protein, whereas the central portion does not interact with heparin (Fig. 1A). These findings are in agreement with an earlier study on the two natural cleavage products of hSlit2 (17). The CT domain of Drosophila Slit has the highest apparent affinity for heparin (compare EG6-LG-EG7-CT and EG6-LG-EG7), but the N-terminal LRR domains of Slit, D1 and D2, also bind to heparin, and their combined presence in D1–4 results in a substantial affinity for heparin.

We also tested whether the Slit-binding ectodomain of Drosophila Robo displays affinity for heparin. Robo IG1–5 bound quite strongly to the heparin affinity column; further dissection...
of this region into IG1–2 and IG3–5 showed that the heparin binding activity is fully contained within IG1–2 (Fig. 1A).

**FIGURE 1.** Heparin-binding sites in Slit and Robo. A, domain structure and heparin binding of Drosophila Slit and Robo constructs (D1–4, leucine-rich repeat domains; EG, epidermal growth factor-like domain; LG, laminin G-like domain; CT, C-terminal domain; IG, IG-like domain). Affinities for heparin are expressed as the molar NaCl concentration required for elution from a heparin HiTrap column (nb, no binding at physiological ionic strength). Full-length Slit and Robo are shown for reference; the domains responsible for their interaction (5, 6) are shaded gray, and the transmembrane segment of Robo is represented by a vertical bar. B, Coomassie Blue-stained SDS-polyacrylamide gel of selected Slit constructs. The molecular masses (in kDa) of selected markers are indicated on the left.

**FIGURE 2.** Heparin enhances the Slit-Robo interaction. Binding of soluble Robo IG1–5 Fc to immobilized Slit D1–4 in the absence and presence of 10 \( \mu \text{g/ml} \) heparin. Bound Robo IG1–5 Fc was detected by alkaline phosphatase-conjugated anti-Fc antibody. 1 \( \mu \text{g/ml} \) Robo D1–5 Fc corresponds to 25 nM Robo monomer. Values are presented as mean ± S.E. (n = 3).

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out a solid-phase binding experiment (Fig. 2) in the absence and presence of soluble heparin. Addition of 10 \( \mu \text{g/ml} \) heparin resulted in a marked (greater than 10-fold) increase of the apparent affinity of Robo D1–5 Fc for Slit D1–4. Thus, heparin enhances the Slit-Robo interaction, most likely via the formation of a ternary Slit-Robo-heparin complex.

To obtain direct evidence for ternary complex formation, we used analytical gel filtration chromatography (Fig. 3A). Drosophila Slit D2 (27.4 kDa + two N-linked glycans) behaved as a monomer, as reported previously (5). The elution volume of Drosophila Robo IG1–2 (24.1 kDa) is also most compatible with a monomer, considering that this construct is likely to adopt an extended conformation and will therefore elute earlier than a globular protein of the same mass. When Slit D2 and Robo IG1–2 were mixed in a 1:1 ratio, a single peak was observed that eluted ahead of the individual proteins, demonstrating formation of a binary complex. SDS-PAGE showed that the peak fractions contained Slit D2 and Robo IG1–2 in comparable amounts (Fig. 3B). The Slit D2-Robo IG1–2 complex must have 1:1 stoichiometry, given that its elution volume of 10.9 ml corresponds to an ~55-kDa globular protein. The close correspondence to the calculated mass (51.5 kDa + two N-linked glycans) indicates a compact shape of the complex. The shoulder at a higher elution volume may result from partial dissociation of the complex on the column.

We next tested whether addition of heparin to the minimal Slit-Robo complex results in the formation of a ternary complex. Because native heparin is too large and polydisperse to be used in these experiments, we used a purified heparin-derived decasaccharide composed of repeating trisulfated disaccharide units, which was expected to be long enough to span the minimal Slit-Robo complex. When Slit D2, Robo IG1–2, and the heparin decasaccharide were mixed in a 1:1:1 ratio, the resulting complex eluted even earlier than the binary Slit-Robo complex, indicating formation of a ternary Slit-Robo-heparin complex (Fig. 3A). SDS-PAGE of the peak fractions again demonstrated the presence of Slit and Robo in comparable amounts (Fig. 3B). Given that a heparin decasaccharide has a similar hydrodynamic radius as an ~20-kDa globular protein (18) and that the elution volume of the ternary complex corresponds to an ~70-kDa globular entity, the most likely stoichiometry of the ternary Slit-Robo-heparin complex is 1:1:1. Interestingly, when the heparin decasaccharide was added to the individual Slit D2 and Robo IG1–2 proteins, no interaction was detected by gel filtration chromatography (data not shown).
suggesting that a high affinity binding site is only formed in the Slit-Robo complex.

**Heparin-dependent Biological Activity of Slit D2**—Our biochemical observation of a minimal Slit-Robo-heparin complex suggests that an important function of heparin/HS in Slit-Robo signaling may be to strengthen the association of Slit domain D2 with Robo receptors. If this is indeed the case, one would expect Slit D2 to display a similar heparin/HS-dependent activity as full-length Slit, even though it is lacking the C-terminal high affinity heparin-binding site of the full-length molecule. In a previous study, we used an endothelial cell migration assay to demonstrate biological activity of Slit D2 (5). A drawback of this assay is that endothelial cells express a divergent member of the Robo family, Robo4, whose role as a Slit receptor is controversial (19–21). In the absence of further studies, it is therefore difficult to attribute the effect of Slit on endothelial cells to a specific Robo receptor. Moreover, all studies into the HS requirement of Slit-Robo signaling so far have focused on (cells of) the nervous system (9–13, 22–24). To facilitate comparison with these studies, we therefore decided to use a chemotropic collapse assay on cultured *Xenopus* retinal growth cones.

In this assay, conditioned medium of cells expressing hSlit2 induces growth cone collapse in a Robo- and heparin/HS-dependent manner (8). We found that recombinant hSlit2 D2 protein (61.5 and 96.6% sequence identity to *Drosophila* and *Xenopus* Slit D2, respectively) applied at 40 nM (the optimal concentration determined in a dose-response experiment; data not shown) elicited a robust collapse response, comparable with that obtained with hSlit2 conditioned medium (Fig. 4, A–C and H). As expected from our previous analysis (5), other Slit domains were inactive (data not shown). Addition of heparin or HS had no effect on the collapse-inducing activity of hSlit2 D2 (Fig. 4, D, E, and H). In sharp contrast, enzymatic removal of HS from the neurites by heparinase treatment abolished the response to hSlit2 D2, demonstrating that Slit-Robo signaling does not occur in the absence of HS chains at the cell surface. Remarkably, addition of heparin to heparinase-digested growth cones fully restored their sensitivity to hSlit2 D2 (Fig. 4, G and H), indicating that, in this assay format, exogenous soluble heparin/HS can substitute for endogenous HS proteoglycans to support productive signaling. Taken together, the results demonstrate that the D2 domain of Slit contains not only the unique Robo-binding site but also a functionally important heparin/HS-binding site.

**Location of the Heparin/HS-binding Site in Slit D2**—We next wanted to determine which residues in Slit D2 contribute to heparin/HS binding. Because the role of HS in Slit-Robo signaling appears to be conserved between invertebrates and vertebrates (9–13, 22, 23), we looked for conserved basic residues...
that might be involved in HS binding. Because we wanted to test the mutants in the growth cone collapse assay, we chose hSlit2 D2 for mutagenesis. Using an alignment of invertebrate and vertebrate Slit sequences (Fig. 5A) and a molecular model of hSlit2 D2 (Fig. 5B), we selected a total of 12 basic residues for mutagenesis (Table 1). Mutants 1–4 target a basic patch in the N-terminal region of hSlit2 D2, and mutants 5–7 target a large concentration of basic charges in the C-terminal cap of the D2 domain (Fig. 5B). Both of these basic patches are adjacent to the Robo-binding site defined in our previous study (5).

All hSlit2 D2 mutants were purified to homogeneity from the conditioned medium of episomally transfected 293 cells (data not shown). Heparin affinity chromatography unexpectedly revealed that hSlit2 D2 has considerably higher affinity for heparin than its Drosophila orthologue (0.85 M NaCl required for elution, compared with 0.28 M for Drosophila Slit D2); the biological relevance of this difference (if any) remains to be established. Mutation of basic residues in the N-terminal half of hSlit2 D2 (mutants 1–4) had no effect on heparin binding, whereas mutation of basic residues in the C-terminal region (mutants 5–7 and the quadruple mutant 5 + 6) resulted in a substantial decrease of heparin affinity (Table 1). Crucially, when we examined the biological activity of two mutants that showed the most reduced heparin binding (mutants 7 and 5 + 6), we found that both mutants had lost the ability to trigger growth cone collapse (Table 1). These results demonstrate that the C-terminal cap region of Slit D2 harbors a functionally critical binding site for heparin/HS.

### DISCUSSION

Several growth factors and cytokines require heparin/HS for productive signaling. The classic example is fibroblast growth factor (FGF), which requires HS as an obligatory co-receptor for signaling (25, 26). HS also binds to the FGF receptor (FGFR) (27), and specific HS sequences support signaling (16). Recent crystallographic studies have confirmed that a ternary complex is formed by these partners (28, 29). Although the structural details of the ternary signaling complex are still a matter of dispute, it is clear that an important role of heparin/HS is to stabilize the relatively weak binary interaction between FGF and FGFR.

In previous studies, HS was shown to be required for Slit-Robo signaling in vitro (9) and in vivo (10–13, 22–24), but the molecular mechanism(s) involved remained unclear. Our previous results have shown that Robo binds to a highly conserved site on the concave face of Slit D2 (5). In this study, we show that
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heparin/HS binding is critical to a conserved basic patch adjacent to the Robo-binding site for the biological activity of Slit D2. Furthermore, we provide direct biochemical evidence for a ternary complex consisting of Slit D2, Robo, and heparin. We conclude that heparin/HS is an integral and essential component of the Slit-Robo signaling complex.

Slit concentrations in vivo are likely to be much lower than the Slit D2 concentration required for growth cone collapse in vitro, and one function of the high affinity heparin-binding site in the C-terminal region of Slit may be to concentrate the Slit protein at the target cell surface or modulate its diffusion through extracellular matrix (12). This interpretation could explain why excess heparin/HS abolishes growth cone collapse through extracellular matrix (12). This interpretation could in the C-terminal region of Slit may be to concentrate the Slit , and one function of the high affinity heparin-binding site.
A Molecular Mechanism for the Heparan Sulfate Dependence of Slit-Robo Signaling

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