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Mini Review

Requirements for and consequences of Rac-dependent protrusion

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ABSTRACT

Small GTPases of the Rac subfamily exert multiple functions, the most prominent of which includes stimulation of dynamic actin rearrangements at the cell periphery. Frequently, these actin reorganizations cause the protrusion of leaflets of plasma membrane, so-called lamellipodia, which remain anchored at flat surfaces during forward protrusion of migrating cells, or develop into ruffles when lifting up- and backwards. Ruffling membranes are also engaged in fluid and particle uptake during pino- and phagocytosis, respectively.

In recent work, we sought to clarify the precise role of Rac GTPases in actin-based protrusion, using a gene disruption approach. Furthermore, we aimed at dissecting the function of its downstream target Arp2/3 complex employing its instantaneous inhibition during simultaneous Rac activation. These complementary approaches allow comparison of the consequences of Rac *versus* Arp2/3 complex loss of function at the cell periphery, and help to formulate a working hypothesis for how the actin network in lamellipodia is initiated and maintained.

Introduction

The formation of actin networks in lamellipodia is most prominently triggered by activation of Rac small GTPases, which operate downstream of activation of cell surface or adhesion receptors both in normal and diseased cells (Cote and Vuori, 2007; Wertheimer et al., 2012). Although seminal studies by Hall and co-workers have early demonstrated the induction of lamellipodia formation and membrane ruffling by Rac (Ridley et al., 1992) and separability of these processes from protrusion of the more finger-like filopodia (Nobes and Hall, 1995), the molecular details of the actin polymerization events triggered downstream of this GTPase are just beginning to emerge. Understanding how actin filaments in lamellipodia are initiated, maintained and disassembled continues to be a challenging task, which is due in part to the multitude of signalling and biochemical activities involved, and to technical limits to study subcellular protrusions (Ridley, 2011; Rottner and Stradal, 2011). Researchers have thus taken advantage of a huge variety of methods to study different types of actin-based protrusions at the plasma membrane, ranging from analyses of dynamics and turnover of their molecular constituents and attempts to determine their molecular inventory (Cho and Klemke, 2002) to functional interference studies of multiple kinds, most frequently RNA interference. An alternative and complementary approach of increasing our understanding of actin assembly pathways at the plasma membrane remains unravelling the mechanisms of exploitation of cellular protrusion pathways by bacterial or viral pathogens. These bugs appear to have evolved remarkable analogies in their mechanisms employed to trigger cellular actin assembly events, which are increasingly appreciated (Welch and Way, 2013). Although the cell surface protrusions triggered by pathogens commonly display a more bundled appearance, reminiscent of filopodia rather than lamellipodia, the molecular mechanisms of their formation and the actin turnover observed in them in most cases and conditions appear analogous to pathways considered essential for lamellipodia. It is not surprising therefore that understanding actin assembly in all these structures is a story of understanding actin filament nucleation and branching by Arp2/3 complex and its activation (Rotty et al., 2013; Welch and Way, 2013). Much has been learned from comparing these different types of actin assembly mechanisms, but here we will focus on discussing our recent efforts to directly dissect the molecular pathways of actin-dependent lamellipodia formation.

Indeed, it is commonly agreed now that the initiation of lamellipodial actin networks requires nucleation and branching by Arp2/3-complex (Suraneni et al., 2012; Vinzenz et al.,

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2012; Wu et al., 2012). One prominent Arp2/3 complex activator was originally termed Scar (suppressor of cyclic AMP receptor) in the social amoeba *Dictyostelium discoideum*, the mammalian homologues of which (3 isoforms) are now more commonly called WAVE (Wiskott-Aldrich syndrome protein-family verprolin homologous protein). Although Arp2/3-complex activators alternative to Scar/WAVE potentially operating in lamellipodia have been described (Zuchero et al., 2009), a significant body of evidence now suggests that pentameric complexes harbouring Scar/WAVE proteins as Arp2/3-interaction and activating component are crucial for lamellipodia formation and membrane ruffling (Innocenti et al., 2004; Kunda et al., 2003; Rogers et al., 2003; Steffen et al., 2004). Furthermore, these WAVE complexes directly connect to upstream Rac GTPases through one of their subunits Sra-1/PIR121 (Kobayashi et al., 1998; Stradal et al., 2004), and this interaction is now established to drive WAVE complex activation, mediated by conformational changes and coincident with additional signals such as phosphoinositol-(3,4,5)-triphosphate (PIP3) (Chen et al., 2010; Ismail et al., 2009; Lebensohn and Kirschner, 2009). Additional contributions to WAVE complex activation and/or recruitment were more recently described for instance for Arf1 (Koronakis et al., 2011) and factors harbouring a consensus sequence termed WIRS (WAVE regulatory complex interacting receptor sequence) (Chen et al., 2014; Chia et al., 2014). Once activated and accumulating at the lamellipodium tip (Steffen et al., 2004), individual Scar/WAVE proteins stimulate continuous, Arp2/3-dependent filament generation, creating a lamellipodial actin network harbouring multiple, Arp2/3-containing branches flowing rearwards at rates coordinated with actin assembly at the tip (Lai et al., 2008).

Here, we summarize our recent efforts to define the precise relevance of Rac isoforms and their effector complexes in actin polymerization events mediating lamellipodium protrusion.

Lamellipodia formation requires at least one Rac isoform and downstream effector signalling to Arp2/3 complex activation

Although it is established since two decades that active, GTP-bound Rac GTPases can induce lamellipodia formation and membrane ruffling in multiple cell types (Hall, 1998), it remained unclear until recently whether the presence of one of the three Rac isoforms in mammals is essential for lamellipodia formation, or whether related small GTPases such as RhoG or Cdc42, which are also capable of inducing lamellipodia (Aspenstrom et al., 2004; Gauthier-Rouviere et al., 1998; Kozma et al., 1995; Nobes and Hall, 1995), are able to bypass Rac signalling to actin assembly in cell protrusions. For instance, using a chemotaxis assay towards platelet-derived growth factor in fibroblasts, Monypenny et al. concluded that Cdc42, Rac1 and RhoG act cooperatively in promoting migration and the formation of different types of protrusions, although Rac1 knockdown was already sufficient to block lamellipodia formation (Monypenny et al., 2009). Moreover, one previous study proposed not only a role for RhoG in Rac-independent migration, but also in Rac-independent membrane ruffling (Meller et al., 2008). Although Cdc42 can contribute to lamellipodia formation and membrane ruffling by multiple means, for instance signalling to Rac activation (Baird et al., 2005; Bosse et al., 2007; Nishimura et al., 2005) or activation of a lamellipodial formin (Block et al., 2012), it is not essential for the formation of these structures (Czuchra et al., 2005). However, it was hitherto difficult to exclude that Cdc42 drives, at least to a certain extent, Rac-independent lamellipodia-like structures.

To clarify these issues, we recently established fibroblast cell lines permanently deleted for the Rac1 gene and lacking any detectable expression of Rac2 and Rac3 (Steffen et al., 2013). Expression of RhoG and Cdc42 in these lines was comparable to the parental control clone (Steffen et al., 2013 and unpublished data), homozygous for the loxP-site flanked Rac1 allele (Chrostek et al., 2006). Importantly, none of five independently generated clones was capable of forming lamellipodia, excluding a clonal artefact, and expression of Rac1, Rac2 or Rac3 equally potently restored lamellipodia formation in Rac1-deficient cells, as judged from quantifying the frequency of cells expressing lamellipodia upon transient transfection of active versions of either small GTPase. In contrast, over-expression of Cdc42 or RhoG in the absence of Rac failed to induce lamellipodia formation (Steffen et al., 2013). These data were fully consistent with pull-down experiments showing that all Rac isoforms but neither Cdc42 nor RhoG were capable of interacting with the effector WAVE complex. Based on these data, we concluded that Cdc42 and RhoG are unable to bypass Rac signalling to WAVE complex and consequently Arp2/3 complex activation during lamellipodia formation, and that they induce formation of these structures in control cells exclusively through Rac (Fig. 1).

Arp2/3 complex is essential for both initiation and maintenance of lamellipodial actin networks

Previous studies established that reducing expression of Arp2/3 complex by RNA interference (Nicholson-Dykstra and Higgs, 2008; Steffen et al., 2006; Wu et al., 2012) or eliminating it by genetic disruption (Suraneni et al., 2012) has multiple effects on actin assembly events at the cell periphery, most prominently lamellipodia and ruffle formation as well as cell spreading. In addition, over-expression of the C-terminal end of Scar/WAVE1 harbouring interaction surfaces for Arp2/3 complex and actin was previously shown to suppress lamellipodia formation (Machesky and Insall, 1998), likely mediated by competing away Arp2/3 complex from its activation at the cell periphery. However, none of these long-term treatments allowed firm statements on the relevance of Arp2/3 complex for the maintenance of already established lamellipodia, since their disappearance upon reduction or elimination of gene expression could have also been caused by the lack of lamellipodia initiation. Alternatively, phenotypes might have also been caused by more indirect effects such as changed Rho GTPase activities as observed for instance upon RNAi-mediated Arp2/3 complex knockdown in neuronal cells (Korobova and Svitkina, 2008).

Notably, the dendritic nucleation model of formation of branched actin networks such as those found in lamellipodia implies continuous, Arp2/3 complex dependent branching (Pollard and Borisy, 2003), although the essential requirement of this branching activity for continuous lamellipodium protrusion has until recently not been experimentally confirmed. Nevertheless, continuous incorporation of Arp2/3 complex was already evident from fluorescence recovery after photobleaching of fluorescently tagged Arp2/3 complex subunits (Lai et al., 2008), consistent with the idea that continuous WAVE complex-mediated branching by Arp2/3 complex is required not only for network initiation, but also for its maintenance. To prove this hypothesis, we recently developed a novel approach to explore the consequences of instantaneous Arp2/3 complex inhibition during lamellipodia protrusion of living cells. To do this, cells were first transfected with constitutively active Rac to generate homogeneous cell populations comprising multiple cells with comparable and experimentally tractable lamellipodia. These cells were subsequently microinjected with constitutively active Rac1 protein as control or the same concentration of constitutively active Rac1 protein mixed with the WCA-domain of Scar1, established previously to sequester Arp2/3 complex in the cytosol (Machesky and Insall, 1998). Cells co-expressing fluorescently-tagged actin or different actin-binding proteins were analyzed by time-lapse microscopy before and after microinjections to carefully document changes in cell behaviour

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