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The Great Escape: Phosphorylation of Ena/VASP by PKA Promotes Filopodial Formation

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The Great Escape: Phosphorylation of Ena/VASP by PKA Promotes Filopodial Formation

The Ena/VASP family of proteins consists of adaptor molecules that localize to subcellular sites of actin polymerization. The role of Ena/VASP proteins in the regulation of cell motility and axon outgrowth has been controversial. Recently, these proteins have been proposed to function as "anticapping" factors, which may have differential effects on filopodial versus lammelipodial actin-based protrusions. A study by Lebrand et al. in this issue of *Neuron* supports this model and identifies PKA as a key regulator of Ena/VASP function downstream of the chemoattractant Netrin.

During neural development, motile growth cones at the tips of growing axons are guided to targets by extrinsic cues. Receptor-ligand binding generates intracellular signals that are integrated and converted into changes in motility. Although great progress has been made in identifying the guidance cues and their receptors, less is known about how intracellular signals are converted into changes in the direction and rate of neurite outgrowth. Growth cone morphology and motility are determined by dynamic changes in cytoskeletal elements such as actin filaments and microtubules, which are regulated by complex interactions with structural and signaling proteins. The local regulation of cytoskeletal dynamics is thought to underlie growth cone turning in response to guidance cues.

Results from mutant screens and analysis of genetic interactions have identified several candidate intermediates between guidance cue receptors and the cytoskeleton. For example, studies in both *Drosophila* and *C. elegans* have reported cytoskeletal regulatory proteins that are required downstream of specific guidance cues for proper pathfinding. One intriguing outcome of these studies is that some cytoskeletal effectors appear to be necessary for both attractive and repulsive responses to guidance cues. For example, *unc-34*, the worm homolog of *Drosophila Enabled* (*Ena*), functions in both UNC-40 (DCC)-mediated attraction (Gitai et al., 2003) and UNC-5-mediated repulsion (Colavita and Culotti, 1998) to Netrin. In addition, Ena has also been shown to mediate the repulsive effects of Slit-Robo signaling (Bashaw et al., 2000). Although it is difficult to define a precise function of Ena/VASP proteins from genetic studies, they do suggest that Ena/VASP activity is modulated by guidance cue receptors.

Cellular studies of Ena/VASP function have suggested these proteins are important regulators of actin assembly and cell motility. Initial work examining the motility of bacterial pathogens in cells concluded that Ena/VASP proteins promote actin polymerization as they increase the speed of *Listeria* movement (reviewed in Machesky, 2000). However, more recently Ena/VASP proteins were found to negatively regulate the migration rate of non-neuronal cells. These apparently conflicting results were reconciled through an elegant series of experiments combining live cell imaging and electron microscopy (Bear et al., 2002). This study concluded that although Ena/VASP activity promoted the rapid extension of lamellipodia, these protrusions were unstable and did not contribute positively to cell translocation. Together with complementary studies, a consensus model for Ena/VASP function in the regulation of cell protrusion has emerged. Briefly, Ena/VASP proteins are targeted to the leading edge of lamellipodial protrusions where they specifically bind to the barbed ends of actin filaments. Ena/VASP interaction with actin filament barbed ends promotes elongation by antagonizing capping proteins, which normally block the addition of actin monomers. Recent studies also suggest that VASP localization to the ends of actin bundles promotes filopodial formation from the lamellipodial actin meshwork (Svitkina et al., 2003). If Ena/VASP proteins function similarly in growth cones, it is of great interest to determine the mechanisms controlling Ena/VASP activity and how these may be modulated by guidance cues.

In this issue of *Neuron*, Gertler and colleagues (Lebrand et al., 2004) test the function of Ena/VASP proteins in outgrowth of primary hippocampal neurites using the sequestration technique previously used to enhance or neutralize the activity of all Ena/VASP proteins in fibroblasts (Bear et al., 2002). In contrast to its effects on fibroblast motility, Ena/VASP proteins appear to promote filopodial formation in growth cones. Inactivating Ena/VASP by targeting these proteins to mitochondria eliminated filopodia, whereas directing Ena/VASP proteins to the plasma membrane increased filopodial length and number. Surprisingly, neither treatment altered the length of axons, suggesting that Ena/VASP proteins and their effect on filopodial protrusion do not alter the rate of process outgrowth. Instead, in primary hippocampal neurons, Ena/VASP proteins appear to promote axon branching, possibly as a result of increased filopodial production or stability.

If Ena/VASP proteins are involved in axonal morphogenesis, then axon guidance cues known to alter growth cone behavior may function by regulating Ena/VASP activity. To test this possibility, soluble Netrin-1, a chemoattractant for hippocampal neurons, was applied to neurons with overactivated or neutralized Ena/VASP proteins. The authors demonstrate that Netrin-1 normally stimulates lamellipodial protrusions and increases the number and length of filopodia within 30–60 min of Netrin addition. However, when Ena/VASP proteins are inhibited by sequestration onto mitochondria or overactivated by targeting to the cell surface, stimulation with soluble Netrin-1 has no effect on filopodial protrusion. Interestingly, even when Ena/VASP proteins are inactivated, Netrin-1 is still capable of promoting lammelipodial protrusions, suggesting that Netrin-1 may activate separate intracellular signaling pathways downstream of DCC receptors. These results are consistent with find-

ings that DCC receptors activate the Rho GTPases Rac1 and Cdc42 (reviewed in Guan and Rao, 2003), which may promote lamellipodial formation independent of Ena/VASP proteins.

What signaling intermediates link DCC receptors to the activation of Ena/VASP proteins in growth cones? This report provides compelling evidence that cAMP-dependent protein kinase (PKA) is both necessary and sufficient to activate Ena/VASP proteins. First, stimulation of filopodial protrusion by Netrin requires PKA activity. Although it was not reported whether lamellipodial protrusion was also dependent on PKA, this is unlikely given that lamellar protrusion was stimulated by Ena/VASP-independent signals. Second, Netrin promotes rapid phosphorylation of Mena at serine 236, which is expected to be a PKA-dependent event. Finally, direct activation of PKA with forskolin induced a rapid increase in the number and length of filopodia, with no effect on lammelipodia. Although it is unknown how phosphorylation of Ena/VASP regulates the activity of these proteins, these results suggest that PKA phosphorylation prevents the capping of actin plus ends.

cAMP/PKA signaling has diverse effects on neurite outgrowth; however, the phospho-targets of PKA that are capable of modulating axon outgrowth have remained elusive (although see Kao et al., 2002). The Ena/VASP proteins are intriguing candidate targets given their subcellular localization to focal adhesions and to the tips of filopodia, their ability to interact with multiple proteins and regulate actin polymerization, as well as their dramatic effects on cell morphology and motility (Reinhard et al., 2001; Lebrand et al., 2004). Ena/VASP proteins such as VASP and Mena have three distinct serine residues that may be phosphorylated with different preferences by either PKA or protein kinase G (PKG). Currently, it is unclear if and how phosphorylation at any of these sites alters the function of Ena/VASP proteins. For example, it is unknown whether generic targeting of Ena/VASP proteins to the plasma membrane (e.g., using "CAAX" motif) promotes filopodial formation independent of PKA phosphorylation, or whether PKA phosphorylation is required after targeting to the plasma membrane. The latter possibility is more satisfying as it provides a second regulatory step through which Ena/VASP may control actin polymerization. This is particularly intriguing since both repulsive (i.e., Robo) and attractive (i.e., DCC) axon guidance cue receptors may bind Ena/VASP proteins either directly (Bashaw et al., 2000) or through adaptor proteins such as Nck (Coppolino et al., 2001). Therefore, association with particular guidance cue receptors may be a first step that functions to both localize Ena/VASP proteins for regulation by PKA or PKG and to position them properly to act on particular downstream targets (Figure 1).

Gradients of chemotropic axon guidance cues across growth cones are believed to promote axon turning through the local activation of intracellular signaling cascades and downstream cytoskeletal effectors (Guan and Rao, 2003). Importantly, cyclic nucleotides have been shown to function as molecular switches for many axons guidance cues, converting them from attraction to repulsion or vice versa. Moreover, recent work suggests that the intracellular ratio of cAMP versus cGMP determines the polarity of growth cone responses to guidance cues

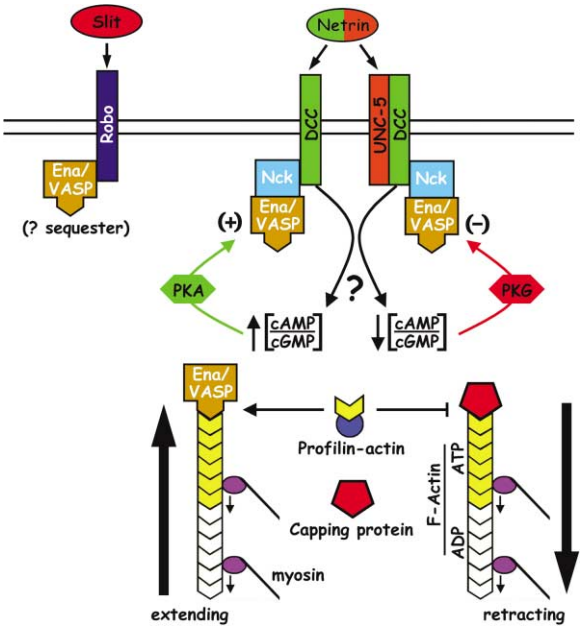

Figure 1. Regulation of Ena/VASP Activity by Cyclic Nucleotides May Act as a Binary Switch to Control Actin-Dependent Extension or Retraction of Filopodia

In this model, Netrin stimulates cAMP or cGMP production depending on the expression of UNC-5 receptors. Activation of Ena/VASP proteins by PKA results in uncapping of actin filament plus ends, which promotes monomer addition in combination with profilin and possibly other nucleating factors not schematized here. In contrast, PKG may preferentially phosphorylate sites of Ena/VASP proteins that reduce or inactivate these proteins. Inactivated Ena/VASP proteins dissociate from actin filaments, allowing capping proteins to bind and block monomer addition to plus ends, resulting in filament retraction. If a basal level of Ena/VASP activity is required for growth cone motility, locally disrupting the activity of these proteins by PKG phosphorylation or sequestration to inhibitory receptors (i.e., Robo receptors) may mediate repulsion.

such as Netrin-1 (Hong et al., 2003). Given that Ena/VASP proteins are phosphorylated by both PKA and PKG at up to three separate serine residues, it is possible that phosphorylation at each site differentially affects the function of Ena/VASP proteins. For example, with PKA inhibited, activation of PKG directly by DCC or other downstream signals could phosphorylate Ena/VASP proteins at sites that inhibit their function, thereby locally reducing actin polymerization (Figure 1). In this model, repulsive axon guidance cues, such as Netrin on UNC-5-expressing neurons, may act in part by elevating cGMP relative to cAMP and thereby preferentially phosphorylating potential inhibitory sites on Ena/VASP proteins. However, this simple model clearly cannot explain all cyclic nucleotide-mediated switching, since increased cGMP signaling has been reported to convert Semaphorin-mediated repulsion into attraction (Song and Poo, 2001).

This study by Lebrand, Dent and colleagues adds an important link to our understanding of the chain of events that occur between the activation of a guidance cue receptor and the alteration of growth cone motility. However, many difficult questions remain unanswered. Guidance cues such as Netrin can activate several intra-

cellular signals, but how such a wide variety of signals is generated and how they work in combination is unknown. For example, Netrin stimulates changes in intracellular calcium, phospholipase C, phosphatidylinositol 3-kinase, mitogen-activated protein kinase, and the small GTPases Cdc42 and Rac1 (reviewed in Guan and Rao, 2003). While some of these signals certainly modulate the growth cone cytoskeleton directly, others likely affect distinct cellular processes such as protein synthesis and degradation, as well as vesicle trafficking and ion channel activity. These additional effects may ultimately feedback to amplify or modulate the intracellular signals generated by receptor activation. Given the complexity of signaling networks that exists for individual guidance cues, it is bewildering to imagine how growth cones in vivo integrate signals generated by simultaneous activation of multiple receptors.

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Lipid Rafts as Organizing Platforms for Cell Chemotaxis and Axon Guidance

Lipid rafts are thought to serve as plasma membrane platforms for localized trafficking and signaling. Recent findings reported by Guirland et al. in this issue of *Neuron* and by Gómez-Moutón in a recent issue of *JCB* support a direct role of lipid microdomains in organizing spatial signaling during axon guidance and cell chemotaxis by concentrating the gradient-sensing machinery at the leading edge.

The existence of discontinuous microdomains in the plasma membrane of eukaryotic cells has been a topic of intense debate in recent years. Discrete plasma membrane domains with different properties could be important for targeting specific components to different locations in the cell and for compartmentalization of signaling pathways. As such, they could contribute to a variety of important biological processes, including endo- and exocytosis, signal transduction, cell polarity, antigen recognition, cell adhesion and migration, axon guidance, and synapse formation and function. The notion that specific lipids, particularly cholesterol and sphingolipids, could serve to organize membranes into distinct microdomains has gained support from studies using model lipid bilayers, detergent extraction, cholesterol depletion, and examination of the cellular distribution of glycosylphosphatidylinositol (GPI)-anchored proteins, widely regarded as markers of such domains. Lipid microdomains are envisioned as discrete platforms of a particular lipid and protein composition floating in an otherwise uniform sea of plasma membrane, and they are therefore usually called lipid rafts. Although their mere existence remains a contentious issue (see Simons and Toomre, 2000, and Munro, 2003, for recent reviews on either side of the controversy), the concept of lipid rafts continues to inspire a great number of researchers to examine how compartmentalization and clustering of different types of molecules in the plasma membrane may contribute to downstream signaling and cell behavior. Among the voluminous literature on lipid rafts (over 1000 Medline hits, 97% from the past 5 years), two recent papers, one of them in this issue of *Neuron*, stand out for their quality and elegance in providing some of the first direct evidence of the role of lipid rafts in organizing spatial signaling during axon guidance and cell chemotaxis (Gómez-Moutón et al., 2004; Guirland et al., 2004 [this issue of *Neuron*]).

In their study in this issue of *Neuron*, Guirland et al. (2004) took advantage of the growth cone turning assay first developed by Mu-ming Poo and colleagues (Lohof et al., 1992) to examine the role of lipid rafts in the chemotropic guidance of axons. In this assay, growth cones on a dish are confronted with chemotropic substances emanating from a micropipette placed at a fixed distance and angle. Chemoattractants make growth cones turn toward the pipette, while chemorepellents deflect growth cones away from it. In their experiments, Guirland et al. found that the chemoattractant effect of a diffusible gradient of brain-derived neurotrophic factor (BDNF) could be eliminated upon disruption of lipid rafts by membrane cholesterol depletion or by treatment with the cholesterol-sequestering agent filipin or with the ganglioside G_{M1}, which perturbs raft stability. Although cholesterol depletion has been shown to have a number of effects on the overall integrity of the plasma membrane, including the release of certain protein compo-