NEW EMBO MEMBER'S REVIEW

Actin cytoskeleton regulation through modulation of PI(4,5)P₂ rafts

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The phosphoinositide lipid PI(4,5)P₂ is now established as a key cofactor in signaling to the actin cytoskeleton and in vesicle trafficking. PI(4,5)P₂ accumulates at membrane rafts and promotes local co-recruitment and activation of specific signaling components at the cell membrane. PI(4,5)P₂ rafts may thus be platforms for local regulation of morphogenetic activity at the cell membrane. Raft PI(4,5)P₂ is regulated by lipid kinases (PI5-kinases) and lipid phosphatases (e.g. synaptojanin). In addition, GAP43-like proteins have recently emerged as a group of PI(4,5)P₂ raft-modulating proteins. These locally abundant proteins accumulate at inner leaflet plasmalemmal rafts where they bind to and co-distribute with PI(4,5)P₂, and promote actin cytoskeleton accumulation and dynamics. In keeping with their proposed role as positive modulators of PI(4,5)P₂ raft function, GAP43-like proteins confer competence for regulated morphogenetic activity on cells that express them. Their function has been investigated extensively in the nervous system, where their expression promotes neurite outgrowth, anatomical plasticity and nerve regeneration. Extrinsic signals and intrinsic factors may thus converge to modulate PI(4,5)P₂ rafts, upstream of regulated activity at the cell surface.

Keywords: actin regulation/anatomical plasticity/nerve sprouting/PI(4,5)P₂/rafts

Introduction

During development, nerve cells establish precise networks of processes that provide the basis for nervous system functioning. To a large extent the organization of such neural networks is predetermined genetically to produce adaptive species-specific behavior. As the young animal becomes autonomous, neural networks produce behavior through circuitry that is thought to be largely anatomically stable. The transition from nervous system construction to nervous system functioning is accompanied by a dramatic downregulation of anatomical growth and growth competence in nerve cells. Anatomical plasticity associated with experience can, however, still take place on a local scale in the adult, where persistent alterations in activity lead to rearrangements of synaptic connections. In addition, lesions of nerve tracts can induce regenerative growth and functional repair. Competence for process outgrowth is thus a regulatable property of neurons. Understanding molecular mechanisms of

anatomical plasticity regulation in the nervous system will provide fundamental insights into how morphogenetic processes are regulated by intrinsic and extrinsic factors. In addition, such knowledge should provide mechanistic leads to develop strategies for repair in the nervous system.

Process outgrowth and synaptic rearrangements coincide with the expression of specific sets of genes in neurons (Skene, 1989). Among these, GAP43-like proteins mediate competence for anatomical plasticity (Skene, 1989; Benowitz and Routtenberg, 1997; Caroni, 1997). During development, these proteins are coexpressed at very high levels in most, and possibly all neurons. In contrast, expression levels in the adult are substantially lower, and restricted to partially overlapping subsets of neurons. Within cells, GAP43-like proteins accumulate at detergent-insoluble, cholesterol-dependent rafts at the inner surface of the cell membrane. At these rafts, they co-distribute with and modulate the lipid second messenger PI(4,5)P₂ (Laux *et al.*, 2000). As outlined below, recent studies have provided evidence that these proteins modulate the assembly of PI(4,5)P₂-containing rafts to regulate actin dynamics at the cell surface (Frey *et al.*, 2000; Laux *et al.*, 2000). Separate studies have uncovered central roles for PI(4,5)P₂ clusters and rafts in the recruiting and modulation of key components that regulate the actin cytoskeleton (Shibasaki *et al.*, 1997; Ma *et al.*, 1998; Higgs and Pollard, 2000; Raucher *et al.*, 2000; Rohatgi *et al.*, 2000; Rozelle *et al.*, 2000) and the endocytotic machinery (Cremona *et al.*, 1999; Gillooly and Stenmark, 2001). These combined findings have raised the possibility that one way of determining a cell's intrinsic competence for morphogenetic activity and anatomical plasticity is by altering the molecular machinery that modulates PI(4,5)P₂ rafts through changes in gene expression. Below, I focus on the possible roles and regulation of PI(4,5)P₂ rafts, and the significance of this regulation for anatomical plasticity in the nervous system.

Broad range of functions for GAP43-like proteins

GAP43-like proteins are mechanistically and functionally related, and include GAP43, CAP23, MARCKS and MacMARCKS (Aderem, 1995; Wiederkehr *et al.*, 1997). GAP43 and MARCKS are the two main PKC substrates in the brain. GAP43 (also known as B50, neuromodulin and F1) was discovered as a protein that is rapidly transported into growing axons, and is expressed by neurons when they grow processes (Skene, 1989). Mice lacking GAP43 exhibit specific defects in growth cone navigation and major deficits in the construction of topographic projections between sensory neurons and their target fields (see for example, Maier *et al.*, 1999). Heterozygous mutants exhibit partial phenotypes, suggesting that the expression

levels of this protein are critical for axon guidance. Gain-of-function experiments in mice have provided evidence that GAP43 is an intrinsic determinant of anatomical plasticity in neurons, where its expression levels define a cell's competence to respond to local signals by anatomical rearrangements and growth (Aigner *et al.*, 1995; Caroni *et al.*, 1997). It has been a puzzling fact that in addition to its well established functions in nerve growth, GAP43 is also involved in stimulus-induced secretion, endocytosis and long-term potentiation of synaptic transmission (Benowitz and Routtenberg, 1997).

CAP23 (also known as NAP22 and BASP1) is a further intrinsic determinant of process outgrowth and anatomical plasticity in neurons (Caroni *et al.*, 1997). Like GAP43, it is expressed at high levels during development and is re-expressed by those lesioned neurons that are competent to regenerate axons. Unlike GAP43, which accumulates selectively in axons, CAP23 decorates all compartments of the neuronal plasmamembrane. GAP43 and CAP23 have closely related functions *in vivo* (Frey *et al.*, 2000). The two proteins synergize in promoting nerve sprouting (Caroni *et al.*, 1997), and expression of both proteins appears to be required to confer competence for long-distance regenerative axon growth in the adult (Bomze *et al.*, 2001).

Finally, the closely related proteins MARCKS (also known as 80K) and MacMARCKS (also known as MRP) (Aderem, 1995) are expressed at very high levels in the nervous system, where their absence leads to dramatic developmental deficits in morphogenesis and process outgrowth (see for example, Stumpo *et al.*, 1995). In addition, and in contrast to GAP43 and CAP23, they are also expressed in a regulated manner by a wide range of non-neural cells. Their function has been studied most extensively in fibroblasts and circulating blood cells, where they promote cell adhesion and migration (Myat *et al.*, 1997). In cultured fibroblasts, MARCKS expression is downregulated at confluency, concomitant with a loss of focal adhesions and actin stress fibers, and is rapidly reinduced by stimuli that overcome contact inhibition.

Taken together, these functional findings suggest that GAP43-like proteins share the property of conferring competence for morphogenetic processes to cells that express them. The wide variety of these processes suggests that the common target of these proteins may be a general mechanism affecting morphogenesis and motility.

Actin cytoskeleton modulation by GAP43-like proteins

GAP43-like proteins are highly hydrophilic, with characteristic biochemical, biophysical and functional properties in common, and no enzymatic activity. Shared properties include the presence of a basic domain that binds acidic phospholipids including PI(4,5)P₂, calcium/calmodulin, actin filaments and PKC in a mutually exclusive manner (Hartwig *et al.*, 1992; He *et al.*, 1997; Wiederkehr *et al.*, 1997; Denisow *et al.*, 1998; Maekawa *et al.*, 1999; Wang *et al.*, 2001). GAP43-like proteins induce comparable dynamic actin-based structures such as filopodia and microspikes at the periphery of non-neuronal cells. A large body of experiments both in transfected cell lines and *in vivo* (see for example Wiederkehr *et al.*, 1997; Laux

et al., 2000) point to raft association and the properties of the basic domains as critical determinants of GAP43-like protein function. With respect to downstream targets of GAP43-like protein function, from phagocytosis to growth cone guidance, the unifying motive appears to be actin cytoskeleton regulation. The combined available evidence therefore suggests that GAP43-like proteins potentiate actin cytoskeleton assembly in a process that requires their accumulation at PI(4,5)P₂-containing rafts and the presence of the basic domain.

PI(4,5)P₂ rafts and actin cytoskeleton regulation

It has long been known that PI(4,5)P₂ can sequester actin-binding proteins such as profilin, gelsolin, CapZ and cofilin *in vitro*, but its possible roles in actin cytoskeleton regulation in living cells remained controversial. This situation has changed recently, as several *in vivo* studies have assigned critical modulatory cofactor roles for PI(4,5)P₂ in signaling pathways promoting actin cytoskeleton recruitment (for example the cdc42→WASP→Arp2/3 pathway) and organization (for example the Rho→ERM-protein pathway). In these processes, PI(4,5)P₂ seems to act through its local concentration to lower pathway activation thresholds in a spatially defined manner. This is achieved by providing a local environment that recruits and activates cdc42, WASP and ERM proteins (Chong *et al.*, 1994; Hirao *et al.*, 1996; MacKay *et al.*, 1997; Ma *et al.*, 1998; Loisel *et al.*, 1999; Macheski and Insall, 1999; Matsui *et al.*, 1999; Rohatgi *et al.*, 1999; Higgs and Pollard, 2000; Lamb *et al.*, 2000; Mullins, 2000; Quang *et al.*, 2000; Rohatgi *et al.*, 2000; Tolia *et al.*, 2000; Wear *et al.*, 2000). Critical local recruitment roles for PI(4,5)P₂ have also been assigned unequivocally to the assembly and budding of endosomes (Cremona *et al.*, 1999; Gillooly and Stenmark, 2001). These findings have thus highlighted the significance of understanding how PI(4,5)P₂ levels and pools are regulated locally (Hinchliffe, 2000; Gillooly and Stenmark, 2001). Although definitive answers to these questions will have to await imaging data from living cells, important potential clues are already available. Thus, PI(4,5)P₂ is concentrated at membrane rafts (Pike and Miller, 1998), and Rho- and Arf-type GTPases can activate PI(4,5)P₂-synthesizing PI5-kinases by inducing recruitment of the kinases at rafts (Chong *et al.*, 1994; Godi *et al.*, 1999; Honda *et al.*, 1999).

What then are rafts, and in what ways may they relate to actin cytoskeleton regulation by PI(4,5)P₂? In membranes rich in cholesterol, lipids with unsaturated fatty acid chains partition into microdomains that resist solubilization by non-ionic detergents. These microdomains have been designated DIGs (detergent-insoluble glycolipids), or rafts (Harder *et al.*, 1998; Ledesma *et al.*, 1999; Brown and London, 2000). Due to the specific distributions of cholesterol and the appropriate lipids in cellular membranes, rafts are predominantly found at the plasma membrane and at early endocytotic and late exocytotic compartments. The combination of highly unsaturated sphingolipids and cholesterol is particularly abundant at the outer leaflet of the plasma membrane, where rafts are best characterized. Inner leaflet plasma membrane rafts, where PI(4,5)P₂ accumulates, are less well understood,

Fig. 1. Pathways of PI(4,5)P₂ raft modulation and actin cytoskeleton regulation at rafts. Top: PI(4,5)P₂ (red) accumulates at subplasmalemmal rafts. Left: Signals through e.g. Rho-GTPases recruit activated PI5-kinases at rafts leading to enhanced contents of PI(4,5)P₂ at rafts (darker red). Right: GAP43-like proteins (green) accumulate at PI(4,5)P₂ rafts. By promoting raft assembly, GAP43-like proteins enhance signal strength at PI(4,5)P₂ rafts. In both cases, elevated signaling by PI(4,5)P₂ at rafts would enhance recruitment and activation of WASP proteins and ERM proteins, to promote actin polymerization and actin filament assembly (blue). Local interactions with extracellular matrix or cell surface components can modulate raft function, e.g. through tyrosine kinase activation, mediating spatial control of actin regulation, and promoting further signal amplification. See text for further details.

and may assemble from phosphatidylethanolamine in the presence of cholesterol. The unique lipid environment at rafts drives the selective accumulation of specific classes of proteins, including GPI-linked, cell-surface proteins, double-acylated cytosolic proteins (e.g. src-family tyrosine kinases) and subgroups of transmembrane proteins (e.g. EphB receptors). These remain associated with small elementary raft units of ~30 nm diameter for at least several minutes (Pralle *et al.*, 2000).

Elementary rafts are thought to play important roles in signaling at the cell membrane and in membrane trafficking, by assembling into raft-based platforms in response to specific stimuli, such as receptor activation or cell adhesion. With respect to PI(4,5)P₂ and actin cytoskeleton regulation, it has recently been shown that actin assembly can be initiated with remarkable efficiency at PI(4,5)P₂ rafts, where it can drive membrane and vesicle movements (Harder and Simons, 1999; Merrifield *et al.*, 1999; Taunton *et al.*, 2000). In signaling, PI(4,5)P₂ rafts may act as effective sites of local PI(4,5)P₂ presentation due to selective recruitment of activated lipid kinases at the rafts. According to this view, PI(4,5)P₂ raft regulation may affect the local synthesis of PI(4,5)P₂, upstream of the role of this lipid second messenger in actin cytoskeleton regulation. In addition, raft assembly may also enhance more directly the activation of cdc42/WASP and ERM proteins by PI(4,5)P₂. PI(4,5)P₂ rafts thus appear to be attractive candidate targets of regulation in the production of local changes in actin dynamics and organization.

Modulation of PI(4,5)P₂ rafts

Raft regulation has been investigated in the most detail in T cell activation. In this context, T-cell receptor engagement triggers raft recruitment through the co-activator CD28, followed by the assembly of a signaling platform of tyrosine kinases and adaptor proteins that greatly enhances signal strength (Lanzavecchia *et al.*, 1999; Penninger and Crabtree, 1999; Viola *et al.*, 1999; Rebres *et al.*, 2001). That strength determines the onset and kinetics of T cell activation. The assembly of subplasmalemmal actin-based structures and platforms such as microspikes or focal adhesions also appears to depend on signal strength, as reflected by the recruitment and activation of e.g. WASP or ERM proteins. Therefore, by analogy to the role of rafts in T cell activation, it is tempting to speculate that modulation of WASP and ERM protein recruitment and activation by PI(4,5)P₂ may involve regulated recruitment or assembly of PI(4,5)P₂ rafts. This may involve two possible types of mechanism: regulation of PI(4,5)P₂ contents at rafts, and regulation of PI(4,5)P₂ raft effectiveness through raft assembly. By further analogy to T cell activation, these regulatory processes may involve both extrinsic signals and intrinsic competence components.

Recent findings on the mechanism of action of GAP43-like proteins suggest that these may indeed modulate the assembly of PI(4,5)P₂ rafts. Thus, GAP43-like proteins promoted PI(4,5)P₂ cluster assembly and partial resistance of the clusters against dispersion by the cholesterol-extracting drug cyclodextrin (Laux *et al.*, 2000). Similarly to their effects *in vivo*, these effects of GAP43-like proteins had a marked dose-dependent component. GAP43-like protein constructs, lacking the basic domain (Δ BD-constructs) that binds PI(4,5)P₂, still accumulated at PI(4,5)P₂ rafts where they interfered with cluster assembly and PI(4,5)P₂ accumulation at rafts (Laux *et al.*, 2000). Thus, GAP43-like proteins may function as positive modulators of PI(4,5)P₂ raft function, and the corresponding Δ BD-constructs may interfere with that function in a dominant-negative manner. Consistent with the notion that the effects on PI(4,5)P₂ rafts reflect the function of GAP43-like proteins, wild-type and mutant constructs had opposite effects on actin recruitment and assembly at the cell membrane, and during cell adhesion, nerve sprouting and nerve regeneration *in vivo* (Laux *et al.*, 2000; Frey *et al.*, 2000). Based on these results, we would like to propose a positive cofactor role for GAP43-like proteins as intrinsic determinants of PI(4,5)P₂ raft function, downstream of PI(4,5)P₂ synthesis but upstream of actin cytoskeleton regulation, and possibly also in membrane trafficking (Figure 1).

The model in Figure 1 emphasizes the potential roles of PI(4,5)P₂ raft regulation in signaling to the actin cytoskeleton and provides a candidate mechanistic frame for understanding the roles of GAP43-like proteins as intrinsic determinants augmenting morphogenetic and motile activity in expressing cells. It also raises a number of questions for future investigation. These include: (i) If PI(4,5)P₂ raft modulation should indeed be a major mechanism in the control of regulation by PI(4,5)P₂, then what further molecular components are involved in this process? What is the composition of PI(4,5)P₂ rafts and how are they regulated? Does raft modulation also

affect PI(4,5)P₂ synthesis at rafts? (ii) Actin cytoskeleton assembly and recruitment is frequently promoted by both PI(4,5)P₂-modulated pathways and enhanced levels of protein tyrosine phosphorylation (Harder and Simons, 1999; Rebres *et al.*, 2001)—is there crosstalk between PI(4,5)P₂ raft modulation and the assembly of src-kinase accumulating rafts? (iii) What molecular mechanisms mediate spatial control of PI(4,5)P₂ raft modulation? For GAP43-like proteins, the basic domains that bind PI(4,5)P₂ are regulated by calcium/calmodulin and phosphorylation by PKC; this regulation may link local signaling through calcium and/or PKC to PI(4,5)P₂ raft modulation. (iv) How do GAP43-like proteins accumulate at PI(4,5)P₂ rafts? Does their basic domain predominantly regulate raft assembly, or does it also directly mask PI(4,5)P₂, thereby regulating its availability? How do GAP43-like proteins synergize in promoting anatomical plasticity?

Conclusion

It has recently become clear that PI(4,5)P₂ is an activating cofactor critical for signaling both to the actin cytoskeleton and in vesicle trafficking. In these pathways PI(4,5)P₂ seems to act by providing a local environment at the membrane that promotes the co-recruitment and activation of signaling components. One therefore needs to know how PI(4,5)P₂ pools are activated locally. Since PI(4,5)P₂ is associated with lipid rafts, regulation likely involves mechanisms controlling raft recruitment. These may affect PI(4,5)P₂ synthesis at rafts, and/or the effectiveness of raft PI(4,5)P₂ in downstream signaling. It now turns out that a class of functionally related proteins that confers intrinsic competence for morphogenetic activity and regulated motility accumulates at and modulates PI(4,5)P₂ rafts. GAP43-like proteins may thus be a first group of PI(4,5)P₂ raft modulators. The fact that GAP43-like proteins can be regulated locally by calcium/calmodulin and PKC provides a possible link between extracellular signals and PI(4,5)P₂ raft regulation that can be explored experimentally. Given the function of GAP43-like proteins in clinically relevant processes such as neural plasticity, nerve regeneration and cell migration, the novel findings potentially have significant clinical implications.

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