ABSTRACT

The second messengers cAMP and cGMP modulate attraction and repulsion mediated by neuronal guidance cues. We find that the Drosophila receptor guanylyl cyclase Gyc76C genetically interacts with Semaphorin 1a (Sema-1a) and physically associates with the Sema-1a receptor plexin A (PlexA). PlexA regulates Gyc76C catalytic activity in vitro, and each distinct Gyc76C protein domain is crucial for regulating Gyc76C activity in vitro and motor axon guidance in vivo. The cytosolic protein dGIPC interacts with Gyc76C and facilitates Sema-1a-PlexA/Gyc76C-mediated motor axon guidance. These findings provide an in vivo link between semaphorin-mediated repulsive axon guidance and alteration of intracellular neuronal cGMP levels.

KEY WORDS: Receptor guanylyl cyclase, Gyc76C, cGMP, Semaphorin-1a, Plexin A, dGIPC, Axon guidance

INTRODUCTION

Both membrane-associated and secreted neuronal guidance cues can attract or repel axons and dendrites during neural development, and several families of guidance cues and receptors perform these functions (Dickson, 2002; Kolodkin and Tessier-Lavigne, 2011). Modulation of guidance cue activities through intracellular signaling components determines how extrinsic factors are interpreted by extending neuronal processes during development (Bashaw and Klein, 2010). For example, growth cone turning experiments in vitro demonstrate that attraction mediated by the guidance cue netrin-1 can be converted to repulsion by lowering intracellular cAMP (Ming et al., 1997), whereas repulsion mediated by the guidance cue Semaphorin 3A (Sema-3A) can be converted to attraction by increasing intracellular cGMP (Song et al., 1998). Elevated cAMP in cultured DRG neurons neutralizes Sema-3A growth cone collapse, whereas elevated cGMP potentiates it (Dontchev and Letourneau, 2002). The ratio of cAMP to cGMP can determine the sign of a growth cone steering response (Nishiyama et al., 2003), and Sema-3A induces cGMP production in neuronal growth cones, activating of cGMP-gated calcium channels (CNGCs), Ca\(^{2+}\) influx and repulsion (Togashi et al., 2008). cAMP and cGMP regulate kinases and phosphodiesterases to direct formation of axons or dendrites in cultured hippocampal neurons (Shelly et al., 2010). Therefore, coordination of cAMP and cGMP signaling regulates cellular responses to different stimuli in the neurons.

Guanylyl cyclases (GCs) include soluble and transmembrane proteins that catalyze the conversion of GTP to cGMP, and they regulate a wide range of diverse cellular and physiological processes (Davies, 2006), including axonal and dendritic guidance (Polleux et al., 2000; Seidel and Bicker, 2000; Gibbs et al., 2001; Nishiyama et al., 2003). The mammalian receptor guanylyl cyclase GC-B and cGMP-dependent kinase I (cGKI) are essential for proper sensory axon afferent guidance into the CNS, and C-type natriuretic peptide is the GC-B ligand that is crucial for murine sensory axon branching, axon outgrowth and axon attraction (Schmidt et al., 2009; Zhao and Ma, 2009). Yet, how GCs are linked to axon guidance signaling to alter intracellular cGMP levels and modulate growth cone responses in vivo is unclear.

The Drosophila transmembrane semaphorin Sema-1a binds to the plexin A (PlexA) receptor to mediate axon-axon repulsion and to control axonal fasciculation in embryonic central and peripheral nervous systems (CNS and PNS) (Winberg et al., 1998b; Yu et al., 1998). The Drosophila receptor GC Gyc76C is required in motoneurons for Sema-1a-PlexA-mediated axon guidance and is dependent on the integrity of the Gyc76C catalytic cyclase domain (Ayoob et al., 2004). Here, we investigate connections between Gyc76C and Sema-1a-PlexA-mediated axon guidance. Our findings support the theory that Gyc76C-generated cGMP within neuronal growth cones facilitates axonal repulsion mediated by Sema-1a and PlexA, allowing for the establishment of Drosophila embryonic neuromuscular connectivity.

RESULTS

Gyc76C suppresses Sema 1a-mediated motor axon repulsion

Gyc76C mutations act as dominant enhancers of a Sema-1a-dependent gain-of-function phenotype that affects CNS commissural axon midline crossing in Drosophila embryos (embryos with this genotype are referred to as ‘PUP’ for the genetic elements in this background) (Ayoob et al., 2004). Altering Gyc76C gene dose modifies a PlexA-dependent gain-of-function phenotype in CNS longitudinal connective axons. Further, Gyc76C mutant embryos exhibit motor axon guidance defects similar to Sema-1a mutant embryos, and Gyc76C genetically interacts with Sema-1a and Plexa (Ayoob et al., 2004). These data suggest Gyc76C functions in Sema-1a-PlexA-mediated motor axon guidance. However, the PUP phenotypes are observed in a Sema-1a-null genetic background in which Sema-1a is ectopically expressed on CNS midline glia. However, the Gyc76C gain- and loss-of-function phenotypes observed previously in motor axons (Ayoob et al., 2004) do not allow for unequivocal assessments of responses to Sema-1a in trans independent of roles Sema-1a and Gyc76C might play in axon-axon interactions. Therefore, we employed a different Sema-1a gain-of-function paradigm to investigate Gyc76C-mediated repulsive signaling in motor axons in response to Sema-1a ligand presented in trans.

Sema-1a is enriched in Drosophila embryonic neurons and mediates axonal repulsion, ensuring proper axon pathfinding (Winberg et al., 1998b; Yu et al., 1998; Ayoob et al., 2004; Cho et
al., 2012; Jeong et al., 2012). During neural development, motor axons exit the CNS in two large bundles that include multiple motor axons which then segregate into smaller motor nerves: the intersegmental nerves (ISNs: ISNb and ISNd) and the segmental nerves (SNs: SNa and SNC) (Landgraf et al., 1997). The fasciclin II antibody 1D4 labels all motor axons, revealing stereotypic embryonic neuromuscular connectivity (Greuningloh et al., 1991; Van Vactor et al., 1993). ISNb axons defasciculate from the main ISN bundle and navigate along ventral longitudinal muscles, including muscles 6, 7, 12 and 13, to innervate appropriate targets (Van Vactor et al., 1993; Landgraf et al., 1997). At each choice point, the ISNb bundle extends nascent projections anteriorly and posteriorly between muscles, establishing initial presynaptic contacts with target muscles (i.e. RP3 and RP5 motor axons leave the ISNb, then innervate muscles 6 and 7, and muscles 12 and 13, respectively) (Fig. 1A).

We ectopically expressed Sema-1a in all embryonic muscles using the Mef2-Gal4 driver (Ranganayakulu et al., 1996). Since Sema-1a is a motor axon repellent (Winberg et al., 1998a; Winberg et al., 1998b; Yu et al., 1998; Yu et al., 2000), we anticipated that Sema-1a-expressing muscles would influence ISNb axons (Fig. 1A, red circles). Removal of a signaling component involved in Sema-1a-mediated axon guidance should suppress, or enhance, gain-of-function phenotypes resulting from muscle-derived Sema-1a. We confirmed expression of Sema-1a in both UAS:Sema-1a/+; Mef2-Gal4/+ embryos (Fig. 1B) and UAS:Sema-1a/+; Mef2-Gal4/+; Gyc76Cex173/+; embryos (K.C. and A.L.K., unpublished) at embryonic stage 16 by immunohistochemistry with anti-Sema-1a (Yu et al., 1998). We observed a range of ISNb stalling and axon pathfinding defects categorized into five distinct phenotypes (see Table 1): (1) aberrant projection onto muscle 12 (M12); (2) stalling between muscles 12 and 13 with no accompanying arborization (M12/13); (3) stalling at muscle 13, or between muscles 13 and 6 (M13, M13/6); (4) no presynaptic arborizations between muscles 6 and 7 (M6/7); and (5) distinct ISNb defects, including bypasses (BPs) and also axon-positioning defects (PDs). UAS:Sema-1a/+; Mef2-Gal4/+ embryos exhibited ISNb defects in 54.0% of hemisegments; the majority of these defects were ISNb axon bundles stalled between muscles 12 and 13 (25.8%) (Table 1). Axon pathfinding defects, including those at M13 and M13/6 or at M6/7, were observed in 7.0% and 9.4% of hemisegments, respectively (Table 1). A significant number of ISNb BPs (8.0% of hemisegments) and PDs (8.4% of hemisegments) were observed in this Sema-1a gain-of-function paradigm (Fig. 1C, open arrowheads). ISNb BP events, including fusion and parallel bypasses, indicate a failure of the ISNb to innervate the entire ventral muscle field, resulting in ISNb dorsal extension along, or directly adjacent to, the ISN (Lin et al., 1994; Desai et al., 1996; Yu et al., 1998; Wills et al., 1999). The observed PDs are distinct ISNb pathfinding defects where the ISNb bundle does not deviate from the ISN bundle but RP3 or RP5 neurons still innervate target ventral muscles (Fig. 1C: open arrowhead, BP; asterisk, muscle innervation). Therefore, Sema-1a presented in trans on muscles acts as a motor axon repellent.

Removing one copy of Gyc76C produced significant reductions in total ISNb defects (Fig. 1C, open arrows), from 54.0% to 29.5% (Fig. 1D; Table 1; P<0.005). Furthermore, both BPs and PDs were suppressed: from 8% to 1.2%, and from 8.4% to 3.2%, respectively, in UAS:Sema-1a/+; Mef2-Gal4/+; Gyc76Cex173/+ embryos. We observed similar suppression of these same phenotypes when one copy of PlexA was removed in this gain-of-function paradigm (UAS:Sema-1a/+; Mef2-Gal4/+; PlexA Df(4)C3/+; (Fig. 1D and Table 1). Furthermore, UAS:Sema-1a/+; Gyc76Cex173/+ embryos did not deviate from the ISN bundle but RP3 or RP5 neurons still innervate target ventral muscles. Z-test for two proportions defines significant differences between genotypes; *(P<0.005 see Table 1 for n values).
exhibit a higher penetrance of motor axon defects than UAS:Sema-1a/+ embryos (Fig. 1D and Table 1), indicating the presence of a modest dominant phenotype resulting from heterozygosity at Gyc76Cex173. The strong suppression of Sema-1a gain-of-function motor axon phenotypes by removing one Gyc76C or PlexA allele suggests Gyc76C participates in Sema-1a-PlexA-mediated repulsive guidance in vivo.

**Gyc76C physically associates with PlexA both in vitro and in vivo**

We next performed co-immunoprecipitation (co-IP) experiments in vitro using a Drosophila embryonic cell line, and in vivo using transgenic fly lines. Myc-tagged full-length (FL) Gyc76C (Myc-FL Gyc76C) and/or hemagglutinin (HA)-tagged FL PlexA (HA-FL PlexA) were overexpressed in the adherent Drosophila S2R+ cell line (Schneider, 1972). Immunoprecipitation of Myc-FL Gyc76C using anti-Myc robustly co-immunoprecipitated HA-FL PlexA (Fig. 2A, lane 1). Immunoprecipitation of HA-FL PlexA using anti-HA precipitated Myc-FL Gyc76C (Fig. 2B, lane 3), and HA-FL PlexA or Myc-FL Gyc76C was not immunoprecipitated by anti-Myc or anti-HA, respectively (Fig. 2A, lane 3 and Fig. 2B, lane 2). We did not detect interactions between HA-FL PlexA and Myc-tagged dumbfounded (Duf), an immunoglobulin domain transmembrane protein required for muscle fusion (Ruiz-Gómez et al., 2000), in developing embryos (supplementary material Fig. S1). In embryonic lysates generated from these transgenic flies, we observed robust co-IP of Myc-FL Gyc76C using anti-HA (Fig. 2C, lane 2) only in flies expressing both HA-FL PlexA and Myc-FL Gyc76C. Therefore, Gyc76C and PlexA can form a protein complex both in vitro and in vivo.

To define region(s) of Gyc76C and PlexA that mediate association between these proteins, we generated three truncated HA-PlexA constructs: the PlexA extracellular region with transmembrane domain (HA-PlexA Ecto(TM)), the PlexA intracellular region with transmembrane domain (HA-PlexA Endo(TM)) and the PlexA intracellular region without transmembrane domain (HA-PlexA Endo). Each truncated PlexA construct was expressed with, or without, Myc-FL Gyc76C in S2R+ cells. Myc-FL Gyc76C immunoprecipitated each of these HA-PlexA constructs (Fig. 2D, lanes 5-8). Since PlexA constructs were expressed at different levels, we normalized each interaction between Gyc76C and PlexA to the corresponding PlexA expression level for that construct; this shows that the extracellular region of PlexA alone interacts with Gyc76C as strongly as FL PlexA, but suggests that the intracellular associations, though present, may be weaker (Fig. 2D; K.C. and A.L.K., unpublished). These experiments show that Gyc76C and PlexA can physically associate and suggest this association involves both extracellular and intracellular regions of these proteins.

**Full-length Gyc76C exhibits guanylyl cyclase activity in vitro, and this activity is influenced by each Gyc76C protein domain**

We generated a series of N-terminally Myc-tagged Gyc76C deletion constructs, each including one or two discrete protein domain deletions (Fig. 3A). Each of these Gyc76C constructs was expressed robustly in S2R+ cells (supplementary material Fig. S2A). Using a direct cGMP enzyme immunoassay (EIA) (Materials and methods), we measured total cGMP levels in S2R+ cells expressing wild-type Myc-FL Gyc76C or Myc-Gyc76C[D945A], in which a crucial amino acid (D945) in the guanylyl cyclase domain required for catalytic activity and semaphorin-mediated axon guidance in Drosophila (Thompson and Garbers, 1995; Ayyob et al., 2004) is mutated. We observed significant cGMP levels in S2R+ cells expressing Myc-FL Gyc76C but no detectable cGMP in cells expressing Myc-Gyc76C[D945A] (Fig. 3B), confirming Drosophila Gyc76C functions as a guanylyl cyclase.

Next, we assessed Gyc76C deletion constructs and found that each Gyc76C protein domain influences Gyc76C-mediated cGMP production in vitro (Fig. 3B). The Myc-Gyc76C construct that lacks the entire cytoplasmic domain (ΔEndo) did not produce cGMP levels above background (Fig. 3B). Deletion of the PDZ-binding motif (PBM) (APBm, deleting the four C-terminal amino acids) completely abolished Gyc76C guanylyl cyclase (GC) activity. Deletion of the entire extracellular domain (ΔEcto) resulted in elevated cGMP production compared with FL Gyc76C, and this was abolished when either the TM, or the PBM, was also deleted. Deletion of the KHD (ΔKHD) resulted in greatly elevated GC activity compared with FL Gyc76C, consistent with previous studies.
demonstrating that the mammalian KHD negatively regulates cGMP production (Chinkers and Garbers, 1989; Potter, 2011). cGMP levels produced by Gyc76C lacking both KHD and PBM domains (∆KHD+PBM) were greatly reduced compared with ∆KHD alone; however, these cGMP levels were significantly higher than FL Gyc76C. Deletion of the dimerization domain (ADD) abolished all GC, like vertebrate receptor GCs (Chinkers and Garbers, 1989). Finally, deletion of the unique Gyc76C C-terminal domain (∆Cterm) yielded very low cGMP levels, and deletion of both Cterm and PBM abolished GC activity (Fig. 2B).

Protein expression levels and localization could contribute to differences in GC activity. We assessed total protein levels for all Gyc76C constructs using western blot analyses (supplementary material Fig. S2A), and also determined cell surface protein expression levels using live cell surface immunostaining (supplementary material Fig. S2B). Some of these modified Gyc76C proteins exhibited different total protein expression levels, and also some showed more robust cell surface localization than others. For example, both ∆Ecto and AKHD proteins are robustly localized to the cell surface (supplementary material Fig. S2B, top) and exhibit high GC activity (~5-10 times FL Gyc76C). However, total protein expression levels produced by these constructs are comparable with, or lower than, FL Gyc76C (supplementary material Fig. S2A, lanes 2, 4 and 7). Several constructs that show low-to-no GC activity do show robust cell surface localization (Gyc76C[D945A], ∆Cterm, ∆Cterm+PBM and ∆Endo; supplementary material Fig. S2B).

Deletion of the PBM alone, or with the Ecto or KHD domain, does not significantly alter total protein expression levels compared with FL Gyc76C (supplementary material Fig. S2A, lanes 2, 5, 8, and 13); however, it does reduce cell surface protein localization (supplementary material Fig. S2B, middle). Nevertheless, ∆KHD+PBM shows robust GC activity (~3 times FL Gyc76C). We observed low cell surface protein localization and total protein for the ADD construct (supplementary material Fig. S2B, middle). For ∆Ecto-TM, total protein levels are high; however, as expected, this variant shows no cell surface localization (supplementary material Fig. S2B, bottom). Biochemical assessments of cell-surface protein levels employing biotinylation of cell-surface proteins revealed protein levels for each Gyc76C deletion variant commensurate with cell-surface labeling experiments (K.C. and A.L.K., unpublished). Therefore, though there are differences in total protein expression and cell-surface localization of Gyc76C variants, except for ADD, these do not account for the differences in GC activity we observe among these Gyc76C proteins. ∆Cterm and ∆Cterm+PBM show robust total
and cell-surface protein expression but produce no cGMP, suggesting that specific molecular mechanisms regulate Gyc76C cyclase activity. These experiments show that each Gyc76C protein domain influences cGMP production and suggest Gyc76C GC activity is regulated by both extracellular and intracellular mechanisms.

Gyc76C protein domain requirements for motor axon guidance rescue in Gyc76C mutant embryos

To investigate Gyc76C protein domain function in vivo, we generated 12 different Gyc76C transgenic fly lines (two independent fly lines per transgene), each expressing an altered Gyc76C lacking a different protein domain (ΔEcto, ΔKHD, ΔDD, ΔCterm, ΔEndo and ΔPBM). Each transgene included an N-terminal Myc, and we drove neuronal expression of these transgenes to determine the ability of each to rescue Gyc76C motor axon defects (Fig. 4A). Gyc76C protein expression levels in each transgenic line, assessed using quantitative western blot analysis, revealed that these constructs vary somewhat in their expression levels. However, each pair of independent transgenic lines harboring the same Gyc76C deletion construct showed comparable expression, and in all cases total protein levels were either equal to, or significantly greater than, those observed for FL Gyc76C (supplementary material Fig. S3A). Therefore, variations in expression of the deletion constructs are not due to positional effects on transgene expression. Similar to ISNb motor axon pathways, SNa axons also display stereotypic neuromuscular connectivity; they navigate past the ventral longitudinal muscle field to innervate lateral transverse muscles 22, 23 and 24 (Landgraf et al., 1997), and together with the ISNb allow for assessment of motor axon guidance in vivo (Araújo and Tear, 2003). Gyc76C mutant embryos show significant defects in these motor axon pathways (Ayoob et al., 2004). These defects are not a secondary consequence of longitudinal muscle defects because overall muscle organization in Gyc76C mutant embryos is apparently normal (supplementary material Fig. S3B,B'). Furthermore, Gyc76C expression in somatic muscles fails to rescue motor axon guidance defects in Gyc76C homozygous mutants (K.C. and A.L.K., unpublished).

The only Gyc76C transgenic flies that rescue the ISNb and SNa defects observed in Gyc76C-null mutants are the two independent FL Gyc76C transgenes [FL(65) and FL(5.2)]. In these flies, ISNb and SNa pathway defects observed in Gyc76Cex173-null mutants were rescued from 42% to 21%, and from 39% to 20%, respectively (Fig. 4A, red highlight; P<0.005; comparable with previous experiments; Ayoob et al., 2004). All other Gyc76C deletion constructs failed to rescue ISNb or SNa defects (Fig. 4A). ΔDD, ΔCterm, ΔEndo and ΔPBM exhibit no GC activity in in vitro GC assays, and so elevated expression levels of proteins encoded by these constructs, compared with FL Gyc76C (supplementary material Fig. S3B, lanes 6-13), are not likely to result in elevated
cGMP production. By contrast, ΔEcto and ΔKHD exhibit the highest in vitro GC activities, yet these two constructs do not rescue the motor axon defects in Gyc76C mutants; ISNb and SNa defects are phenotypically comparable, both quantitatively and qualitatively, with those observed in Gyc76C-null mutants (Ayoob et al., 2004), suggesting that motor axon pathfinding defects in unrescued embryos are not the result of dominant-negative effects from Gyc76C variants.

Taken together, these results suggest that Gyc76C cyclase activity is regulated by both extracellular and intracellular protein domains. The unique Gyc76C C terminus, and also the PBM, are crucial for normal cyclase activity in vitro, suggesting that signaling components exist that interact with Gyc76C and regulate its function.

PlexA augments cGMP levels produced by Gyc76C in vitro

We next investigated whether the PlexA receptor influences Gyc76C GC activity, employing the direct cGMP EIA assay described above and a different Drosophila cell line, DmBG2, to assess whether or not a functional relationship exists between Gyc76C and PlexA. Unlike S2R+ cells, which are derived from a macrophage-like lineage, DmBG2 cells are derived from the Drosophila third instar larval CNS (Yanagawa et al., 1998). A constant amount of the construct encoding FL Gyc76C DNA (or CD8-GFP as a control), 0.04 μg, was co-transfected into DmBG2 cells with 0.2 μg (5×), 0.04 μg (1×) or 0.008 μg (1/5×) of the construct encoding FL PlexA DNA. The amount of PlexA DNA transfected into DmBG2 cells in these experiments directly correlates with PlexA protein levels (supplementary material Fig. S4, top panel). When fivefold more PlexA DNA compared with Gyc76C DNA was transfected into DmBG2 cells, total cGMP levels were increased by ~2.4-fold over what we observed for Gyc76C alone (Fig. 5A, representative experiment; Fig. 5B, average fold change, four independent experiments). In the absence of transfected FL Gyc76C, FL PlexA alone had no significant effect on cGMP levels in DmBG2 cells. Different amounts of PlexA DNA transfected into DmBG2 cells in these experiments did not affect Gyc76C protein expression levels (supplementary material Fig. S4, bottom panel, lanes 1-4). We performed similar GC assays with ΔEcto, ΔKHD or ΔPBM; however, 5×PlexA did not increase GC activity of these constructs (K.C. and A.L.K., unpublished). PlexA-mediated augmentation of Gyc76C GC activity is consistent with our genetic analyses showing that Gyc76C influences Sema-1a gain-of-function phenotypes in the PNS (this study), and also PlexA-dependent gain-of-function phenotypes in the CNS (Ayoob et al., 2004). Supporting a model whereby Gyc76C facilitates Sema-1a/PlexA-mediated axon repulsion.

A PDZ domain-containing protein, dGIPC, interacts with Gyc76C and increases cell surface expression of Gyc76C in vitro

Our in vitro and in vivo results suggest that there may be PDZ (PSD95/Dlg1/ZO-1) domain-containing proteins that interact with...
Gyc76C and regulate its function. We used the C-terminal 75 amino acids of Gyc76C, which include the PDZ-binding motif, as a bait (KC1) to search for Gyc76C-interacting proteins, screening a Drosophila embryonic (0-24 hours) yeast two-hybrid (Y2H) cDNA library (supplementary material Fig. S5A; Materials and methods). Clones encoding interacting proteins were further examined using another bait (KC2) that is similar to KC1 but lacks the PDZ-binding motif (supplementary material Fig. S5A). We found that clones that interacted with KC1 but not KC2 encoded the Drosophila GAIP interacting protein, C terminus homolog (dGIPC; Kermit – FlyBase) (Djiane and Mlodzik, 2010).

There are three mammalian, two Xenopus and one Drosophila GIPC. GIPC is an intracellular protein with a centrally located PDZ domain and no other conserved sequence motifs. GIPC interacts with RGS-GAIP (regulator of G protein signaling-GTPase activating protein for Gαi) (De Vries et al., 1998) and also with other GIPCbinding partners (Cai and Reed, 1999; Wang et al., 1999; Lou et al., 2001). GIPC is implicated in regulating the distribution of the Sema-5A protein (Wang et al., 1999) and NMDA receptor trafficking (Yi et al., 2007) in vitro. The Drosophila GIPC homolog, dGIPC, was first described in a gain-of-function screen designed to identify planar cell polarity genes in Drosophila (Djiane and Mlodzik, 2010). dGIPC is also important for locomotor activity and longevity, possibly through the regulation of dopamine (DA) receptor trafficking (Kim et al., 2010).

We performed in vitro co-IP experiments, expressing HA-dGIPC with Myc-FL Gyc76C, Myc-ΔPBM, Myc-ΔCterm, Myc-ΔCterm+PBM, Myc-ΔEndo or GFP in S2R+ cells. Immunoprecipitation of HA-dGIPC with anti-HA revealed a robust interaction between Gyc76C and dGIPC. This was greatly attenuated when the Gyc76C PBM domain was removed (Fig. 6A, lanes 1 and 2, asterisk), and was completely abolished when the Gyc76C C-terminal region, the Gyc76C C-terminal region plus the PBM or the entire intracellular region of Gyc76C was deleted (Fig. 6A, lanes 3-5). These experiments demonstrate that dGIPC interacts with Gyc76C in vitro, requiring the PBM domain and, to a much lesser extent, other Gyc76C C-terminal sequences.

Since mammalian GIPC regulates the distribution of several transmembrane proteins (Wang et al., 1999; Tan et al., 2001; Yi et al., 2007), we asked whether dGIPC influences cell surface localization of Gyc76C. Using biotinylation cell surface protein labeling assays, we found a significant increase in the levels of Gyc76C protein localized to the plasma membrane of S2R+ cells overexpressing dGIPC (Fig. 6B, lanes 2 and 4). This result suggests that dGIPC regulates Gyc76C GC activity by influencing Gyc76C cell surface distribution.
dGIPC is required for embryonic motor axon guidance and genetically interacts with Gyc76C, Sema1a and PlexA

We next examined dGIPC function in motor axon guidance and observed significant defects in the ISNb (46.1%) and SNa (31.5%) motor axon pathways in embryos trans-heterozygous for two chromosomal deficiency lines (Df7890 and Df8941) that remove nine genes, including dGIPC (K.C. and A.L.K., unpublished). The dGIPC<sup>ex31</sup> allele does not express dGIPC protein (Djiane and Mlodzik, 2010), and dGIPC<sup>ex31</sup> homozygous embryos show pathfinding defects in ISNb (35.5%) and SNa (30.4%) pathways; dGIPC<sup>ex31</sup>/Df8941 embryos exhibit 46.2% and 20.8% pathfinding defects in ISNb and SNa pathways, respectively. These phenotypes are qualitatively and quantitatively similar to those observed in dGIPC<sup>ex31</sup> homozygous embryos. Furthermore, the predominant pathfinding defects are qualitatively similar to those we observed in Gyc76C homozygous mutant embryos, though the penetrance of SNa defects is somewhat lower. Together, these results show that the dGIPC<sup>ex31</sup> allele is likely a null, or strong hypomorphic, allele of dGIPC.

Embryos heterozygous for dGIPC and either Gyc76C, Sema-1a or PlexA, were assessed for trans-heterozygous genetic interactions to determine whether or not these genes function in the same genetic pathway (Artavanis-Tsakonas et al., 1995; Winberg et al., 1998b). dGIPC<sup>ex31</sup>+/+/ Gyc76C<sup>ex173</sup>+/+ embryos show 48.5% and 23.1% defects in ISNb and SNa pathways, respectively (Fig. 7A). dGIPC also genetically interacts with Sema-1a and PlexA, as observed in dGIPC<sup>ex31</sup>+/+; Sema-1a<sup>Δ3</sup>/+ and dGIPC<sup>ex31</sup>+/+; PlexA<sup>Δ9(45)C</sup>/+ embryos (Fig. 7A). In these embryos, ISNb axons often fail to innervate their muscle targets with a penetrance comparable to that observed in dGIPC<sup>ex31</sup> homozygotes.

Qualitatively and quantitatively these trans-heterozygous mutant embryos display ISNb phenotypes similar to dGIPC or Gyc76C homozygous mutant embryos, including stalling defects at M12/13 and at M6/7 (Fig. 7A). However, in these embryos we observe only mild SNa defects (Fig. 7A), suggesting that alterations in signaling in these trans-heterozygous embryos are not strong enough to affect all motor axon pathways. We also analyzed motor axon pathways in dGIPC<sup>ex31</sup>; Gyc76C<sup>ex175</sup> double null mutants and observed that the penetrance of ISNb defects is similar to that observed in dGIPC<sup>ex31</sup> or Gyc76C<sup>ex173</sup> homozygous single mutants, whereas the penetrance of SNa defects is similar to dGIPC<sup>ex31</sup> homozygous mutants (Fig. 7A, red box). These data suggest that dGIPC and Gyc76C function in the same genetic pathway, and together our genetic analyses support dGIPC functioning with Gyc76C in Sema-1a/PlexA-mediated repulsion.

Neuronal dGIPC is required for embryonic motor axon pathfinding

dGIPC exhibits strong expression in CNS midline glia of the Drosophila ventral nerve cord (VNC) (Djiane and Mlodzik, 2010). In situ hybridization (ISH) with a dGIPC-specific cRNA antisense probe, and immunohistochemistry (IHC) using an antibody directed against dGIPC, revealed enriched dGIPC in the midline region of VNC wild-type embryos. This midline staining was absent when a dGIPC sense probe was used in ISH (K.C. and A.L.K., unpublished), or when dGIPC<sup>ex31</sup>-null embryos were used in IHC experiments (Djiane and Mlodzik, 2010). However, we also detected weak, but significant, dGIPC protein expression in ventral nerve roots exiting the VNC (supplementary material Fig. S5B, red arrowheads) that is not present in dGIPC-null mutants (supplementary material Fig. S5B), dGIPC-null mutants display motor axon defects, and so dGIPC could function in neurons, non-neuronal cells, or both. In the Drosophila brain, dGIPC is predominantly expressed in glial cells but is also expressed in DA neurons, and DA neuronal expression of dGIPC is crucial for locomotor activity (Kim et al., 2010).

To determine dGIPC cell type requirements, we employed Gal4 drivers for in vivo rescue experiments dGIPC<sup>ex31</sup> homozygous mutant embryos. We expressed dGIPC in all neurons using elav-Gal4 in dGIPC<sup>ex31</sup>-null mutant embryos and found significant rescue of both ISNb and SNa motor axon guidance defects (Fig. 7B,B′): from 35.5% to 17.7% and from 30.4% to 15.1%,
respectively (Fig. 7B). When dGIPC was expressed in all glial cells using the repo-Gal4 (Sepp et al., 2001) driver, or in midline glial cells and MP1 CNS neurons using the single-minded GAL4 (sim-GAL4) driver (Hidalgo and Brand, 1997), no rescue of ISNb defects (36.3% for repo-Gal4; 31.9% for sim-Gal4), were observed in repo-Gal4; dGIPCex31; 20.5% for sim-Gal4 (21.8% for sim-GAL4)) driver (Hidalgo and Brand, 1997), no rescue of ISNb mutants. However, neuronal expression of dGIPC driven by cell type-specific Gal4 drivers. **Significantly different from dGIPCex31 homozygous mutants. Z-test for two proportions; P<0.005. N.S., not significantly different from values for dGIPCex31 homozygous mutants. (B’;B″) Filleted late stage 16 embryos stained with 1D4 revealing motor axons in abdominal segments. In a dGIPCex31 homozygous mutant embryo, axons within the ISNb (black arrows) and SNa (black arrowheads) pathways often fail to reach their proper targets. Scale bar: 5 μm. (B) Quantification of rescue experiments using dGIPC driven by cell type-specific Gal4 drivers. **Significantly different from dGIPCex31 homozygous mutants. Z-test for two proportions; P<0.005. N.S., not significantly different from values for dGIPCex31 homozygous mutants. (B’;B″) Filleted late stage 16 embryos stained with 1D4 revealing motor axons in abdominal segments. (B’) Neuronal dGIPC in a dGIPCex31 mutant restores proper ISNb (black arrows) and SNa (black arrowhead) muscle innervation. (B”) Glial expression of dGIPC does not rescue ISNb (open arrows) or SNa muscle innervation (open arrowhead). Scale bar: 5 μm. Anterior is leftwards; dorsal is upwards.

**DISCUSSION**

We provide here support for the Drosophila receptor guanylyl cyclase Gyc76C being a component of the Sema-1a-PlexA signaling cascade in vivo. Each discrete Gyc76C protein domain is essential for Gyc76C catalytic activity in vitro and for motor axon guidance in vivo. Furthermore, the cytosolic protein dGIPC interacts with Gyc76C and functions in Gyc76C-mediated motor axon guidance. These results provide an in vivo link between semaphorin-mediated repulsive guidance and alteration of intracellular cGMP levels.

**Gyc76C is part of the Sema-1a-PlexA signaling complex**

The Gyc76C-Sema-1a gain-of-function genetic interactions we observe here are consistent with previous observations showing that Gyc76C loss and gain of function modifies aberrant CNS midline crossing by FasII” longitudinal axons in a PlexA gain-of-function genetic background (Ayoob et al., 2004). Furthermore, we observe robust physical interactions between Gyc76C and PlexA both in vitro and in vivo, raising the possibility that PlexA regulates Gyc76C-mediated signaling. Co-expressing PlexA at high levels in vitro augments cGMP levels produced by Gyc76C. Future work will...
establish whether extracellular, intracellular, or both, types of protein-protein associations between Gyc76C and PlexA are essential for regulating Gyc76C enzymatic activity.

Each Gyc76C domain is required for cyclase activity and proper motor axon guidance

Our Gyc76C structure-function analyses are consistent with the idea that PlexA binds to the extracellular and intracellular regions of Gyc76C to relieve inhibitory effects on GC activity from of Gyc76C intramolecular interactions, increasing cGMP levels within extending motor axon growth cones and affecting growth cone guidance. This is reminiscent of Sema-3A bath application increasing intracellular cGMP levels in Xenopus spinal neurons in vitro (Togashi et al., 2008), and our results suggest that intracellular cGMP produced by Gyc76C is required for Sema-1a-mediated repulsion. However, it is possible that signaling by intracellular cGMP is coupled with intracellular cAMP in Sema-1a-mediated repulsive guidance events (Nishiyama et al., 2003), and future work will determine whether varying the cAMP-to-cGMP ratio modulates Sema-1a-mediated repulsion, or converts it to attraction. Bath application of Sema-1a did not affect Gyc76C-PlexA physical associations or Gyc76C-PlexA-mediated cGMP production (K.C. and A.L.K., unpublished), suggesting that Sema-1a-dependent regulation of intracellular cGMP levels could involve ligand-gated, dynamic, spatiotemporal regulation of GC activity; visualizing this signaling event will require real-time imaging of cGMP during repulsive growth cone steering.

The small GTPase Rac and its downstream effector p21 activated kinase (PAK) can regulate receptor GCs to raise cellular cGMP levels in fibroblasts in vitro (Togashi et al., 2008), and our results suggest that intracellular cGMP produced by Gyc76C is required for Sema-1a-mediated repulsion. However, it is possible that signaling by intracellular cGMP is coupled with intracellular cAMP in Sema-1a-mediated repulsive guidance events (Nishiyama et al., 2003), and future work will determine whether varying the cAMP-to-cGMP ratio modulates Sema-1a-mediated repulsion, or converts it to attraction. Bath application of Sema-1a did not affect Gyc76C-PlexA physical associations or Gyc76C-PlexA-mediated cGMP production (K.C. and A.L.K., unpublished), suggesting that Sema-1a-dependent regulation of intracellular cGMP levels could involve ligand-gated, dynamic, spatiotemporal regulation of GC activity; visualizing this signaling event will require real-time imaging of cGMP during repulsive growth cone steering.

The small GTPase Rac and its downstream effector p21 activated kinase (PAK) can regulate receptor GCs to raise cellular cGMP levels in fibroblasts in vitro, and the kinase domain of PAK interacts with the cyclase domain of receptor GC-E (Guo et al., 2007; Guo et al., 2010); PAK, therefore, may associate with Gyc76C and regulate this receptor GC during axon pathfinding.

dGIPC interacts with Gyc76C and is required for motor axon guidance in Drosophila

The deletion of the Gyc76C PBM strongly suppresses cGMP production by FL Gyc76C, and Gyc76C variants lacking the PBM motif exhibit low cell-surface expression levels, suggesting dGIPC regulates Gyc76C cell-surface localization. PDZ-containing proteins could form a protein scaffold required for plasma membrane localization of the Sema-1a-PlexA/Gyc76C signaling complex, and we find that the PDZ domain-containing dGIPC protein interacts with Gyc76C. In vertebrates, GIPC regulates protein trafficking, subcellular localization and various signaling events (De Vries et al., 1998; Cai and Reed, 1999; Wang et al., 1999; Lou et al., 2001; Tan et al., 2001; Yi et al., 2007). Gyc76C cell-surface localization is enhanced in vitro in the presence of dGIPC, and this may serve to regulate Gyc76C-mediated signaling. dGIPC genetic analyses show that dGIPC plays a neuronal role in motor axon guidance, and this likely occurs through interactions that modulate Gyc76C-mediated cGMP signaling. Mammalian GIPC forms dimers and multimeric complexes (Bunn et al., 1999; Gao et al., 2000; Hirakawa et al., 2003; Jeanneteau et al., 2004; Kedlaya et al., 2006; Naccache et al., 2006; Varsano et al., 2006). dGIPC may be a part of a molecular scaffold that couples Gyc76C to cell membrane trafficking machinery or anchors Gyc76C to the plasma membrane. Alternatively, dGIPC may be essential for activating or transducing Gyc76C-mediated cGMP signaling in axon guidance events. Future genetic and biochemical analyses will reveal the downstream signaling components that respond to changes in cGMP levels in vivo and direct discrete neuronal growth cone steering responses.

MATERIALS AND METHODS

Drosophila strains and phenotypic characterization

Culturing Drosophila was performed as described previously (Yu et al., 1998; Terman et al., 2002; Ayyob et al., 2004). Stocks Df(2L)8860 and Df(3R)8941 were from the Bloomington Stock Center. Gal4 drivers were: elav(2)-GALA, elav(3E)-GALA (Yao and White, 1994), Mef2-Gal4 (Ranganayakulu et al., 1996), sim-Gal4 (Hülsmeier et al., 2007), repo-Gal4 (Seppe et al., 2001), OK171-Gal4 (Ramadan et al., 2007) and Sca-GAL4 (Klaes et al., 1994). Analyses of axon guidance defects performed as described previously (Yu et al., 1998).

Gyc76C deletion constructs

Gyc76C (amino acids 1-1525) domains are defined as Ecto (1-492), TM (494-514), KHD (517-812), DD (815-870) and Cterm (1105-1521) respectively. dGIPC from a cDNA clone was C-terminally tagged with Igk-leader sequence, followed by 5\*Myc (EQLEISEEDL) or 2\*HA (YPYDVPDYA), respectively. dGIPC from a cDNA clone was C-terminally tagged with Igk-leader sequence, followed by 5\*Myc (EQLEISEEDL) or 2\*HA (YPYDVPDYA), respectively. dGIPC from a cDNA clone was C-terminally tagged with Igk-leader sequence, followed by 5\*Myc (EQLEISEEDL) or 2\*HA (YPYDVPDYA), respectively.

Cell culture

S2R+ cells were grown in Schneider’s medium (Invitrogen) with 10% heat-inactivated fetal bovine serum (FBS); ML-DmBG2 cells were grown in Shields and Sang M3 medium with 10% heat-inactivated FBS and 10 μg/ml insulin. Both cells types were cultured at 25°C [procedures can be found at DRSC (http://www.flyrnai.org/DRSC-PRC.html)].

Co-immunoprecipitation assays

Drosophila S2R+ cells were transfected with pUAST:5\*Myc- FL Gyc76C with or without, full-length pUAST:2\*HA-FL- PlexA using Effectene (Qiagen). Forty-eight hours later, cell lysates were immunoprecipitated with anti-Myc monoclonal antibody (9E10; Sigma) and blotted with anti-HA monoclonal antibody at 1:2500 (12CA5; Roche); alternatively, cell lysates were immunoprecipitated using anti-HA (12CA5) and immunoblotted using anti-Myc (9E10) at 1:2500. For in vitro co-immunoprecipitation, UAS:HA-PlexA, elav-Gal4;cyo flies were crossed to either UAS:Myc-FL Gyc76C or wild-type flies. Embryonic lysates were isolated and immunoprecipitated with anti-Myc (9E10) (Terman et al., 2002). Immunoprecipitates were probed using anti-HA (12CA5) at 1:2500 and anti-Myc (9E10) at 1:2500.

Guanylyl cyclase activity assay

In vitro cGMP concentrations determined from cell lysates with the Direct Guanylyl cyclase activity assay kit (Assay Designs, # 900-014). S2R+ cells and ML-DmBG2 cells were transfected for 36 hours or 72 hours, respectively. Each well of a 24-well plate was plated with 0.5×10^6 cells. Transfected cells were incubated with 0.5 mm IBMX in serum-free medium for 30 minutes at room temperature with gentle rocking, washed once with 1×PBS, lysis in 0.1 M HCl for 15 minutes at room temperature, and spun at 600 g for 5 minutes. Supernatants were diluted four- or fivefold in 0.1 M HCl for cGMP concentration determination. Expression of FL Gyc76C and the Gyc76C construct variants in western blots were quantified using ImageJ and normalized using actin controls. Raw cGMP levels produced by each Gyc76C variant were then normalized to the protein expression level of FL-Gyc76C (raw cGMP levels × [FL-Gyc76C protein expression/corresponding actin loading control]).

Immunohistochemical analyses

Embryo collections and staining were performed as described (Yu et al., 1998; Ayyob et al., 2004). Primary antibodies used were: anti-FasII mAb ID4 (1:4; Van Vactor et al., 1993), rabbit anti-Sema-1a (1:500; Yu et al., 1998), mouse anti-MHC (1:1000; Sigma), mouse anti-dGIPC (1:250; Djiane and Mlodzik, 2010), anti-Myc 9E10 (1:500; Sigma) and anti-HA 12CA5 (1:500; Roche). HRP-conjugated goat anti-mouse and anti-mouse IgG/M
incubated with 50 mM glycine on ice for 10 minutes, incubated with anti-Myc 9E10 (1:100/10% FBS/s2 medium on ice for 10 minutes with washed with ice-cold 3% sucrose/PBS. Cells were fixed in 4% paraformaldehyde/3% sucrose for 10 minutes, permeabilized with 0.5% triton/PBS for 5 minutes, blocked in 10% NGS/PBS for 15 minutes and incubated with rabbit anti-Myc 71D10 (1:50; Cell Signaling) overnight at 4°C. Secondary antibodies were Alexa 488-conjugated goat anti-mouse IgG (1:500) and Alexa486-conjugated goat anti-rabbit IgG (1:500; Molecular Probes). In biotinylation cell surface protein assays, transfected cells were washed twice with ice-cold PBS, incubated with 1 mg/ml EZ-link Sulfo-NHS-SS-Biotin (Thermo Scientific) on ice for 20 minutes, washed twice with ice-cold PBS and then incubated with 50 mM glycine on ice for 10 minutes prior to lysis in RIPA buffer and sonication. Homogenates were centrifuged at 23,000 g for 20 minutes at 4°C, supernatants were incubated with NeutrAdvin beads (Thermo Scientific) for 2 hours at 4°C and washed four times with Wash buffer [150 mM NaCl, 50 mM Tris (pH 8.0), 1 mM MgCl₂ and 1% NP40]. Precipitates were analyzed by western blot using anti-Myc 9E10 (1:2000).

Yeast two-hybrid screen


