Son of Sevenless Directly Links the Robo Receptor to Rac Activation to Control Axon Repulsion at the Midline

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Summary
Son of sevenless (Sos) is a dual specificity guanine nucleotide exchange factor (GEF) that regulates both Ras and Rho family GTPases and thus is uniquely poised to integrate signals that affect both gene expression and cytoskeletal reorganization. Here, using genetics, biochemistry, and cell biology, we demonstrate that Sos is recruited to the plasma membrane, where it forms a ternary complex with the Roundabout receptor and the SH3-SH2 adaptor protein Dreadlocks (Dock) to regulate Rac-dependent cytoskeletal rearrangement in response to the Slit ligand. Intriguingly, the Ras and Rac-GEF activities of Sos can be uncoupled during Robo-mediated axon repulsion; Sos axon guidance function depends on its Rac-GEF activity, but not its Ras-GEF activity. These results provide in vivo evidence that the Ras and RhoGEF domains of Sos are separable signaling modules and support a model in which Robo recruits Sos to the membrane via Dock to activate Rac during midline repulsion.

Introduction
Correct wiring of the nervous system depends on precisely coordinating the distribution and activity of a diverse set of axon guidance cues and their neuronal receptors. Studies of both invertebrate and vertebrate nervous systems have begun to define the signaling mechanisms that function downstream of guidance receptors to regulate growth cone steering and motility (Patel and Van Vactor, 2002; Yu and Bargmann, 2001). The Rho family of small GTPases (Rac, Rho, and Cdc42) have emerged as central regulators of actin cytoskeletal dynamics in neurons and have been implicated in diverse axon guidance receptor signaling pathways (Dickson, 2001; Luo, 2000; Yuan et al., 2003). Increasing evidence indicates that the positive and negative regulators of the Rho GTPases (GEFs and GAPs) can couple axon guidance receptors to the Rho GTPases to regulate actin dynamics in the growth cone. For example, activation of RhoA downstream of guidance receptors is mediated by another Rho family GEF—Vav (Cowan et al., 1999; Kramer et al., 1993). Several proteins that regulate the actin cytoskeleton, including the cytoplasmic tyrosine kinase Abelson (Abl) and its substrate Enabled (Ena), contribute to the Robo signaling pathway in Drosophila and C. elegans (Bashaw et al., 2000; Hsouna et al., 2003; Wills et al., 2002; Yu et al., 2002). In addition, genetic interaction and biochemical experiments in Drosophila and biochemical experiments in mammalian cell culture indicate that activation of Slit-Robo signaling leads to activation of Rac and Rho, and inactivation of Cdc42 (Fan et al., 2003; Fritz and VanBerkum, 2002; Matsuura et al., 2004; Wong et al., 2001).

It is clear from studies of Slit-mediated neural precursor cell migration in rats that inactivation of Cdc42 by Robo is mediated by Slit-Robo GAP (SrGAP1) (Wong et al., 2001). However, how Slit leads to the activation of Rac—RhoGEF domains of Sos are separable signaling modules and support a model in which Robo recruits Sos to the membrane via Dock to activate Rac during midline repulsion.

Among the 22 Rho family GTPases in the Drosophila genome, Sos is a good candidate to play this role for the following reasons. First, sos is among eight RhoGEFs that are enriched in the Drosophila embryonic central type III repeats, a single transmembrane domain, and a long cytoplasmic tail that contains four blocks of conserved cytoplasmic (CC) sequences (CC0, CC1, CC2, CC3) (Bashaw et al., 2000; Kidd et al., 1998). Robo is required to prevent axons from inappropriately crossing the CNS midline in both invertebrates and vertebrates, and it has also been implicated in controlling cell migration in other cell types (Kidd et al., 1998; Kramer et al., 2001; Long et al., 2004). In Drosophila, mutations in robo and its midline-expressed ligand slit result in too many axons crossing and staying at the midline (Kidd et al., 1999; Seeger et al., 1993). Several proteins that regulate the actin cytoskeleton, including the cytoplasmic tyrosine kinase Abelson (Abl) and its substrate Enabled (Ena), contribute to the Robo signaling pathway in Drosophila and C. elegans (Bashaw et al., 2000; Hsouna et al., 2003; Wills et al., 2002; Yu et al., 2002). In addition, genetic interaction and biochemical experiments in Drosophila and biochemical experiments in mammalian cell culture indicate that activation of Slit-Robo signaling leads to activation of Rac and Rho, and inactivation of Cdc42 (Fan et al., 2003; Fritz and VanBerkum, 2002; Matsuura et al., 2004; Wong et al., 2001).

It is clear from studies of Slit-mediated neural precursor cell migration in rats that inactivation of Cdc42 by Robo is mediated by Slit-Robo GAP (SrGAP1) (Wong et al., 2001). However, how Slit leads to the activation of Rac in either Drosophila or vertebrate systems is still unknown. Recent work in Drosophila suggests that the SH3-SH2 adaptor protein Dock may play a role in recruiting Rac to the Robo receptor. Slit stimulation recruits Dock and p21-activated kinase (Pak) to the Robo receptor, and Pak is a downstream target of Rac. It has been proposed that Dock recruits Pak to specific sites at the growth cone membrane, where Pak, activated by Rac, regulates the recycling and retrograde flow of actin filaments (Fan et al., 2003; Hing et al., 1999). Despite these observations, it still remains unclear how Rac is activated in this context. One possible mechanism would be by negative regulation of a Rac-specific GAP(s) upon Slit stimulation. Indeed, a genome-wide analysis in Drosophila has identified a Rac-specific GAP, CrossGAP/Vilse (CrGAP), which interacts directly with the CC2 motif of Robo (Hu et al., 2005; Lundstrom et al., 2004). Overexpression of crGAP mimics the robo mutant phenotype, which suggests that it plays a negative role in Slit-Robo signaling. However, crGAP/vilse mutants do not have major midline axon guidance defects; in fact, loss of crGAP/vilse actually leads to mild robo-like defects (Lundstrom et al., 2004). Thus, it would appear that downregulating crGAP alone in the Robo signaling pathway is not sufficient to lead to activation of Rac.

Since Rho GTPases are directly activated by GEFs, we wondered whether Slit-dependent upregulation of a Rac-specific GEF leads to the activation of Rac. Among the 22 Rho family GEFs in the Drosophila genome, Sos is a good candidate to play this role for the following reasons. First, sos is among eight RhoGEFs that are enriched in the Drosophila embryonic central

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nervous system (Hu et al., 2005). Second, sos was previously shown to genetically interact with slit during midline guidance (Fritz and VanBerkum, 2000); no other GEF has been shown to genetically interact with slit or robo. Third, mutations in sos partially suppress the commissureless mutant phenotype, where elevated robo function results in a complete absence of axon commissures, suggesting that sos functions in the robo pathway (Fritz and VanBerkum, 2000). Finally, mammalian Sos is a Rac-specific GEF and it directly binds to Nck, the mammalian homolog of Drosophila Dock (Hu et al., 1995; Nimmul et al., 1998; Okada and Pessin, 1996).

Sos was identified in Drosophila as a GEF for Ras in the sevenless signaling pathway during the development of the Drosophila compound eye, where it activates the Ras signaling cascade to determine R7 photoreceptor specification (Bonfini et al., 1992; Simon et al., 1991). Studies in mammalian cell culture demonstrated that Sos functions as a GEF for both Ras and Rac in the growth factor-induced receptor tyrosine kinase (RTK) signaling cascade (Nimmul and Bar-Sagi, 2002; Nimmul et al., 1998). Upon RTK activation, the SH3/SH2 adaptor protein Grb2/Drk recruits Sos to autophosphorylated receptors at the plasma membrane, where Sos activates membrane-bound Ras. In a later event downstream of RTK activation, Sos is thought to be targeted to submembrane actin filaments by interaction with another SH3 adaptor, E3b1(ABI-1), where Sos activates Rac (Innocenti et al., 2002, 2003; Scita et al., 1999, 2001). Whether the activation of Rac by Sos is strictly dependent on prior activation of Ras remains controversial, nor is it clear how Sos coordinates the activity of its two GEF domains in vivo.

Here we present evidence that Sos functions as a Rac-specific GEF during Drosophila midline guidance. Sos is enriched in developing axons, and sos exhibits dosage-sensitive genetic interactions with slit and robo. Strikingly, genetic rescue experiments show that the Dbl homology (DH) RhoGEF domain of Sos, but not its RasGEF domain, is required for its midline guidance function. Biochemical experiments show that Sos physically associates with the Robo receptor through Dock in both mammalian cells and Drosophila embryos. Furthermore, Slit stimulation of cultured cells results in the rapid recruitment of Sos to membrane Robo receptors. These results provide a molecular link between the Robo receptor and Rac activation, reveal an independent in vivo axon guidance function of the DH RhoGEF domain of Sos, and support the model that Slit stimulation recruits Sos to the membrane Robo receptor via Dock to activate Rac-dependent cytoskeletal changes within the growth cone during axon repulsion.

**Results**

**Sos Is Enriched in CNS Axons and Interacts Genetically with slit and robo**

It has been reported that Sos protein is enriched in the axons of stage 16 Drosophila embryos (Fritz and VanBerkum, 2000), a stage when most axons have already made their midline guidance decisions. Therefore, we examined Sos expression at earlier stages. In stage 12 wild-type embryos, when axons start to project, Sos is broadly expressed in most cells but begins to be enriched in developing axons as revealed by double-staining with BP102 (Figures 1A–1C). By stage 17, there is a strong enrichment of Sos in all CNS axons, including longitudinal axons and commissural axons (Figures 1D–1F). In sos zygotic null mutants, the overall Sos protein level is significantly reduced, but there is still a considerable amount of Sos remaining in CNS axons (Figures 1G and 1H). Interestingly, Sos is also enriched in tracheal cells (Figures 1D and 1F), whose migration is regulated by Slit-Robo signaling as well (Englund et al., 2002).

To investigate the potential role of Sos in Slit-Robo-mediated Drosophila midline axon guidance, we analyzed the phenotype of sos mutants and tested for genetic interactions between sos, slit, and robo. In wild-type embryos, Fasciclin II (FasII)-positive axons project longitudinally and never cross the midline (Figures 2A and 2F). In robo mutants, FasII-positive axons inappropriately cross the midline many times (Figure 2B). Consistent with previous results (Fritz and VanBerkum, 2000), two independent sos null mutants have mild midline crossing defects that are strongly enhanced by reducing the gene dose of slit or robo (Figures 2C–2F). Importantly, these interactions are dosage sensitive: removing one copy of sos and one copy of slit or robo did not cause any axon guidance defects, while removing both copies of sos and one copy of slit or robo did. Here it is important to recall that, even though we are removing both zygotic copies of sos, there is still a considerable amount of Sos protein remaining in these embryos (Figure 1G). The midline crossing defects observed in sos mutants together with these dosage-sensitive genetic interactions suggest that sos may contribute to Slit-Robo repulsion.

Since Drosophila sos is involved in cell fate specification, the guidance defects in sos mutants might be due to malfunctions in other processes rather than axon guidance itself. To rule out this possibility, a genetic rescue approach using the Gal4/UAS system was taken (Brand and Perrimon, 1993). Since sos mutants have only weak axon guidance defects, we performed rescue experiments in the stronger sos<sup>230</sup>, slit<sup>2</sup>isos<sup>60</sup> mutant background by expressing UASSosmyc under the control of a neuronal-specific fushi-tarazu neurogenic Gal4 (ftz<sup>0β</sup>Gal4) driver. The ftz<sup>0β</sup>Gal4 driver expresses Gal4 in subsets of ipsilaterally projecting neurons. We found that the ectopic midline crossing phenotype of sos<sup>230</sup>, slit<sup>2</sup>isos<sup>60</sup> was significantly rescued in embryos that carried the wild-type sos transgene (Figure 2F). Using several different transgene inserts, we found that the extent of rescue correlated with the level of transgene expression (data not shown). Taken together, these results strongly suggest that the neuronal-specific function of Sos is important for Slit-Robo-mediated midline axon repulsion.

It seems likely that the strong maternal deposit of sos in developing embryos could account for the relatively weak midline axon guidance defects observed in sos zygotic mutants. Due to the requirement of Sos during early development, embryos lacking both maternal and zygotic sos have severe patterning defects (Luschnig et al., 2004; Silver et al., 2004), making it difficult to determine the full contribution of Sos to axon guidance. RNAi experiments to more completely block sos
function did lead to much stronger midline crossing defects; however, these manipulations also resulted in additional patterning defects that complicate the interpretation of these results (L.Y. and G.J.B., unpublished data).

**Drosophila** Sos Functions as a Rac-Specific GEF In Vivo

*Drosophila* Sos is highly homologous to mammalian Sos and contains all the conserved functional domains. To test if *Drosophila* Sos, like its mammalian homolog, can activate small Rho family GTPases in vivo and to determine its substrate preference, we took advantage of the fact that when *rac1*, *rhoA*, or *cdc42* is overexpressed in the developing eye, a “rough eye” phenotype is induced because of a disruption of ommatidial cell development (Figures S1C, S1E, and S1G in the Supplemental Data available with this article online) (Hariharan et al., 1995; Nolan et al., 1998). The “rough eye” phenotype caused by overexpression of *rac1* was strongly enhanced by coexpression of sos (Figures S1C and S1D). This enhancement is specific for *rac1*, as coexpressing *sos* with *rhoA* or *cdc42* did not modify the rough eye phenotype (Figures S1E–S1H). This result indicates that *Drosophila* Sos can specifically activate Rac in the developing eye, which is consistent with the in vitro data that mammalian Sos only displays GEF activity toward Rac, but not RhoA and Cdc42 (Nimnual et al., 1998).

Next, we asked which small GTPase is likely to be the in vivo target of Sos during midline axon guidance, since all three members of the Rho family of small Rho GTPases (Rac, Rho, Cdc42) have been implicated in Slit-Robo repulsion. Both genetic and biochemical evidence indicates that activation of Slit-Robo signaling leads to activation of Rac and Rho, and inactivation of Cdc42 (Fan et al., 2003; Wong et al., 2001). Since the *sos* mutant phenotype and its genetic interaction with *slit* and *robo* (Figure 2; Table S1) suggest that Sos may play a positive role in Slit-Robo signaling, we focused on determining whether Rac or RhoA is the in vivo target for Sos during midline axon guidance. Panneuronal overexpression (using *elavGal4*) of *RacDN* (*UASRacN17*) or *RhoADN* (*UASRhoAN19*), in an otherwise wild-type background did not result in significant ectopic midline crossing. However, both the *RacDN* and the *RhoADN* showed strong enhancement of midline guidance defects seen in sos homozygous mutants (Table S1). This result is consistent with the previous finding that overexpressing either *RacDN* or *RhoADN* with the *ftzGα4* driver enhances the sos mutant phenotype to the same extent (Fritz and VanBerkum, 2002), suggesting
that Sos could activate both Rac and Rho during midline guidance.

A major concern of using this type of dominant-negative approach is its nonspecificity. Dominant-negative forms of the Rho GTPases are thought to act by sequestering endogenous GEFs. Since different Rho GTPases may share common GEFs, it is not clear whether the observed genetic interactions could reflect simultaneous downregulation of multiple Rho GTPases. To resolve this issue, we examined the effects of "loss-of-function" mutations of rac1 or rhoA in sos homozygous mutant embryos. Loss-of-function mutants of rac1 (rac1(t1111)) or rhoA (rhoA220/220) alone did not result in obvious midline guidance defects (Figures 3B and 3D; Table S1). However, removing only one copy of rac1 strongly enhanced the ectopic midline crossing phenotype seen in sos mutants (Figures 3A and 3C). Considering that there are three redundant rac genes (rac1, rac2, and mtl) in Drosophila (Hakeda-Suzuki et al., 2002; Ng et al., 2002), it is particularly striking that sos22/Nie22, rac1(t1111)/+ mutants exhibit such strong defects (Figure 3G). Indeed, the greater than 5-fold increase in ectopic midline crossing is likely to underestimate the total defects, as some segments are fused in these mutants, and fused segments were only counted as one ectopic cross in our quantification scheme (Figure 3C).

In contrast, removing one copy of rhoA did not show any effect on the sos mutant phenotype, and removing two copies of rhoA resulted in only a modest enhancement of the ectopic crossing defects, which were much weaker than sos22/Nie22, rac1(t1111)/+ mutants (Figures 3A, 3D, 3F, and 3G). Based on this highly sensitive loss-of-function genetic interaction between sos and rac1, but not rhoA, we conclude that Rac is the preferred substrate for Sos during midline guidance; however, these observations do not formally exclude the possibility that Sos also activates Rho to a lesser extent during midline guidance.

Sos Functions in Opposition to CrGAP/Vilse

Since CrGAP/Vilse is a Rac-specific GAP in the Robo signaling pathway (Hu et al., 2005; Lundstrom et al., 2004), we would predict that Sos plays an opposing role to CrGAP/Vilse. Consistent with published results (Hu et al., 2005), low-level overexpression of crGAP/vilse (one copy of UAScrGAP and one copy of elavGal4 driver) results in a wild-type FasII axon projection pattern (Figure 4A). High-level overexpression of crGAP/vilse (two copies of UAScrGAP and two copies of elavGal4 driver) results in a series of defects, including extensive ectopic midline crossing in some segments, which is reminiscent of the robo mutant phenotype, and axon outgrowth defects in some segments, which is similar to triple loss-of-function rac mutants (Hakeda-Suzuki et al., 2002; Ng et al., 2002) (Figure 4C). If Sos does play an opposing role to CrGAP/Vilse in regulating Rac activity, we would expect that reducing the levels of sos should enhance the axon guidance defects in embryos with low-level overexpression of crGAP/vilse, while overexpressing sos should suppress the axon guidance and outgrowth defects caused by high-level overexpression of crGAP/vilse. This is indeed the case. Removing two copies of sos in embryos expressing low levels of crGAP/vilse led to a striking enhancement of ectopic midline crossing and axon outgrowth.
defects (Figures 4A, 4B, and 4E; Table S1). On the other hand, panneuronal overexpression of UASSosmyc in embryos expressing high levels of crGAP/vilse rescued both of these defects (Figures 4C–4E; Table S1). Together, these observations suggest that activation of Rac downstream of Robo is tightly regulated by both GEF and GAP activities.

The Guidance Function of Sos Is Dependent on Rac, but Not Ras Activation

Although the results we have presented support the idea that Sos functions as a Rac-GEF during Robo repulsive axon guidance, it remains unclear whether the sos axon guidance function is also dependent on Ras activation. Although previous genetic studies failed to detect dose-dependent genetic interactions between ras and robo (Fritz and VanBerkum, 2000), there are several reasons to give serious consideration to this possibility. First, studies of other repulsive axon guidance receptor signaling pathways have revealed an important role for Ras activation. For example, Plexin-B1 mediates Sema4D-induced repulsive axon guidance signaling in part through the activation of Ras (Onuma et al., 2004a, 2004b). Second, it is not clear whether the Rac-GEF activity of Sos is gated by Sos-dependent Ras activation. For example, in the canonical RTK→Sos→Ras→Sos→Rac pathway, Sos couples Ras activation to the subsequent activation of Rac (Innocenti et al., 2002, 2003; Nimnual et al., 1998). Alternatively, other studies suggest that Rac activation after growth factor treatment could occur in a Ras-independent manner, RTK→Sos→Rac, in which the Ras-GEF activity of Sos seems not to be required for its Rac-GEF activity (Scita et al., 2000; Sini et al., 2004). Our genetic model system provides an excellent opportunity to test in vivo whether the Ras and Rac-GEF activities of Sos can be functionally uncoupled.

To test whether or not the Ras-GEF activity of Sos is required for the activation of Rac, we took advantage of the observation that the axon guidance defects caused by removing one copy of slit in a sos mutant background can be rescued by overexpressing wild-type Sos (Figures 5A, 5B, and 5J). Two mutant versions of Sos, UASSos::DHmyc and UASSos::RasGFPmyc, were generated and examined for their ability to rescue sos<sup>a31</sup>, slit<sup>2/sose4G</sup> mutants (Figure 5I). The expression level of transgenes was determined by western blot (Figure S2D), and transgene localization was determined by anti-myc staining (Figures S2A–S2C). Mutant transgenes with comparable expression levels and...
localization were used in the rescue experiments. Overexpression of UASSosD\textit{DHmyc} could not rescue the axon defects in sos\textsuperscript{sose2H}, slit\textsuperscript{2}/sos\textsuperscript{sose4G} mutants (Figures 5A, 5C, and 5J). To exclude the possibility that the differential rescue effects of truncated Sos transgenes are dependent on particular genetic backgrounds or Gal4 drivers, we performed the same genetic rescue experiment in the sos, rhoA double mutant background using a panneuronal driver (elavGal4). Similar rescue effects were observed (Figure 5E, 5F, and 5J). Taken together, these results reveal that the Rac-GEF activity of Sos, but not its Ras-GEF activity, is required for its function in Robo repulsive signaling and provide strong in vivo evidence that the Ras and Rac-GEF activities of Sos can be functionally uncoupled during signal transduction.

Dock Physically Couples Sos to the Robo Receptor

While the genetic results we have presented suggest that Sos functions to activate Rac in the Robo signaling pathway, it remains unclear how and whether Sos is linked to the Robo receptor. We first examined the possibility that Sos directly binds to Robo, but we failed to detect any physical interaction by coimmunoprecipitation from 293T cells coexpressing Robo and Sos (Figure 6C). We next considered the possibility that other components of the Robo signaling pathway could serve as links between Robo and Sos. Since \\textit{ena} and \\textit{dock} are both implicated in Robo signaling (Bashaw et al., 2000; Fan et al., 2003; Yu et al., 2002), and they appear to function independently of each other, we tested whether either of these genes displayed dose-dependent genetic interactions with sos. Reducing the dose of \\textit{dock} enhanced the sos mutant phenotype, while similar reduction of \\textit{ena} had no effect (Table S1). These genetic data suggest that Sos might function as a Rac-GEF in Dock-dependent repulsive signal transduction downstream of Robo. Other lines of evidence also support this idea. First, Dock has been implicated in the increase in Rac activity caused by Slit stimulation, since the ΔCC2ΔCC3 mutant version of Robo that cannot bind to Dock is unable to mediate the Slit-dependent increase in Rac activity (Fan et al., 2003). Second, mammalian Sos has been shown to directly bind to Nck, the homolog of Dock (Hu et al., 1995; Okada and Pessin, 1996).

Therefore, we examined the physical association between Sos and Dock in 293T cells. Both GST pull-down and coimmunoprecipitation assays revealed that Sos physically associates with Dock (Figures 6A and 6B). In a yeast two-hybrid assay, the direct binding of Dock to Sos was also detected, and the binding domain for each protein was determined. We found that the C-terminal portion of Sos that contains the PXXP motif directly interacts with the SH3-2 and SH3-3 domains of Dock (Figure 6D and 6E). Since the SH3-1 and SH3-2 domains of Dock are required for binding to Robo (Fan et al., 2003), we would predict that a Robo-Dock-Sos ternary complex can form, in which Dock bridges the interaction between Robo and Sos. This is indeed the case. In 293T cells, when Dock is coexpressed with Robo and Sos, formation of a Robo-Dock-Sos protein complex is detected by coimmunoprecipitation (Figure 6F). Furthermore, in Drosophila embryo lysates, endogenous Sos and Dock are detected in a complex with Robo (Figure 6G), suggesting that this protein
complex exists under physiological conditions, and that it is not just an artifact when proteins are overexpressed in mammalian cells.

**Slit Stimulation Triggers the Recruitment of Sos to Membrane Robo Receptors and Induces Membrane Ruffles and Lamellipodia Formation**

The genetic and biochemical data that we have presented, together with previously published data indicating that Slit stimulation leads to enhanced Dock recruitment and increased Rac activity (Fan et al., 2003), support the model that Sos is recruited to the Robo receptor where it activates Rac (and possibly Rho) to promote midline repulsion. If this model were true, we would predict that Slit activation of Robo at the plasma membrane should lead to the recruitment of Sos to membrane Robo receptors and induce changes in actin morphology. To test this prediction, we turned to a mammalian HEK293T cell culture system where both the subcellular distribution of Sos and actin morphology have been well characterized. Studies from mammalian cell culture reveal that the Rac-GEF activity of Sos is tightly linked to the midline guidance function of Sos.
Figure 6. Dock Physically Couples Sos to the Robo Receptor
(A and B) Interactions between Sos and Dock in 293T cells. (A) 293T cell lysates coexpressing His-Sos-Myc and HA-Dock, or HA-Dock alone, were precipitated by Ni-NTA beads. The right two lanes show the coprecipitated proteins, while the left two lanes indicate Sos and Dock expression in cells. (B) 293T cell lysates expressing His-Sos-Myc were precipitated by Glutathione Sepharose 4B beads bound to GST or GST-Dock. The right two lanes show the coprecipitated proteins, while the leftmost lane indicates Sos expression in cells. (C) 293T cell lysates coexpressing His-Sos-Myc and HA-Robo, or HA-Robo alone, were precipitated by Ni-NTA beads. The right two lanes show that Robo is not coprecipitated with Sos, while the left two lanes indicate Sos and Robo expression in cells. (D) Different sized forms of Sos were fused to the B42 transcription activation domain. Full-length Dock was fused to the LexA DNA binding domain. Our analysis indicates that the C-terminal part of Sos, which includes the conserved proline-rich regions, is important for the Sos-Dock physical interaction. PRR, proline-rich region. (E) The same strategy was used to identify the potential interacting domain in Dock for Sos. LexA-fused truncated forms of Dock were tested with B42-Sos. Strong binding was mediated by the SH3-2 or SH3-3 domains. Yeast turned dark blue (++++; strong interaction), blue (+++), light blue (++), or white
regulated, and that it is dependent on the precise subcellular localization of Sos. In resting cells, Sos is predominantly localized to the cytoplasm, and its Rac-GEF activity is inhibited. In growth factor-treated cells, Sos is recruited to the membrane, where its Rac-GEF activity induces a specific actin morphology—membrane ruffles and lamellipodia, a hallmark of Rac activation. In human Robo1-expressing 293T cells after control treatment, endogenous Sos shows a similar predominantly cytoplasmic localization (Figures 7E–7H) as in non-hRobo1-expressing cells after control (Figures 7A–7D) or hSlit2 treatment (Figures 7I–7L). In contrast, 5 min of human Slit2 treatment leads to a dramatic redistribution of Sos to the plasma membrane (Figures 7M, 7P, and 7G) and induces membrane ruffling (arrows in Figure 7O). In addition, on the membrane, Sos proteins are partially colocalized with hRobo1 receptors and F-actin (Figure 7P).

Similar results are observed in a Drosophila cell culture system. In Robo-expressing Drosophila S2R+ cells, an embryonic Drosophila cell line, endogenous Sos is localized in the cytoplasm (Figures S3A and S3B). In control-treated cells, very little colocalization between Robo and Sos is observed (Figures S3C and S3H). In contrast, Slit treatment leads to a considerable redistribution of Sos to the plasma membrane (Figures S3D and S3F). In these Slit-treated cells, we observe a striking increase in Robo and Sos colocalization in plasma membrane swellings that bear morphological similarity to membrane ruffles (Figures S3F and S3H). In addition, in Slit-treated cells Sos and Robo colocalization is observed in a large internal vesicular structure that may correspond to endocytosed receptor: the significance of this site of Sos and Robo colocalization is not clear (Figures S3D–S3F). Together with our biochemical data, these observations support the model that Slit triggers the formation of a protein complex on the plasma membrane consisting of Robo, Dock, and Sos. Once at the membrane, Sos would be poised to locally activate Rac to regulate the actin cytoskeleton, and in turn growth cone behavior.

Discussion

In this paper we present genetic and biochemical evidence that Sos is an important component of the Robo receptor signaling pathway. Specifically, our data support the idea that Sos provides a direct molecular link between the Robo receptor and the activation of Rac during Drosophila midline guidance. Genetic interactions between sos, robo, dock, crGAP/vilse, and the Rho family of small GTPases strongly suggest that Sos functions in vivo to regulate Rac activity during Robo signaling. Genetic rescue experiments indicate that sos is required specifically in neurons to mediate its axon guidance function. Furthermore, our genetic data establish that, in the context of midline axon guidance, the Ras-GEF and Rac-GEF activities of Sos can be functionally uncoupled. Biochemical experiments in cultured cells and Drosophila embryos show that Sos is recruited into a multiprotein complex consisting of the Robo receptor, the SH3-SH2 adaptor protein Dock, and Sos, in which Dock bridges the physical association between Robo and Sos. Finally, experiments in cultured cells support the idea that Slit activation of Robo can recruit Sos to the submembrane actin cytoskeleton to regulate cell morphology. Together, these results suggest a model in which Slit stimulation recruits Sos to the Robo receptor via Dock to regulate Rac-dependent cytoskeletal changes within the growth cone during axon repulsion.

Rho GTPase Substrate Specificity of Sos in Robo Signaling

Based on previous work implicating rac in Robo repulsion, as well as in vitro studies demonstrating that Sos exhibits GEF activity for Rac, but not Rho or Cdc42, Rac seemed the most likely Sos substrate. However, rho has also been implicated in mediating Robo repulsion (Fan et al., 2003; Fritz and VanBerkum, 2002), and genetic interactions between sos and dominant-negative Rho have been interpreted to suggest that Sos could act as a GEF for Rho. We have investigated this question further and have presented two types of genetic evidence that suggest that indeed Rac is the favored substrate of Sos. First, ectopic expression experiments in the eye reveal interactions exclusively between sos and rac. Second, genetic interaction experiments using loss of function mutations in rac and rho (rather than the more problematic dominant-negative forms of the GTPases) reveal strong dose-dependent interactions between sos and rac, but not sos and rho during midline axon guidance. Together, our observations argue in favor of Rac as the primary in vivo Sos substrate. Nevertheless, we cannot exclude the possibility that Sos also contributes to Rho activation and that the combined activation of Rac and Rho is instrumental in mediating the Robo response.

Sos as the Direct Molecular Link between Robo and Rac Activation

Previous studies from our lab and others have demonstrated that Slit stimulation of the Robo receptor leads to a rapid increase in Rac activity in cultured cells. However, the mechanism by which Rac is activated downstream of Robo was not clear. Here we provide direct genetic and biochemical evidence that Sos is coupled to the Robo receptor through the Dock/Nck SH3-SH2 adaptor, where it can regulate local Rac activation. Studies in cultured mammalian cells have highlighted the importance of distinct Sos/adaptor protein complexes in controlling the subcellular localization and activity of Rho family GTPases.
substrate specificity of Sos. In the context of Rac activation, the E3b1 (Abi-1) adaptor has been shown to play a critical and rate-limiting role in Sos-dependent Rac activation and subsequent formation of membrane ruffles (Innocenti et al., 2002). Could Sos regulation of Rac activity during Robo repulsion be similarly limited by the availability of specific adaptor proteins? It is interesting to note in this context that overexpression of dock does not lead to ectopic axon repulsion, suggesting that Dock may not be limiting for Robo signaling. However, although dock mutants do have phenotypes indicative of reduced Robo repulsion, their phenotype is considerably milder than that seen in robo mutants, raising the possibility that there may be additional links between Robo and Sos.

A Ras-GEF-Independent Function of Sos in Axon Guidance

A number of studies in cultured mammalian cells have suggested that Rac activation induced by activated growth factor receptors requires the prior activation of Ras. For example, PDGF-induced membrane ruffling can be promoted or inhibited by expression of constitutively active or dominant-negative Ras, respectively (Nimnual et al., 1998; Scita et al., 1999). However, other studies have suggested that in Swiss 3T3 cell lines RTK activation of Rac is Ras independent (Ridley et al., 1992). In addition, the observation that Ras activation and Rac activation display very different kinetics, with Rac activation persisting long after Ras activity has returned to basal levels, has been used to argue against an obligate role for Ras in Rac activation (Innocenti et al., 2002). Here, using a genetic rescue approach, we have directly tested whether the ability of Sos to activate Rac during axon guidance in an intact organism requires its Ras-GEF function. Our genetic data indicate that the RasGEF domain of Sos is dispensable for axon guidance, while the DH RhoGEF domain is strictly required (Figure 5). This observation argues strongly in favor of the model that in vivo Sos activation of Rac does not strictly require Sos activation of Ras.

How Is the Rac-GEF Activity of Sos Regulated?

It is clear that subcellular localization plays a major role in regulating Sos activity and that different protein complexes containing Sos exist in different locations in the cell. Here we have shown that activation of the Robo receptor by Slit triggers the recruitment of Sos to Robo receptors at the plasma membrane. Our biochemical data argue that the adaptor Dock/Nck is instrumental in...
bridging this interaction, and given the diverse interactions between Dock/Nck and guidance receptors, it seems likely that Dock/Nck could fulfill this bridging role in many guidance receptor contexts. This bridging function of Dock/Nck and guidance receptors is analogous to the role of Grb2 for growth factor receptors only insomuch as it brings signaling molecules to the receptor—the mechanism of interaction is distinct, since it is mediated through SH3 domain contacts rather than SH2/phosphotyrosine interactions. Our observations suggest that there may be an additional pool of Sos that can function in a distinct adaptor protein/guidance receptor complex to regulate cell morphology in response to extracellular guidance cues.

Could Abl Regulate the Rac-Specific GEF Activity of Sos?

Is regulating subcellular localization the only mechanism by which Sos activity is controlled? This seems unlikely. Indeed, a recent study has implicated tyrosine phosphorylation of Sos by Abl as an additional mechanism to activate the Rac-specific GEF activity of Sos in vertebrate cell culture models (Sini et al., 2004). This raises the intriguing possibility that Abl may fulfill a similar role for Robo signaling. This is a particularly appealing idea given the well-documented genetic and physical interactions between Robo and Abl (Brushaw et al., 2000; Hsouna et al., 2003; Wills et al., 2002). Indeed, we have observed that sos and abl exhibit dose-dependent genetic interactions during midline axon guidance (L. Y. and G. J. B., unpublished data). A clear genetic test of whether Abl activates the Rac-GEF activity of Sos downstream of Robo may be complicated by the fact that Abl appears to play a dual role in Robo repulsion: both increasing and decreasing abl function lead to disruptions in Robo function. Nevertheless, it should be possible in the future to generate mutant versions of Sos that are refractory to Abl activation and to test whether these alterations disrupt the Sos guidance function. It will also be of great interest to determine whether the redistribution of Sos can also be observed in response to guidance receptor signaling in navigating growth cones, and if so, then what changes in actin dynamics and growth cone behavior are elicited.

Experimental Procedures

Molecular Biology

Drosophila Sos was PCR amplified from EST clone #GH01796 (DGRC) and subcloned into the pUAST vector. A 6-myc tag was PCR amplified and subcloned to the C terminus of Sos to generate a pUAST-Sosmyc construct. Sos.Dhm2 was lacking the DH domain (aa 251–432) and Sos.srasGEFmyc lacking the RasGEF domain (aa 824–1066) were generated by PCR mutagenesis and subcloned into the pUAST vector. Sosmyc was also subcloned into the pCDNA3.1-His vector (Invitrogen) to generate pCDNA3.1-His-Sosmyc. Different fragments of Sos (aa 1–600; aa 601–1079; aa 1080–1595) were cloned into the pJG4-5 vector. All constructs were sequenced.

Genetics

The following fly strains were used: sos^{+/+}CyO/Wg^{+};Gal, (3) sos^{+/+}CyO/Wg^{+};Gal, (4) sos^{+/+}CyO/Ela^{+};Gal, rac^{+/+}TM6B/Ubx^{+};Gal, (5) sos^{+/+}CyO/Wg^{+};Gal, UASRac^{+/+}, (6) sos^{+/+}CyO/Wg^{+};Gal, UASRho^{+/+}, (7) sos^{+/+}CyO/Wg^{+};Gal, elavGal4, (8) sos^{+/+}CyO/Wg^{+};Gal, fts^{+/+}Gal4/I-TM2, (9) sos^{+/+}, rhoA^{+/+}CyO/Wg^{+};Gal; elavGal4, (10) sos^{+/+}/CyO/Ela^{+};Gal, UASCrGAP, elavGal4/I-TM6B/Ubx^{+};Gal, (11) UASSosmyc; UASGAP, elavGal4/I-TM6B/Ubx^{+}Gal, (12) sose^{2H}, dock^{+/+}CyO/Wg^{+};Gal, (13) sose^{2H}, ena^{+/+};CyO/Wg^{+};Gal, sose^{2H} and sose^{4G} contain premature stop codons at amino acid positions 579 and 421, respectively, and have previously been demonstrated to be null mutants. To generate transgenic fly strains, UASSos, UASSosmyc, UASSos.Dhm2myc, and UASSос jrasGEFmyc were transformed into w^{1118} flies using standard procedures. Independent transformant lines on the second and third chromosomes were obtained. The Gal4-UAS system was used to express transgenes in the Ftz ipsilateral neurons (ftsz^{+/+}Gal4) or in all neurons (elavGal4). Crosses to GMGf4Gal4 to generate “rough eye” phenotypes were conducted at 18°C. All other crosses were conducted at 25°C.

Immunohistochemistry

HRP immunohistochemistry was performed as previously described, and images were obtained using a Zeiss Axiocam and Openlab software (Improvision). Fluorescent staining for FasII guidance defects was performed using mouse MAb 1D4 (1:100) and antibodies against β-gal (mouse anti-β-gal 1:150, rabbit anti-β-gal 1:10,000; Roche), β-gal staining allowed the identification of genotypes in embryos. Cy3 secondary antibody (Molecular Probes) was used at 1:10,000, and Alexa Fluor 488 secondary antibody was used at 1:500–1000. Fluorescence double-staining for Sos and BP102 was performed using rabbit anti-Sos (1:500; a gift from Dr. U. Banerjee) and mouse MAb BP102 (1:100). Fluorescent images were taken using a Leica Confocal TCS TL microscope and processed with NIH Image J software.

Immunoprecipitation

293T cells were transfected with plasmid DNA at 90% confluency using Effectene Transfection Reagent (Qiagen). Twenty-four hours posttransfection, cells were lysed in lysis buffer containing 1% Triton X-100, 150 mM NaCl, 50 mM Tris (pH 8.0), 10 mM Imidazole, 2 μM protease inhibitor (Roche), and 1 mM NaN3. Cell lysates were incubated with Ni-NTA resin (Qiagen) at 4°C for 2 hr. The beads were washed three times with lysis buffer and heated at 100°C for 10 min. The precipitates were resolved on SDS-PAGE gels and blotted with mouse anti-HA antibody (1:1000; Covance) and mouse anti-myc antibody (9E10; 1:1000). For in vivo IP, lysates were prepared from 100 μl of embryos overnight expressing one copy of UASRobomyc in all neurons. Embryos were smashed in lysis buffer containing 0.5% Triton X-100, 1% PBS, 2 μM protease inhibitor, and 1 mM NaN3, and were incubated with mouse anti-myc antibody and protein A Sepharose 4B beads at 4°C for 3 hr. Beads were washed three times with lysis buffer, heated at 100°C for 10 min, resolved on SDS-PAGE gels, and blotted with rabbit anti-Sos antibody (1:2000), rabbit anti-Dock antibody (1:4000), and mouse anti-myc antibody.

GST Pull-Down

GST and GST-Dock were expressed in E. coli and purified using the Bulk and RediPack purification modules (Amersham). His-Sosmyc was expressed in 293T cells, and cells were lysed in lysis buffer containing 1% Triton X-100, 150 mM NaCl, 50 mM Tris (pH 7.5), 2 μM protease inhibitor, and 1 mM NaN3. Cell lysates were incubated with Ni-NTA resin (Qiagen) at 4°C for 2 hr. The beads were washed three times with lysis buffer and heated at 100°C for 10 min. Precipitated proteins were resolved on SDS-PAGE gels and blotted with mouse anti-myc antibody.

Cell Immunofluorescence

HEK293T Cells

293T cells were seeded on glass coverslips coated with poly-L-lysine and transfected with plasmid DNA at 40% confluency using Effectene Transfection Reagent (Qiagen). Twenty-four hours after transfection, cells were starved in serum-free DMEM for 12–16 hr and then stimulated with conditioned medium of Hsl12-stably expressing 293T cells (a gift from Dr. Y. Rao) for 5 min. Treated cells
were washed with 1× PBS once and immediately fixed in 4% paraformaldehyde/1× PBS for 20 min. Fixed cells were permeabilized in 0.1% Triton X-100/1× PBS for 2 min and blocked with 3% BSA/1× PBS for 5 min. Cells were then incubated with primary antibody (rabbit anti-hSos 1:50, mouse anti-myc 1:1000) overnight at 4 °C, and secondary antibody (rabbit Alexa Fluor 488, mouse Cy3 secondary antibody) for 30 min at RT, respectively. Finally, cells were stained with Alexa 568-conjugated phalloidin for 1 hr at RT. Fluorescent images were taken using a Leica Confocal TCS SL microscope and processed by NIH Image J software.

Quantification of Sos Membrane/Cytosol Fluorescence Intensity

For each group of cells, ten random cells were selected for quantification. Fluorescence intensity is calculated by area (pixel numbers) multiplied by average fluorescence intensity using NIH Image J software. In 293T cells, actin staining was processed to generate a membrane mask for all cells. The overlapping area of membrane mask and Sos staining is used to calculate Sos membrane fluorescence intensity. The cytoplasmic area is calculated by nonoverlapping Sos staining area minus nucleus area.

Supplemental Data

The Supplemental Data include Supplemental Experimental Procedures, three supplemental figures, and one supplemental table and can be found with this article online at http://www.neuron.org/cgi/content/full/52/4/595/DC1/.

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