DRK/DOS/SOS converge with Crk/Mbc/dCed-12 to activate Rac1 during glial engulfment of axonal debris

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Nervous system injury or disease leads to activation of glia, which govern postinjury responses in the nervous system. Axonal injury in *Drosophila* results in transcriptional up-regulation of the glial engulfment receptor Draper; there is extension of glial membranes to the injury site (termed activation), and then axonal debris is internalized and degraded. Loss of the small GTPase Rac1 from glia completely suppresses glial responses to injury, but upstream activators remain poorly defined. Loss of the Rac guanine nucleotide exchange factor (GEF) Crk/myoblast city (Mbc)/dCed-12 has no effect on glial activation, but blocks internalization and degradation of debris. Here we show that the signaling molecules downstream of receptor kinase (DRK) and daughter of sevenless (DOS) (mammalian homologs, Grb2 and Gab2, respectively) and the GEF son of sevenless (SOS) (mammalian homolog, mSOS) are required for efficient activation of glia after axotomy and internalization/degradation of axonal debris. At the earliest steps of glial activation, DRK/DOS/SOS function in a partially redundant manner with Crk/Mbc/dCed-12, with blockade of both complexes strongly suppressing all glial responses, similar to loss of Rac1. This work identifies DRK/DOS/SOS as the upstream Rac GEF complex required for glial responses to axonal injury, and demonstrates a critical requirement for multiple GEFs in efficient glial activation after injury and internalization/degradation of axonal debris.

reactive glia | engulfment signaling | Draper pathway | Wallerian degeneration

Activation of glia is a hallmark of nearly all neurodegenerative diseases and neural injuries. When brain insults have occurred, glia rapidly change their morphology and gene expression profiles to invade the injury site and clear pathogens and/or neuronal debris by phagocytic engulfment (1, 2). Failure to clear debris from the CNS can result in prolonged neuroinflammation and hamper the recovery of the CNS (3, 4). However, the genetic pathways promoting glial activation after neural injuries remain poorly defined.

Genetic studies of Wallerian degeneration in *Drosophila* have provided important insights into glial responses to axotomy (5). Olfactory neuron axotomy results in the degeneration of axons projecting into the antennal lobe of the fly brain, where local glia sense degenerating axons, and initiate a multistep process of reactivity. Reactive glia up-regulate the transcription of the engulfment receptor Draper (*dpr*) and extend membranes to degenerating axons (5, 6). When at the injury site, glia internalize axonal debris and degrade it through the phagolysosomal pathway (7). Finally, glia terminate their responses by withdrawing from the injury site and down-regulating Draper, and finally return to a resting state (6).

Draper is essential for all glial responses to axonal injury. In *dpr*-null mutants, glia fail to respond morphologically to axonal injury, and axonal debris lingers in the brain for weeks after axotomy (5). Downstream of Draper, the small GTPase Rac1 appears to be critical in executing glial activation to axon injury, as loss of Rac1 phenocopies *dpr*-null mutants (7). The only Rac guanine nucleotide exchange factor (GEF) known to be required for glial engulfment of axonal debris is the noncanonical GEF Crk/myoblast city (Mbc)/dCed-12. However, in contrast to loss of

Rac1, animals lacking Crk/Mbc/dCed-12 signaling exhibit relatively normal activation of glia after axotomy, with glia increasing Draper expression and extending membranes to degenerating axons, but glia then fail to internalize and degrade axonal debris (7). These data argue for a specific role for the Crk/Mbc/dCed-12 complex at the internalization/degradation phase of the glial response, and suggest that an additional Rac1 GEF must act earlier during initial activation of glial responses to axonal injury.

In an RNAi-based screen for new engulfment genes, we identified downstream of receptor kinase (*drk*) as a gene required for efficient glial clearance of degenerating axons. *drk* is best known for its role in signaling downstream of the Sevenless (Sev) receptor tyrosine kinase (RTK), where it functions with daughter of sevenless (*dos*) and son of sevenless (*sos*) to activate the small GTPase Ras (8–13). More recent studies have also linked SOS to the activation of Rac1 in the regulation of axon guidance during *Drosophila* embryonic CNS development (14), indicating that DRK/DOS/SOS can act upstream of multiple small GTPases. Here we show that the DRK/DOS/SOS complex plays a critical role in activation of glial responses to injury and internalization of axonal debris. Moreover, we provide genetic evidence that, at the earliest stage of glial activation, DRK/DOS/SOS function redundantly with Crk/Mbc/dCed-12 to promote Rac1 activation and initiate all steps in glial responses to axonal injury.

Results

DRK, DOS, and SOS Are Required for Glial Engulfment of Axonal Debris. To identify new pathways required for glial engulfment of degenerating axons, we performed an RNAi-based screen for genes required in glia for clearing axonal debris after axotomy. We expressed each of ~500 UAS-RNAi constructs from the Vienna *Drosophila* Resource center (15) by using the pan-glial driver *repo-Gal4* (16). For animals that did not survive to adulthood, we

Significance

Neuronal cell death or injury leads to the production of neuronal cell corpses and cellular debris that must be cleared from the nervous system to avoid inflammation or toxicity. Glia are the primary cell type responsible for clearing neuronal debris, but precisely how these cells recognize, phagocytose, and destroy this material remains poorly defined. Here we use a simple nerve injury assay to identify genes downstream of the engulfment receptor Draper that are required for efficient glial engulfment of degenerating axons. Based on the molecular and functional conservation of this pathway in mammals, this study sheds new light on pathways potentially used by mammalian glia to react to brain injury or neurodegeneration.

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which was absent when we drove *drk^{RNAi}* in glia (Fig. 2A), indicating that glial DRK is recruited to severed axons. Moreover, we found that Draper is required for the recruitment of DRK after injury because we could not detect up-regulation of DRK 1 d after axotomy in *drpr*-null (*drpr^{A5}*) animals (Fig. S4), suggesting that the activation of DRK/DOS/SOS requires Draper.

We next sought to determine if DRK expression was increased after antennal ablation, which leads to a dramatic increase in

Draper expression and hypertrophy of glial membranes (5). We labeled glial membranes with mCD8::GFP by using the *repo-Gal4* driver, and assayed glial morphology and DRK expression in control animals and animals where the third antennal segments had been ablated 1 d earlier. Consistent with our findings in the SOG, DRK immunoreactivity was dramatically increased around the antennal lobe 1 d after antennal ablation in control flies (Fig. 2B). The increase in DRK is likely a result of recruitment of DRK to glial membranes at sites of axon injury rather than up-regulation of *drk* gene transcription and/or translation, as DRK protein levels did not increase significantly after ablation of olfactory organs (Fig. S5). When we drove *drk^{RNAi}* in glia, there was a significant decrease of DRK immunoreactivity in the hypertrophic ensheathing glia (Fig. 2B, arrows) but not in neurons, confirming that the increase of DRK immunoreactivity comes from glia. Together, these results are consistent with the model that DRK acts downstream of Draper to promote engulfment of axonal debris.

DRK-SOS Activation Helps Glia to Internalize Axonal Debris and Activate the Phagolysosomal Program. After axotomy, glial membranes are recruited to severed axons, where they internalize and degrade axonal debris (7). As the first step to determine where DRK impinged on glial engulfment of axonal debris, we examined glial membrane dynamics after mp injury. In healthy, uninjured animals, glia usually wrap each glomerulus with their fibrous membranes. After axon injury, glia form large numbers of membranous vesicles throughout the field of degenerating axons (Fig. 3A); these vesicles often contain internalized axonal debris, presumably to become acidic thereafter for degradation (7). However, we found that, unlike in control animals, knocking down DRK in glia by using *repo-GAL4* resulted in a failure of glia to efficiently form enclosed vesicles 1 d after injury, with total vesicle number in *drk^{RNAi}* animals being reduced to ~30% of that found in the controls (Fig. 3B). DRK activity in glial membrane vesicle formation appears to act through SOS, as the *Sos^{JC2}/+* was sufficient to suppress the loss of glial membrane vesicles in the *drk^{RNAi}* background.

We next sought to determine whether reduced DRK function affected the ability of glia to internalize axonal debris. We labeled ensheathing glia with *UAS-mCD4::tdTomato* by using the *TIFR-Gal4* driver (7) and compared internalization of *OR85e-mCD8::GFP*-labeled ORN axons in control and *drk^{RNAi}* animals. Internalized axonal debris was scored when GFP⁺ axonal debris was found within tdTomato⁺ membrane-enclosed vesicles. Before injury, tdTomato-labeled glial membrane vesicles were not detectable in the OR85e⁺ glomerulus. However, in controls, 1 d after mp ablation, glial membranes invaded the glomerulus and tdTomato-labeled vesicles containing mCD8::GFP⁺ axonal materials were found throughout this glomerulus (Fig. 3C, arrows). Expression of *drk^{RNAi}* in TIFR⁺ glia significantly reduced the number of glial vesicles containing axonal debris compared with the control (Fig. 3D). Activating SOS signaling in the *drk^{RNAi}* background through GOF *Sos^{JC2}* resulted in glial internalization of axonal debris at WT levels. *Sos^{JC2}* itself did not increase the total number of glial vesicles, nor did it alter the number of vesicles internalizing degenerating axons, arguing that the rescuing effect did not result from a *Sos^{JC2}*-dependent general increase in vesicle number. We therefore concluded that DRK-SOS signaling plays an important role in forming phagocytic vesicles during glial engulfment of axonal debris.

After internalization of axonal debris, glia activate the phagolysosomal pathway to degrade engulfed axonal materials (23). To explore if activation of the phagolysosomal pathway was also affected by altered DRK-SOS signaling, we examined the formation of acidified phagolysosomes surrounding severed axons by using LysoTracker red DND-99. In control uninjured brains, the area surrounding OR85e⁺ axons did not exhibit detectable

Fig. 2. DRK is recruited to glial membranes surrounding the injured axons. (A) Endogenous DRK (red, α -DRK) in glia was recruited to the severed mp nerves labeled with *OR85e-mCD8::GFP* (green, α -GFP) 1 d after axotomy. Before injury, DRK expression was evenly distributed in the SOG but not colocalized with the OR85e⁺ maxillary nerve. One day after mp injury, DRK expression was increased around the degenerating maxillary nerves (arrowheads), which was not seen in *drk^{RNAi}* animals. Representative images (single slice) are shown. (Scale bar: 10 μ m.) (B) Endogenous DRK expression in glia was increased 1 d after axotomy. Glial membranes were labeled with mCD8::GFP by glia-specific *repo-Gal4* driver. Antennal ablation (removal of the third segment of antennae) was performed to induce a greater extent of axon degeneration in the antennal lobe (AL). Normally, thin glial membranes ensheath (arrows) the antennal lobe and each glomerulus (no injury, control). However, 1 d after antennal ablation, ensheathing glia became hypertrophy (1 d after injury, dashed lines), and strong DRK immunoreactivity (red) was found in hypertrophic region of ensheathing glia (yellow), but not when *drk^{RNAi}* was expressed by *repo-Gal4*, indicating that the increase of DRK is glia-specific. Representative images (single slice) are shown. C, cortex. (Scale bar: 10 μ m.)

Fig. 4. SOS modulates Rac1 activity in glial response to axonal injury. (A) *Sos^{JC2}* suppressed the inhibitory effects of DN Rac1 (*Rac1^{N17}*) on glial clearance of axonal debris. The clearance assay was performed as described in Fig. 1. (Scale bar: 30 μ m.) (B) Quantification of data in A ($n = 10$ for all). (C) *Sos^{JC2}* partially rescued *Rac1^{N17}* and increase glial membrane (red, α -Draper) recruitment to severed OR85e⁺ glomerulus (green, α -GFP) 1 d after axotomy. (Scale bar: 10 μ m.) (D) Quantification of Draper immunoreactivity at OR85e⁺ glomerulus in C. a.u., arbitrary unit. $n = 10$ for all.

a complementary GEF acting upstream of Rac1 might also play a role in glial response to axon injury. Based on the similarity of the phenotypes associated with inhibition of Crk/Mbc/dCed-12 and DRK/DOS/SOS (i.e., reduced vesicle formation, failure to activate the phagolysosomal pathway, and an axonal debris clearance defect), we speculated that Crk/Mbc/dCed-12 and DRK/DOS/SOS activation might act redundantly downstream of Draper to activate Rac1 not only at the early phases of the glial response (when glial membranes are recruited to severed axons), but also at later phases (during internalization and degradation of axonal debris). This predicts that simultaneous depletion of Crk/Mbc/dCed-12 and DRK/DOS/SOS should result in a complete suppression of glial activation. To test this model, we simultaneously knocked down SOS and MBC, both of which possess the enzymatic GEF activity for Rac1, specifically in adult glia, and examined the recruitment of glial membranes to the severed OR85e⁺ axons 1 d after injury (Fig. 5A). Interestingly, we found that *sos^{RNAi}* or *mbc^{RNAi}* resulted in a slightly reduced recruitment of Draper-decorated glial membranes to severed axons. However, when *sos^{RNAi}* and *mbc^{RNAi}* were simultaneously expressed, the recruitment of glial membrane to injured OR85e⁺ glomerulus was completely blocked 1 d after injury. The additive nature of the *sos^{RNAi}* and *mbc^{RNAi}* phenotypes also extended to clearance of axonal debris as well as glial hypertrophy response (Fig. S8). Thus, simultaneous blockade of DRK/DOS/SOS and Crk/Mbc/dCed-12 signaling phenocopied *drpr*-null mutants (5), and inhibition of Rac1 signaling (7).

We further explored if SOS activation could partially substitute for Crk/Mbc/dCed-12 during glial clearance of axonal debris by crossing *Sos^{JC2}* into *dCed-12^{RNAi}* background. As

previously shown (7), glial knockdown of dCed-12 function strongly suppressed glial clearance of axonal debris 5 d after injury (Fig. 5C). However, in a *Sos^{JC2/+}* background, the effect of *dCed-12^{RNAi}* was attenuated (Fig. 5D), indicating that activation of SOS can partially compensate for the reduced activity from Crk/Mbc/dCed-12 to promote glial clearance of axonal debris. We conclude that Crk/Mbc/dCed-12 and DRK/DOS/SOS act in a partially redundant fashion downstream of Draper to activate Rac1 and thereby glia, and promote glial clearance of axonal debris.

Discussion

In this work, we identify *Drosophila* DRK, DOS, and SOS as new molecules required for glial responses to axonal injury. We show that glial depletion of DRK, DOS, or SOS results in a delay in glial responses to ORN axotomy and reduced efficiency of glial internalization and digestion of axonal debris. We observe no obvious alterations in glial morphology or expression of engulfment machinery (e.g., Draper), and demonstrate that adult-specific knockdown of DRK/DOS/SOS leads to defects in glial clearance of degenerating axons. These data indicate that DRK/DOS/SOS promote engulfment signaling in mature glia, and argues against a developmental defect causing the phenotypes we observe. Based on our observation that a dominant GOF allele of SOS (*Sos^{JC2}*) can partially suppress depletion of DRK and DOS, we propose that SOS acts genetically downstream of DRK and DOS.

Our previous work demonstrated a key role for the *Drosophila* GEF Crk/Mbc/dCed-12 in glial engulfment activity, with elimination of this signaling complex from glia resulting in normal

Fig. 5. DRK/DOS/SOS and Crk/Mbc/dCed-12 are redundant pathways in activating glial clearance of axonal debris. (A) Simultaneous knockdown of SOS and Mbc completely blocked glial membrane (red, α -Draper) recruitment to injured OR85e⁺ glomerulus (green, α -GFP) 1 d after injury, whereas individual RNAi expression showed mild inhibitory effects. (Scale bar: 30 μ m.) (B) Quantification of data in A ($n = 10$ for all). (C) *Sos^{JC2}* partially rescued the clearance defect caused by *dCed-12^{RNAi}*. The clearance assay was performed as described in the other figures, and the results were quantified in D ($n = 10$ for all).

glial activation (e.g., recruitment of glial membranes to axonal debris), but a failure to engulf axonal debris (7). Here we provide strong evidence that DRK/DOS/SOS and Crk/Mbc/dCed-12 act redundantly downstream of Draper at two key steps in the engulfment process (Fig. S9). First, based on the fact that simultaneous depletion of both signaling complexes phenocopies Rac1 loss of function, we propose that these complexes act redundantly to activate Rac1 and glial responses, including Draper up-regulation and extension of glial membranes to degenerating axons. Second, after glia have arrived at axonal debris, both complexes are required for the elimination of axonal debris. At this step, DRK/DOS/SOS and Crk/Mbc/dCed-12 appear to act in a nonredundant fashion to promote glial internalization of axonal debris and activation of the phagolysosomal program for degradation of internalized axonal material.

DRK/DOS/SOS signaling has been studied most intensively for its role downstream of the RTK Sev, where it acts to activate small GTPase Ras (8–13). However, consistent with our findings, in vitro and in vivo studies have also demonstrated a role for SOS in activating Rac1. In cell culture studies, SOS stimulates guanine nucleotide dissociation from Rac1 but not Cdc42 (21). In some cases, such as axon guidance in the *Drosophila* embryo, SOS action as a Rac1 GEF is independent of Ras activation (14), but, in other situations, SOS activation of Rac1 is coupled to stimulation of Ras (21). Based on our observation that glial expression of a DN Ras only very weakly suppresses clearance of degenerating axons, SOS activation of Rac1 during glial responses to axonal injury is likely largely independent of Ras activation.

The increase of DRK localization to glial membrane processes engulfing axonal debris, and the consequences of DRK depletion by RNAi all argue for an early role for DRK (and likely DOS and SOS) in engulfment events. Although DRK is expressed in neurons and glia (see Fig. 2), our glial-specific RNAi clearly demonstrates that DRK function is required in glia during engulfment. Western blot analysis of the brain lysates before and after axotomy suggests the increase of DRK in the glial membrane processes is likely a result of recruitment of DRK other

than up-regulation of gene transcription/translation (Fig. S5). We also found that Draper is responsible for DRK localization to the site of injury, as DRK does not localize to severed nerves in *drpr*-null animals (Fig. S4), suggesting DRK/DOS/SOS activation requires Draper. It seems Draper activation or Draper-mediated recruitment of DRK is necessary for DRK/DOS/SOS to execute their functions, as we found *Sos*^{C2} failed to rescue the debris clearance defect in *drpr*-null animals (Fig. S10). Does DRK interact directly with Draper? Despite considerable efforts, we were unable to detect physical interactions between DRK and Draper. DRK may therefore interact very transiently or indirectly with Draper or DRK might associate with another yet unknown glial receptor that localizes to degenerating axonal materials.

The requirements we describe for DRK/DOS/SOS appear to be conserved in mammals in the context of phagocytic activity. Mammalian DRK (growth factor receptor-bound protein 2) is accumulated at the phagocytic cup during leukocyte phagocytosis (27). Mammalian DOS (Grb2-associated binding protein 2) also plays a critical role during Fcγ receptor-mediated phagocytosis (28). Recently, MEGF10 (mouse Draper) is expressed in mammalian astrocytes and essential for synaptic pruning during refinement of dorsal lateral geniculate nucleus connectivity (29). The work we present here also argues that DRK/DOS/SOS and Crk/Mbc/dCed-12 are exciting candidates downstream of MEGF10 to promote synaptic pruning.

Materials and Methods

Olfactory axon injury, adult brain dissection, sample preparation, and image analysis were previously described in ref. 5. Lysotracker staining in *Drosophila* adult brain was previously described in ref. 7. Fly strains and antibodies used in this study are provided in [SI Materials and Methods](#).

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