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Research Focus

R-Ras fills another GAP in semaphorin signalling

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Plexins are cell-surface receptors for the semaphorin family of neuronal guidance cues. Following semaphorin binding, the plexin cytoplasmic region initiates poorly understood signal-transduction events that lead to modifications of the cytoskeleton. Recent findings shed new light on the signalling network downstream of semaphorins and plexins by demonstrating that one of the plexins, plexin-B1, possesses an intrinsic GTPase-activating protein (GAP) activity towards R-Ras. Inactivation of R-Ras by the plexin-B1 GAP domains is required for plexin-B1-mediated effects on the cytoskeleton. These results indicate that plexins not only bind to but also regulate directly the activity of some of their downstream effectors.

Introduction

During nervous-system development, neurons extend axonal processes that use molecular gradients in their outside environment for navigating to their target structures. These gradients are composed of axon-guidance molecules (see Glossary) and are detected by a sensory structure at the leading tip of each axon: the growth cone. High-affinity receptors at the cell surface of the growth cone enable extending axons to sample a vast number of different axon-guidance molecules. Following ligand binding, these receptors activate intracellular signalling cascades that, in turn, modulate cytoskeletal dynamics. Ultimately, these cytoskeletal changes lead to alterations in growth-cone motility and growth-cone steering.

The semaphorins comprise a large family of axon-guidance molecules. Many semaphorins function as axon repellents (i.e. growth-cone-collapse proteins), influencing axon steering, axon fasciculation and axon branching *in vivo* [1]. Thus far, all repulsive semaphorin signalling has been attributed to receptor complexes that contain plexins as obligatory signal-transducing subunits [2,3]. The cytoplasmic region of plexins is required for semaphorin signalling but it displays no homology to known catalytic domains. Several intracellular proteins have been implicated in plexin-mediated axon repulsion, some of which interact physically with the plexin cytoplasmic domain [4]. However, it remains largely unknown how these cues are activated by plexin receptors and how they work together to form a functional signalling network.

The plexin cytoplasmic region shows sequence similarity to a group of Ras-family-specific GTPase-activating proteins (GAPs) [4]. This observation supports the exciting possibility that plexins might directly regulate the activity of GTPases. However, since their original identification in 1995 [5], no intrinsic GAP activity has been demonstrated for plexins. Inspired by recent experimental and methodological advances, Oinuma *et al.* have reported on the identification of this long-sought activity [6]. The work of Oinuma *et al.* provides novel mechanistic insight into plexin signalling and helps to further the knowledge of how semaphorin signalling pathways intersect with other signalling cascades.

Plexins as GAPs?

One of the key regulators of actin dynamics is the Rho family of small GTPases. RhoGTPases serve as molecular switches by cycling between an inactive GDP-bound state

Glossary

Axon branching: the formation of axon collaterals from the primary axon shaft.
Axon fasciculation: the process during which individual axons converge to form larger axon bundles.
Axon-guidance molecules: molecules that attract or repel extending axons during neural development, thereby instructing them to grow in a specific direction.
Axon steering: directed extension of axons in response to axon-guidance molecules.
COS-7 cell collapse: the abrupt contraction of COS-7 cells, transfected with semaphorin receptor constructs, in response to addition of semaphorin ligand to the culture medium. This is considered to be a heterologous system that mimics growth-cone collapse and that can be used to study the properties of semaphorin receptors.
Growth cone: hand-like structure at the tip of extending axons that senses the outside environment for molecular signals.
Growth-cone collapse: the abrupt retraction of extending growth cones. Observed *in vitro* when neuronal cells are exposed to repulsive axon-guidance molecules such as semaphorins.
Growth-cone motility: rate of extension of growth cones.
Growth-cone steering: changes in the direction of growth-cone extension in response to axon-guidance molecules.
PDZ domain: protein–protein interaction module that is found in a wide variety of cytoplasmic molecules associated with cell-surface proteins.
PDZ-RhoGEF and LARG: Rho-specific guanine-nucleotide-exchange factors that bind to the C-terminal PDZ-binding motif of plexin-B1 and that function in Sema4D–plexin-B1 Rho activation.
Plexins: transmembrane proteins that serve as signal-transducing subunits in semaphorin receptor complexes. Eleven plexins have been identified and categorized into four subclasses (A–D) on the basis of sequence similarities.
Plexin-B1-GGA: a mutant plexin-B1 that cannot interact with Rnd1 owing to mutations in its Rnd1-binding site (contains L1849G, V1850G and P1851A substitutions) [17].
Plexin-B1-RA: a plexin-B1 molecule with mutations in the conserved arginine residues of its RasGAP homology domains (Arg is mutated to Ala at amino acids 1677, 1678 and 1984) [6].
Semaphorins: a large family (>25 members) of secreted and membrane-associated proteins, many of which function as axon attractants or repellents. They are categorized into eight classes based on sequence similarity and distinctive structural features.

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and an active GTP-bound state. After activation, they can interact with their effectors and initiate specific signalling pathways. Three classes of proteins regulate the nucleotide-binding state of GTPases: guanine-nucleotide-exchange factors (GEFs), which facilitate the exchange of GDP for GTP; GAPs, which stimulate the hydrolysis of bound GTP to GDP; and GDP-dissociation inhibitors (GDIs), which inhibit the release of GDP [7].

The evidence that RhoGTPases participate in semaphorin signalling is compelling [8]. By contrast, the molecular mechanisms by which semaphorin receptors modulate RhoGTPase activity are less clear. Thus far, only plexin-B1 has been found to regulate GTPase activity (indirectly). The RhoGEFs PDZ-RhoGEF (postsynaptic density protein 95 kDa, discs large, zona occludens-1) and leukemia-associated RhoGEF (LARG) bind to the PDZ-domain-binding motif of plexin-B1. Activation of plexin-B1 by its ligand semaphorin4D (Sema4D) enhances the activity of these RhoGEFs, and leads to RhoA activation and growth-cone collapse [9–13]. Initial support for the hypothesis that plexins posses an intrinsic GAP activity came from work by Rohm *et al.* [14]. The plexin-A1 cytoplasmic domain includes two essential conserved arginine residues that correspond to the invariant catalytic residues of RasGAPs [15]. Mutations of these residues abolished the ability of plexin-A1 to induce COS-7 cell collapse in response to semaphorin3A (Sema3A) [14]. In another study, Püschel and coworkers showed that, from a large number of RhoGTPases tested, only Rnd1 and RhoD bound to the cytoplasmic domain of plexin-A1. However, no GAP activity of plexin-A1 towards Rnd1 or RhoD could be detected [16].

Stimulation of R-Ras GTPase activity by plexin-B1 requires Rnd1

The failure of several groups to show that plexins can function as GAPs for individual GTPases was explained by the observation of Oinuma *et al.* that this activity involves a simultaneous or sequential interaction of the plexin cytoplasmic domain with two different GTPases [6]. Similar to plexin-A1, Rnd1 binds to plexin-B1, and plexin-B1–Rnd1 interactions are required for Sema4D-induced COS-7 cell collapse [17]. Within the cytoplasmic domain of plexins, two subdomains show sequence homology to R-RasGAPs [6,14]. Because the Rnd1-binding site in plexin-B1 is located in the linker region that connects these two GAP homology domains [16,17], Oinuma *et al.* hypothesized that Rnd1 functions to modulate plexin-B1 GAP activity [6]. The authors showed first that Rnd1 is required for R-Ras binding to plexin-B1. Interactions between R-Ras and plexin-B1 were observed only in the presence of Rnd1, and a mutant plexin-B1 (plexin-B1-GGA [17]) that could not interact with Rnd1 failed to bind to R-Ras. Site-directed mutagenesis of the GAP homology domains (plexin-B1-RA) abolished binding of R-Ras to plexin-B1, although Rnd1–plexin-B1 interactions remained intact. Another Ras family member, H-Ras, did not interact with the plexin-B1–Rnd1 complex [6]. These results show that plexin-B1 interacts specifically and directly with active R-Ras through its GAP homology domains. Interestingly, interactions between R-Ras and plexin-B1 occur only in the presence of another GTPase: Rnd1.

But, in addition to binding to R-Ras, can plexin-B1 also promote the intrinsic GTPase activity of this GTPase (i.e. function as an R-RasGAP)? After stimulation with Sema4D, COS-7 cells expressing exogenous plexin-B1, Rnd1 and R-Ras showed a rapid decline in GTP-bound R-Ras [6]. By contrast, following co-expression of plexin-B1-GGA or plexin-B1-RA, levels of GTP-bound R-Ras were unchanged [6]. This indicates that plexin-B1 GAP activity towards R-Ras is ligand dependent, and requires Rnd1–plexin-B1 interactions and an intact GAP homology region. In addition to cell collapse, Sema4D can also induce plexin-B1-dependent growth-cone collapse [12]. Both expression of a constitutively active form of R-Ras and knockdown of Rnd1 in hippocampal neurons prevented growth-cone collapse by Sema4D, whereas R-Ras knockdown triggered ligand-independent collapse responses [6]. Thus, collapse responses in both non-neuronal cells and growth cones are likely to require R-Ras inactivation by plexin-B1 in an Rnd1-dependent manner.

Overall, the experiments by Oinuma *et al.* suggest that R-Ras inactivation is essential for Sema4D-induced collapse responses. But how does this observation fit with the previously established requirement for RhoA activation by PDZ-RhoGEF or LARG to mediate Sema4D-induced collapse [9–13,18]? Inactivation of plexin-B1 GAP activity has no effect on the ability of this receptor to activate RhoA, and plexin-B1 GAP activity is intact following suppression of PDZ-RhoGEF–plexin-B1 and LARG–plexin-B1 interactions [6]. These observations suggest that inactivation of R-Ras and activation of RhoA by plexin-B1 are separate events that are both required to regulate different aspects of Sema4D-induced collapse. Because R-Ras activation is known to promote cell adhesion by activating integrins [19] and because Sema4D inhibits integrin-mediated cell adhesion through unidentified mechanisms [20], R-Ras inactivation by Sema4D–plexin-B1 interactions might function to reduce cell adhesion, thereby enabling RhoA-mediated collapse (Figure 1). Alternatively, the molecular basis of plexin-B1 signalling might differ in non-neuronal cells (e.g. COS-7 and PC12) and neurons [20]. In other words, plexin-B1-mediated collapse of growth cones and COS-7 cells might require (partially) different protein–protein interaction events. The observations that constitutively active R-Ras blocks Sema4D growth-cone collapse and that R-Ras knockdown by itself leads to collapse do not truly establish a requirement for R-Ras inactivation in neuronal plexin-B1 signalling. Also, in COS cells, Rnd1 binding to plexin-B1 is essential for PDZ-RhoGEF and LARG activation. This implies that the suppression of Sema4D-induced growth-cone collapse following Rnd1 knockdown might not only be a result of impaired GAP activity but could also result from defective RhoA signalling. Future experiments that introduce plexin-B1 mutants (e.g. plexin-B1-GGA and plexin-B1-RA) into plexin-B1-deficient neurons will clarify the role of Rnd1 and GAP activity in neuronal plexin-B1 signalling.

The data described show that binding of Rnd1 to plexin-B1 at a specific Rnd1-binding site is required for collapse

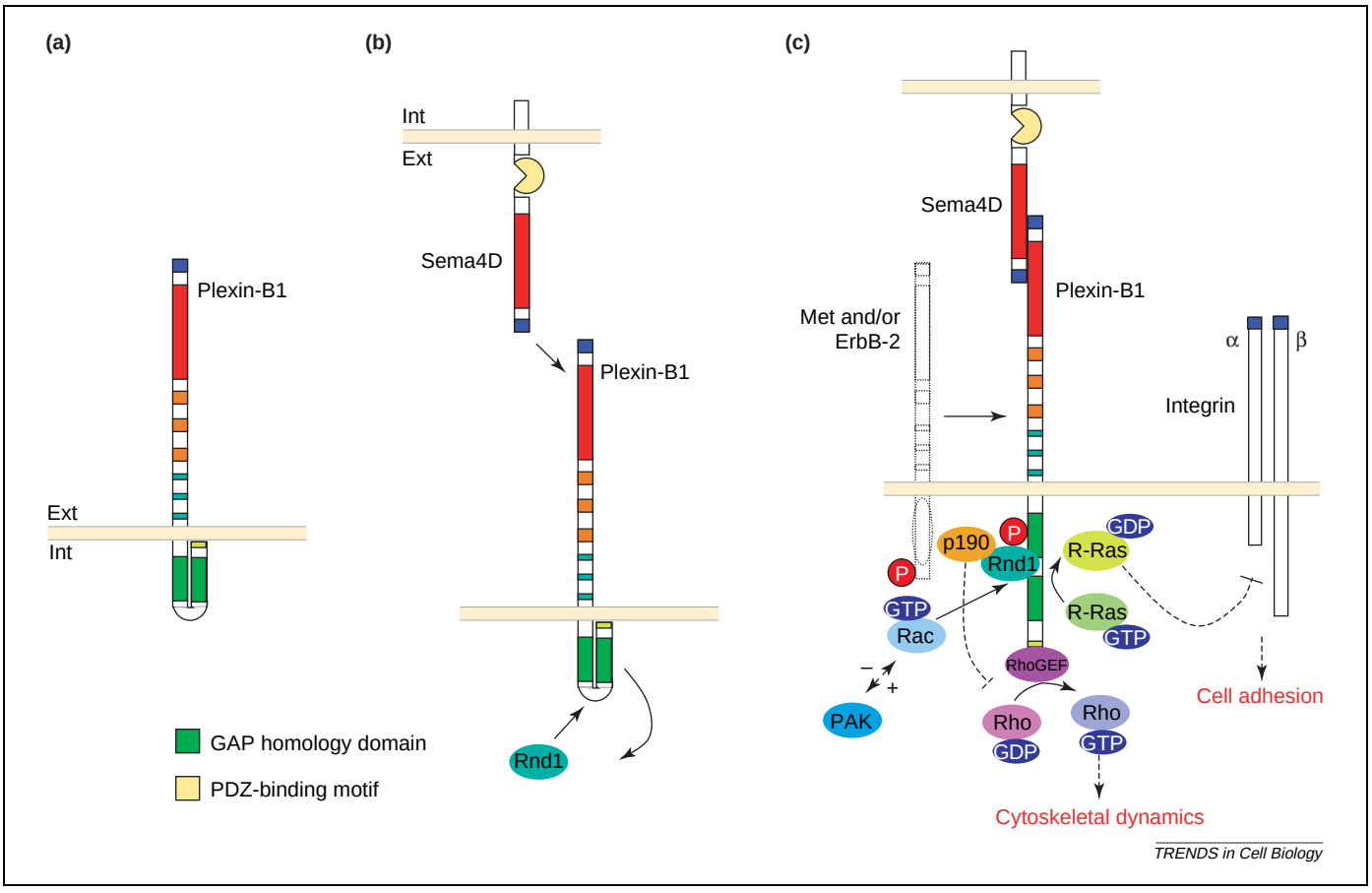

Figure 1. Model of Sema4D–plexin-B1 signalling in cell and growth-cone collapse. **(a)** In the absence of Sema4D, the cytoplasmic region of plexin-B1 is closed owing to autoinhibitory intramolecular interactions. **(b)** Following ligand binding, the RhoGTPase Rnd1 binds to plexin-B1 and relieves the 'closed' conformation of the plexin-B1 cytoplasmic region. **(c)** In an 'open' conformation, plexin-B1 can induce several cytosolic signalling events: first, plexin-B1 sequesters active Rac1 away from its downstream targets, including p21-activated kinase (PAK); second, plexin-B1 functions as an Rnd1-dependent GAP for R-Ras. Inactivation of R-Ras suppresses integrin activation and, consequently, reduces cell adhesion; third, plexin-B1 associates with the transmembrane kinases Met and ErbB-2. These interactions lead to the tyrosine phosphorylation of plexin-B1, Met, ErbB-2 and unidentified downstream effectors; fourth, The C-terminal PDZ-binding motif of plexin-B1 binds to the RhoGEFs PDZ-RhoGEF and LARG. Sema4D–plexin-B1 and ErbB-2–Rnd1 interactions increase PDZ-RhoGEF and LARG activity, leading to RhoA activation and collapse responses. Interestingly, Rnd proteins can bind to and stimulate p190 RhoGAP: an event shown to antagonize RhoA activation. Abbreviations: α, α subunit; β, β subunit; Ext, extracellular; Int, intracellular; P, phosphorylation.

responses in COS-7 cells and, perhaps, neurons. Interestingly, other authors have shown that active Rac1 and Rnd1 bind to plexin-B1 using a similar binding site [18,21]. In addition, Rnd2 and Rnd3 also bind to plexin-B1 [17]. These different RhoGTPases could compete for the same binding site and, thereby, modulate plexin-B1 activity, as has been shown for Rnd1 and RhoD in the case of plexin-A1 [16]. However, for Rnd1 and Rac1 at least, this 'competition model' seems unlikely because both GTPases contribute to repulsive plexin-B1 signalling. Binding of plexin-B1 to GTP–Rac1 is thought to sequester Rac1 from its downstream effectors and enhance the affinity of plexin-B1 for Sema4D [18,21,22]. The data of Oinuma *et al.* suggest that Rnd1 is required for RhoA activation and plexin-B1 GAP activity [6,17]. One possibility is that Rac1 and Rnd1 binding to plexin-B1 are sequential events that both regulate different aspects of plexin-B1 function. Alternatively, Rac1- and Rnd1-binding sites, although close, might be distinct and enable simultaneous binding. Interestingly, a recent study showed that the cytoplasmic region of plexin-A1 undergoes autoinhibitory intramolecular interactions that can be relieved after binding to active Rac1 [23]. Based on these observations and the data of Oinuma *et al.*, it seems possible that the constitutively active GTPase Rnd1 functions to open the closed formation of the plexin-B1 cytoplasmic region, thereby facilitating recruitment of active Rac1 and enabling other signalling events, including R-Ras inactivation and plexin-B1 tyrosine phosphorylation by ErbB-2 [6,18,21,24] (Figure 1). These signalling events might also include the binding of Rnd1 to p190 RhoGAP. Rnd proteins have been shown to antagonize RhoA activation by enhancing the activity of this RhoGAP [25]. Rnd1-dependent RhoGAP stimulation could function to regulate tightly RhoA-activation events downstream of plexin-B1 (Figure 1).

Concluding remarks

Several transmembrane and cytosolic proteins have been shown to bind to plexin-B1 and to be required for plexin-B1 signalling. The novelty of the observations of Oinuma *et al.* [6] is that, for the first time, plexins have been found not only to bind to but also to regulate directly the activity of one of their downstream effectors. The GAP homology domains of plexins are well conserved, suggesting that direct regulation of (R-)Ras activity might be an important

signalling pathway for different subclasses of semaphorins. This idea is supported by the observation that mutations in the GAP homology domains of plexin-A1 and the expression of constitutively active Ras prevent Sema3A-mediated growth-cone collapse [6,14]. Remarkably little is known about the nature and extent of molecular cross-talk between semaphorin and other signalling cascades. Intriguingly, the work of Oinuma *et al*. suggests that plexins, through the regulation of small GTPases, can suppress signalling pathways that normally counter semaphorin-induced collapse. Future research will, undoubtedly, refine the role of GTPases in plexin signalling and will provide molecular insight into the signalling cascades downstream of plexin–GTPase interactions.

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The cadherin superfamily and dendrite development

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Dendrite morphogenesis is a highly dynamic process that involves constant extension and retraction of branches, and stabilization of these dynamics has pivotal roles in determining the number and length of branches. The classic cadherin N-cadherin is involved in stabilization of dendritic spines and branches. Recent work in mammals by Shima *et al.*, and earlier work in *Drosophila* by Gao *et al.* and Sweeney *et al.* have determined the presence of a non-classic, seven-pass transmembrane cadherin in this process.

Introduction

Dendrites and axons are the major input and output apparatus of a neuron. How the tremendous diversity of

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