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Drinking problems: Mechanisms of macropinosome formation and maturation

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Abbreviations

BMDMs- bone marrow-derived macrophages

CME- clathrin-mediated endocytosis

EGF- epidermal growth factor

GAPs- GTPase activating proteins

GDI- GDP dissociation inhibitor

GEFs- guanine nucleotide exchange factors

M-CSF- macrophage colony stimulating factor

mTORC1- mechanistic target of rapamycin complex 1

PAE cells- primary aortic endothelial cells

PDGF- platelet-derived growth factor

PIP- phosphoinositide/phosphatidylinositol phosphate

SNX- sorting nexin

WASH- WASP and SCAR homologue

Keywords

Macropinocytosis, phagocytosis, macropinosome, vesicle trafficking, phosphoinositides

Abstract

Macropinocytosis is a mechanism for the non-specific bulk uptake and internalisation of extracellular fluid. This plays specific and distinct roles in diverse cell types such as macrophages, dendritic cells and neurons, by allowing cells to sample their environment, extract extracellular nutrients and regulate plasma membrane turnover. Macropinocytosis has recently been implicated in several diseases including cancer, neurodegenerative diseases and atherosclerosis. Uptake by macropinocytosis is also exploited by several intracellular pathogens to gain entry into host cells.

Both capturing and subsequently processing large volumes of extracellular fluid poses a number of unique challenges for the cell. Macropinosome formation requires co-ordinated three-dimensional manipulation of the cytoskeleton to form shaped protrusions able to entrap extracellular fluid. The following maturation of these large vesicles then involves a complex series of membrane rearrangements to shrink and concentrate their contents, whilst delivering components required for digestion and recycling.

Recognition of the diverse importance of macropinocytosis in physiology and disease has prompted a number of recent studies. In this article we summarise advances in our understanding of both macropinosome formation and maturation, and seek to highlight the important unanswered questions.

Introduction

Macropinocytosis is a mechanism for the non-specific bulk uptake and internalisation of extracellular fluid. This ancient and conserved process shares many similarities with other types of endocytosis, such as phagocytosis and clathrin-mediated endocytosis, yet plays specific and distinct roles that are important in normal cellular function, various diseases, as well as potential therapeutic use for drug delivery [1-3].

Macropinocytosis occurs in many different cell types and organisms, where it has specialised functions. Both amoebae and cancer cells use macropinocytosis to obtain nutrients from the environment [4], whilst immune cells such as macrophages and dendritic cells use it to survey their external environment and capture antigens for presentation to T-cells. In contrast, in neurons macropinocytosis enables modulation of synapse signalling by regulating the amounts of cell surface receptors [5].

Recently, interest in macropinocytosis has been piqued due to the discovery of its importance in several diseases such as the cell-to-cell transmission of prions in neurodegenerative diseases, and pathogenesis of atherosclerosis and cancer [4, 6-8]. Macropinocytosis also offers a way for infectious agents to enter the cell and is thus inevitably subverted by several pathogens to establish intracellular niches - enhancing survival and driving infection [9, 10].

Whilst macropinocytosis has historically been the ugly duckling of endocytic pathways, largely ignored in comparison to its cousins, its importance is fast becoming recognised. This has led to many advances in our understanding of the mechanisms that drive macropinosome formation, and subsequently regulate their maturation.

How do you construct a macropinocytic cup?

Generating protrusions that will efficiently internalize extracellular fluid requires complex spatial regulation of the cytoskeletal machinery. Cells generally achieve this by generating a circular ring or ruffle of protruding membrane encircling a region that remains stationary[11] (Figure 1). The formation of this 3-dimensional shape requires additional spatial information and regulation over that required for less complex projections such as pseudopodia and lamellipodia. There also needs to be temporal control allowing cups to switch from phases of extension to closure. How this is achieved has been the subject of several recent studies, leading to significant advances in our understanding of how macropinosomes form.

Macropinocytic cup formation is highly related to that of phagocytic cups and although most studies point to involvement of conserved cytoskeletal machinery, there are several important differences. Firstly, the two pathways differ in their initiation; whilst phagosomes are induced locally upon engagement of surface receptors with a target particle, macropinosomes form spontaneously. Indeed, whilst the frequency of macropinosome formation can be dynamically regulated in response to factors such as immune cell activation and growth factor stimulation [2], these signals are diffuse and do not provide local information. In the context of macropinosome initiation therefore, cup formation is stochastic, with the plasma membrane acting as an excitable medium.

Secondly, whilst solid particles provide a template for phagosome formation, macropinocytic cups form independent of any physical guide or localized receptor activation (Figure 1). Current models of Fc receptor-mediated phagocytosis both predict and depend on a zippering mechanism by which receptors bind to the surface of the particle, driving extension along its surface [12-15]. During macropinocytosis however, intrinsic mechanisms alone can be sufficient for cup formation and internalization. Whether this means that models of phagocytosis are incomplete, or macropinosome self-assembly uses a different mechanism is unclear and requires further study.

Thirdly, macropinocytic cups are not necessarily formed *de novo* each time. Recent analysis of macropinosome dynamics in *Dictyostelium* found that cups were frequently produced by the splitting of pre-existing ones [16]. Similar behavior has been described for pseudopods, which spontaneously self-assemble and proliferate by splitting in both amoebae and immune cells [17-19]. The underlying mechanism of excitable actin polymerization and extension is therefore likely to be conserved between both types of protrusion, and indeed macropinosome and pseudopod formation appear to be in direct competition [20].

It is important to note that macropinosomes may be assembled differently in specific contexts. For example, a comparison of constitutive and macrophage colony-stimulating factor (M-CSF)-induced macropinocytosis in primary macrophages found that not only were the stimulated macropinosomes much larger, they were insensitive to removal of extracellular calcium, which completely blocked the constitutive pathway [21]. Although different in initiation and size, how mechanistically dissimilar the biomechanics of cup construction is in these two conditions remains unclear. A number of recent studies have however begun to establish a more detailed understanding of the spatio-temporal events that lead to macropinosome formation in a range of experimental systems.

Temporal signals during cup formation

Multiple roles for Rho and Ras family small GTPases

Although many of the major players such as small GTPases, cytoskeletal proteins and inositol phospholipids have been identified, how they are spatially and temporally organized over the large distances involved in macropinocytic cup formation is poorly understood [22]. Perhaps the best characterized components are the Rho family of small GTPases, which switch between inactive GDP-bound to active GTP-bound forms by the activity of specific Guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs).

In particular, the Rho family member Rac1 is crucial for ruffle and macropinosome formation in diverse cell types including dendritic cells, macrophages, amoebae and fibroblasts [23-25]. The recent development of optogenetic tools to transiently manipulate Rac1 activity has elegantly demonstrated that Rac1 activation is sufficient to drive ruffle formation in macrophages [26]. However, the authors also found that the subsequent deactivation of Rac1 is equally important

as it allows ruffle collapse and closure, which are essential for successful fluid capture. This is supported by quantitative microscopy studies that demonstrate that Rac1 is physiologically deactivated just prior to closure [27, 28].

The Ras family of small GTPases are also important in macropinocytosis. Expression or injection of constitutively activate (oncogenic) Ras is sufficient to induce ruffling and macropinocytosis in fibroblasts [4, 29-31] and active Ras localizes to macropinocytic cups in both macrophages and *Dictyostelium* [16, 32, 33]. Ras functionally sits between growth factor receptors and activation of class I PI-3 kinases via their Ras-binding domains, providing a direct mechanism for stimulated macropinocytosis [34, 35]. However, how Ras is stochastically activated and spatially restricted during constitutive macropinocytosis is less well understood.

Complex regulation of phosphoinositide dynamics

Macropinosome formation needs to be temporally regulated so that cups know when to stop extending and start closing. This is currently not well defined, however many of the events appear to be coordinated by phosphoinositide (PIP) signaling. Interconversion of PIP species by a family of kinases and phosphatases allows specific effector proteins to be recruited in a highly regulated manner and is exploited by a wide range of plasma membrane and membrane trafficking pathways (review in depth in [36, 37]. Transient and sequential peaks of different phosphoinositides occur during macropinosome formation, for example in both growth-factor stimulated cancer cells (A431) and M-CSF stimulated macrophages, cup formation starts with a localized ~2-fold elevation of $PI(4,5)P_2$ at membrane ruffles, before a much stronger accumulation of $PI(3,4,5)P_3$, peaking just before closure [28, 32, 38].

The role of $PI(3,4,5)P_3$ appears to be complex; whilst in PDGF-stimulated PAE cells it appears to be required for ruffle formation [39], others report a much later role during cup closure in both EGF-stimulated A431 cells and M-CSF-stimulated BMDMs [38, 40]. Surprisingly, both functions can be observed in *Dictysotelium* cells, with distinct class I PI-3-kinases responsible for either ruffle formation or cup closure [41]. How these kinases exert distinct functions is not known, but may depend on interactions with the enzymes themselves rather than $PI(3,4,5)P_3$ production. As many of the mammalian studies rely on global class I PI-3-kinase inhibitors, the role of PI-3-kinases and $PI(3,4,5)P_3$ may be similarly complex and nuanced in these cells.

In addition to its production, $PI(3,4,5)P_3$ breakdown is implicated in macropinocytic cup closure, leading to subsequent peaks of $PI(3,4)P_2$ then PI(3)P, as observed in M-CSF stimulated macrophages and *Dictyostelium* [28, 32, 42]. Depletion of the inositol-5-phosphatase SHIP2, 4-phosphatase INNP4B, or 3-phosphatases MTMR6 and 9 leads to defective fluid entrapment but not ruffling in EGF-stimulated A431 cells [43, 44]. Therefore progressive dephosphorylation of $PI(3,4,5)P_3$ appears to be important for cup closure in these cells. Whether $PI(3,4)P_2$ plays a direct role is not clear, however PI(3)P was shown to directly activate the Ca^{2+}-activated K^+ channel KCa3.1 at ruffles, driving closure by an unknown mechanism [43].

The spatial organisation of macropinocytic cups

The formation of a macropinocytic cup requires the self-assembly of a circular protrusion many hundreds of nanometers in diameter, whilst blocking extension in the interior (Figure 1). This membrane protrusion is driven by localized actin polymerization, which must be tightly regulated by the large-scale spatial regulation of factors such as those described above.

Perhaps the clearest description of the organization of macropinocytic cups comes from recent studies in *Dictyostelium*. Laboratory strains of this amoeba contain mutations in the RasGAP Neurofibromin (NF1) causing excessive Ras activation and the formation of oversized macropinocytic cups [33]. This demonstrates the instructive role of RasGAPs in cup formation, and provides a convenient system to interrogate cup structure due to the large and frequent macropinosomes produced by these cells.

Using lattice light-sheet microscopy to watch cups form in 3-dimensions Veltman *et al.* describe a mechanism whereby self-organising patches of active Ras and $PI(3,4,5)P_3$ recruit a ring of SCAR/WAVE complex to their periphery [16]. SCAR drives local actin polymerisation via activation of the Arp2/3 complex producing a circular ruffle. These patches of Ras/$PI(3,4,5)P_3$ therefore instruct and define the non-protruding inner surface of the cup and differentiate them from pseudopodia that are also generated by SCAR, but lack $PI(3,4,5)P_3$ (Figure 2) [16, 20].

How SCAR activity and protrusion is restricted to the cup lip however is not understood. SCAR is downstream of Rac1, but probes for activated Rac1 indicate a localization that mirrors active Ras and PI(3,4,5)P_3 - being uniformly present throughout the cup interior in both *Dictyostelium* and macrophages [16, 28]. Rac1 may therefore be permissive in driving protrusion, however other, as yet unknown signals must be required that either provide positive reinforcement of SCAR recruitment at the lip or suppress it at the cup base.

SCAR is not the only actin regulator to be implicated in macropinosome formation. In contrast to the branched networks of actin filaments generated by Arp2/3 activation, nucleation and elongation of linear filaments can be driven by the activity of Formin dimers [45]. Whilst SCAR is restricted to the lip, a *Diaphanous* related formin, ForG, plays a reciprocal role by localizing to and directing actin polymerisation at the base of *Dictyostelium* macropinocytic cups [46]. This implies structural differences in the actin network of the base versus the rim, and may allow formins to generate a stabilizing network parallel to the membrane at the sides to support the cup shape, whilst Arp2/3-derived perpendicular filaments at the extending rim drive protrusion (Figure 2).

Another important question is how the localized signaling of rapidly diffusible factors such as lipids and membrane associated proteins is maintained. Lipid diffusion barriers have been demonstrated in both phagosome and macropinosome formation in macrophages [47, 48]. In macropinosome formation, it has been proposed that this barrier is due to the encircling, actin-rich ruffle [32]. However experiments in *Dictyostelium* indicate that defined patches of Ras and PI(3,4,5)P_3 are still maintained after actin depolymerisation, implying that additional mechanisms are involved [16]. How this is achieved remains elusive and deserves further attention.

Many advances have been made in our understanding of macropinosome formation. Whilst universal principles are emerging, disparities exist between observations in different experimental systems highlighting that, like phagocytosis, macropinosome formation is more than a single process. Whether this represents multiple independent mechanisms or variations of a core mechanism, remains to be determined. In particular it is unclear how much the dramatic bursts of ruffling and fluid-phase uptake caused by stimulation of macrophages or fibroblasts correspond to the constitutive pathway in antigen presenting cells or amoebae. The

picture is also muddied by the fact that fluid can seemingly be captured by the collapsing of any sheet-like projection, and the catch-all term "ruffle" is perhaps applied too generally to structures that may appear physically similar, but may be generated by different mechanisms. In every case however, the ultimate result is the production of a large fluid-filled intracellular vesicle. How the cell subsequently processes this has also been the subject of much recent progress, and is discussed below.

Macropinosome maturation

Closure and fission of the macropinocytic cup results in the formation of a large, aqueous-filled vesicle that must be processed by the cell. These may not be processed in the same way by all cells, but macropinosomes destined for antigen presentation or used to feed the cell must undergo a defined sequence of maturation steps in order to digest and process their contents. In contrast macropinosomes in A431 carcinoma cells traffic directly back to the surface without lysosomal fusion [49, 50]. The basis for this difference is unknown, and the majority of studies are in cells that produce degradative macropinosomes, which are the focus of this section. Like other endocytic pathways, macropinosome maturation is highly regulated and although much less studied, has become much better defined in recent years.

Early phases of macropinosome maturation

Over the first 10-20 minutes of maturation, macropinosomes undergo dramatic remodelling. During this period, they undergo fission and rapidly shrink, concentrating their contents. This happens at the same time as vesicles containing the vacuolar (V)-ATPase and digestive enzymes are delivered, producing the acidic and hydrolytic environment required for efficient digestion. These complex trafficking steps are orchestrated by the sequential recruitment of regulatory molecules such as members of the Rab family of small GTPases and PIPs, to deliver specific proteins to endocytic vesicles at defined times [51, 52].

Rab5 is the first such protein to be recruited to macropinosomes, and its activity increases during early maturation before exchanging with Rab7 [53]. Rab5 also recruits the class III PI3 kinase Vps34, which generates PI(3)P by phosphorylation of PI and is present on macropinosome for around ten minutes post-internalisation [2, 54]. PI(3)P then also recruits

various effector proteins, via either FYVE- or PX/PH lipid-binding domains [55-57]. Other Rab GTPases such as Rab20 and Rab21, and presumably their effectors, also sequentially and transiently associate over this period [58, 59] indicating that macropinosome identity undergoes gradual, graded changes as it matures (summarized in Figure 3 and reviewed in detail by [52]).

Whilst this stereotyped maturation cascade is becoming better defined (Figure 3), how macropinosome fission from the plasma membrane is detected, and how the initial Rab5 recruitment is mediated are important unanswered questions. Interestingly, whilst the recruitment and function of Rab5 is restricted to internal vesicles during CME, Rab5 has been reported in the surface ruffles of both Ras-activated COS-7 cells and M-CSF stimulated macrophages prior to cup closure [28, 30, 53]. A direct functional role or activation of Rab5 during cup closure however awaits further clarification, and has yet to be examined during constitutive macropinocytosis in mammalian cells.

Recycling of plasma membrane proteins

Macropinosome formation results in the non-specific internalisation of large portions of the plasma membrane [2, 60, 61]. Alongside membrane, surface proteins such as phagocytic receptors will also be internalised and subject to degradation. This poses a significant problem, particularly in cells undergoing constitutive macropinocytosis such as macrophages which are estimated to internalise their entire cell surface by this pathway in ~30mins [61]. To maintain steady state level of cell surface proteins, they must be rapidly recycled from the macropinosome to prevent their degradation.

Recently, we showed that recycling from early macropinosomes is driven by the activity of the Wiscott-Aldrich and SCAR homologue (WASH) complex and the retromer sorting complex [62]. Like SCAR, WASH is an activator of the Arp2/3 complex, but is responsible for generating patches of actin on intracellular vesicles [63, 64]. The retromer complex is made up of three Vps subunits (Vps35, Vps26 and Vsp29) and a sorting nexin heterodimer (SNX1/SNX2 and SNX5/SNX6), and mediates retrieval from several endocytic compartments [65-67]. The WASH and retromer complexes directly interact, sequestering the retromer and its cargos into actin subdomains on the surface of vesicles and driving their retrieval into recycling vesicles [68].

Rescuing cell surface proteins from macropinosomes needs to occur very early during maturation. Consistent with this, in *Dictyostelium* both WASH and retromer are recruited immediately after internalisation, with a burst of activity that lasts only 2 minutes [62]. This transient flurry of recycling is essential for cells to maintain surface levels of proteins such as integrin receptors, and maintain their phagocytic capacity.

How such an acute recruitment of WASH and the retromer is achieved is unclear. The SNX1 and SNX5 components of the retromer complex are recruited to early macropinosomes by binding to PI(3)P [69-71] but this signal persists much longer than the 2 minutes where WASH and retromer activity are observed [55-57]. Other studies found that the Vps subunits of the retromer require active GTP-bound Rab7 for recruitment to endosomes [72, 73]. However Rab7 localisation in macropinosomes also peaks far later than WASH or retromer [74]. Additional regulatory mechanisms must therefore exist.

In *Dictyostelium* the situation is further complicated by the presence of a second, much later phase of WASH and retromer recruitment to macropinosomes. In these cells, after digestion is complete, WASH and the retromer drive neutralisation and hydrolase retrieval from macropinosomes before they fuse with the plasma membrane, expelling any indigestible material [75-77]. There are therefore discrete phases and targets for WASH and retromer activity during maturation, under complex temporal and functional control.

Tubulation and fission of macropinosomes

As they mature, macropinosomes shrink and become more concentrated, presumably to facilitate acidification and digestion. This occurs by tubulation and fission, in which portions of the membrane are pinched off from tubular protrusions, allowing both proteins and membrane to be extracted. How tubulation and fission are mediated and regulated is still under-studied, but a number of important players have been identified.

Obvious candidates for driving tubule formation on macropinosomes are the sorting nexins (SNX) family, which have the ability to oligomerise and physically induce membrane curvature via their BAR domains [78]. Tubule formation was described to require the sorting nexin SNX5, which colocalises with Rabankyrin-5, a Rab5 effector protein, on macropinosomes [69, 71, 79]. In agreement with previous findings, localisation of SNX5 to macropinosomes was dependent

on SNX1, which binds to PI(3)P [79, 80]. Shortly after internalisation SNX5-positive tubules were visible projecting from the macropinosomes that later subsided, consistent with the early nature of protein recycling.

More recently, it was shown that $PI(3,5)P_2$ also plays an important regulatory role in early macropinosome maturation. $PI(3,5)P_2$ is formed by the phosphorylation of PI(3)P by the kinase PIKfyve (Fab1 in yeast), which is also recruited by PI(3)P via its FYVE domain [81]. PIKfyve is therefore recruited to early macropinosomes and is required for both shrinkage, and nutrient export in both macrophages and fibroblasts [57, 82].

Depletion of PIKfyve leads to the formation of enlarged vesicles in all cell types and organisms used to study it, implying a general role in vesicular fission [57, 82-87]. However the precise function of $PI(3,5)P_2$ and identity of its effector proteins remains subject to much debate. A major problem has been the lack of reliable reporters. One reported effector is the calcium channel TRPML1 (mucolipin), which is activated by $PI(3,5)P_2$ [88]. Whilst the lipid binding domain of TRPML1 has begun to be used as a reporter [89-91] others report that this probe is not completely specific [92]. The precise dynamics of $PI(3,5)P_2$ during macropinosome maturation are therefore not entirely clear.

Nonetheless, disruption of TRPML1 caused similar swollen endosomal defects, and over-expression of active TRPML1, or treatment with a synthetic TRPML1 agonist alleviates the swollen vesicle phenotype in $PI(3,5)P_2$ depleted cells [82]. This suggests that at least some of the macropinosome maturation defects caused by $PI(3,5)P_2$ depletion are via TRPML1, although whether or not this is the sole effector protein involved remains to be determined.

How PIKfyve and TRPML1 mediate macropinosome shrinkage is not well understood. TRPML1 is a Ca^{2+} efflux channel that localises to late endosomes/lysosomes, and under some conditions to phagosomes [88-90, 93]. Recently however it was demonstrated that TRPML1 regulates interactions between lysosomes and the microtubule minus-end motor dynein, mediating lysosomal movement along microtubules [94]. Microtubules also associate with SNX5-containing tubules during macropinosome maturation [79]. Little is known about how macropinosomes interact with microtubules, but the $PI(3,5)P_2$-TRPML1-dynein pathway provides a plausible mechanism to drive fission.

Regulation of macropinosome fusion

Whilst macropinosomes are shrinking, vesicle fusion is also important for delivery of key components of maturation to macropinosomes, for example the recruitment of the V-ATPase and delivery of hydrolases and proteases. Interestingly, whilst disruption of either PIKfyve or TRPML1 cause defects in phago-lysosomal fusion and degradation [57, 93], acidification and digestion of macropinosomes appears to be unaffected [82]. Whether this implies a fundamental difference between the regulation of phagosome and macropinosome maturation, or different requirements for cells to process solid particles versus aqueous vesicles is not clear.

Whilst the role of TRPML1 in macropinosome fusion is unclear, a recent paper identified a member of the septin family, SEPT2, as being involved in macropinosome-lysosome fusion in mammalian epithelial cells [95]. Septins are filamentous GTPases that form higher-order cytoskeletal structures on various membranes [96]. SEPT2 localises to sites where macropinosomes contact other vesicles and appears to facilitate the fusion event, as SEPT2 knockdown caused accumulation of clusters of unfused, but docked macropinosomes. SEPT2 localised more to compartments containing Rab7 than on Rab5 and its recruitment was reduced by inhibition of PIKfyve, suggesting a specific role in later macropinosome fusion [95]. Whilst septins and TRPML1 clearly play important roles in fusion, how this is mechanistically achieved is unclear but could involve similar proteins to those involved in docking and vesicle fusion in endosomes such as SNARE and VAMP proteins, but have yet to be investigated in the context of macropinocytosis [97].

Macropinosomes as nutrient sensors

Linked to its role in nutrient capture, there have been several studies describing roles for macropinocytosis in nutrient sensing. As they are sampling the external environment, macropinosomes are ideally placed to detect and mediate responses to changes in extracellular nutrient availability. A core metabolic regulator is the mechanistic target of rapamycin complex 1 (mTORC1), which both activates autophagy and inhibits protein synthesis upon starvation or growth factor stimulation [98]. Recently it was shown that macropinocytosis is both regulated by mTORC1 [99], and is required for growth-factor dependent activation of mTORC1 by amino acids [100].

Several recent reports have also pointed to a role for $PI(3,5)P_2$ in mTORC1 regulation as PIKfyve inhibition leads to a decrease in mTORC1 activation, and decreased association with endolysosomes [82, 100, 101]. Whether mTORC1 directly associates with macropinosomes is unclear, although mTORC1 is also required for macropinosome fission and shrinkage independently of PIKfyve and TRPML1 [82].

Late stages of maturation

The later stages of macropinosome maturation, beyond $PI(3,5)P_2$ synthesis, are much less well defined. Macropinosomes acquire late lysosomal markers such as LAMP1, potentially via TRPML1-mediated vesicle fusion, and become Rab7 positive and Rab5 negative [2]. This exchange marks the later stages of macropinosome maturation and requires Rab5-GTP hydrolysis and release from the membrane, along with recruitment and activation of Rab7 [102].

On mammalian endosomes, this transition is mediated by the Mon1-Ccz1 complex [103]. Mon1a preferentially interacts with Rab5 and together with Ccz1 forms a complex which can dissociate Rab7-GDI, allowing recruitment and activation Rab7 through the complex's GEF activity. Loss of Mon1 leads to drastic acidification defects akin to those seen in cells overexpressing dominant negative Rab5 or Rab7.

Recently the dynamics of Rab5-Rab7 exchange during macropinocytosis was described [74]. Rab7 gradually accumulates on macropinosomes, reaching an intermediate level at 10 minutes post-internalisation, and continues to increase until it peaks 20-40 minutes after internalisation. This suggests that moderate amounts of Rab7 and some Rab5 are present during intermediate phases of maturation, whereas high levels of Rab7 and negligible Rab5 are present during late stages. In agreement with this the Mon1-Ccz1 complex also increases gradually, peaking at 10 minutes, suggesting an increasing gradient of Rab7 activity. Overlapping gradients of different Rab proteins are also important for tubule formation and retromer recruitment during endosome maturation [104], and could also be important for coordinating specific hydrolase delivery.

As of yet, the PIP composition of late macropinosomes is somewhat mysterious. PI(3)P, the first phospholipid to localise to macropinosomes is lost around ten minutes after internalisation, most likely by the myotubularin family of inositol 3-phosphatases [55-57]. Two recent papers have shown that PI(4)P is present at later stages of phagosome maturation [105, 106]. The PI(4)P present on late phagosomes and lysosomes is synthesised by phosphorylation of PI by class II PI4Kα and corresponds with the loss of PI(3)P and emergence of Rab7 [106]. Inhibition of PI(4)P formation prevents late phagolysosomal fusion, and by analogy, may play a similar role in macropinosome maturation [105].

Both TRPML1 and PI(4)P have also been observed transiently localising to phagocytic cups where TRMPL1 at least appears to be important for providing membrane required for cups to engulf large particles [90, 106]. It is therefore interesting to speculate that TRPML1-positive lysosomes might also be PI(4)P positive, marking them as fully matured and ready for fusion. Whether PI(4)P is present on mature macropinosomes or local exocytosis is required for macropinocytic cup formation however remains to be determined.

Future perspectives

Recent years have seen a significant increase in our understanding of macropinocytosis, in a variety of physiological settings. It is now well established that macropinocytosis is an important and highly regulated endocytic pathway. A deeper understanding of the mechanisms underlying cup formation and macropinosome processing will be crucial to determine how they are subverted by pathogens and diseases such as cancer.

The formation and processing of these large vesicles also provides an amenable system to study the fundamental mechanisms regulating the actin cytoskeleton and membrane trafficking. At all stages there is significant crossover with other pathways such as cell motility, phagocytosis and clathrin-mediated endocytosis. The lessons learned from macropinocytosis will therefore have implications beyond bulk fluid uptake.

Macropinocytosis offers significant promise both as a therapeutic target and a mechanism for drug delivery [107-110]. However, the vast majority of studies are restricted to cultured cells. Whilst this had been critical for the mechanistic advances seen in recent years, the role of macropinocytosis *in vivo* remains virtually unexplored. This leaves a significant gap in our understanding of the broader physiological significance of macropinocytosis that must be addressed by future studies. Nonetheless, the rate of progress has been rapid, and improvements in genetic tools and imaging will doubtless continue to further our understanding in the years to come.

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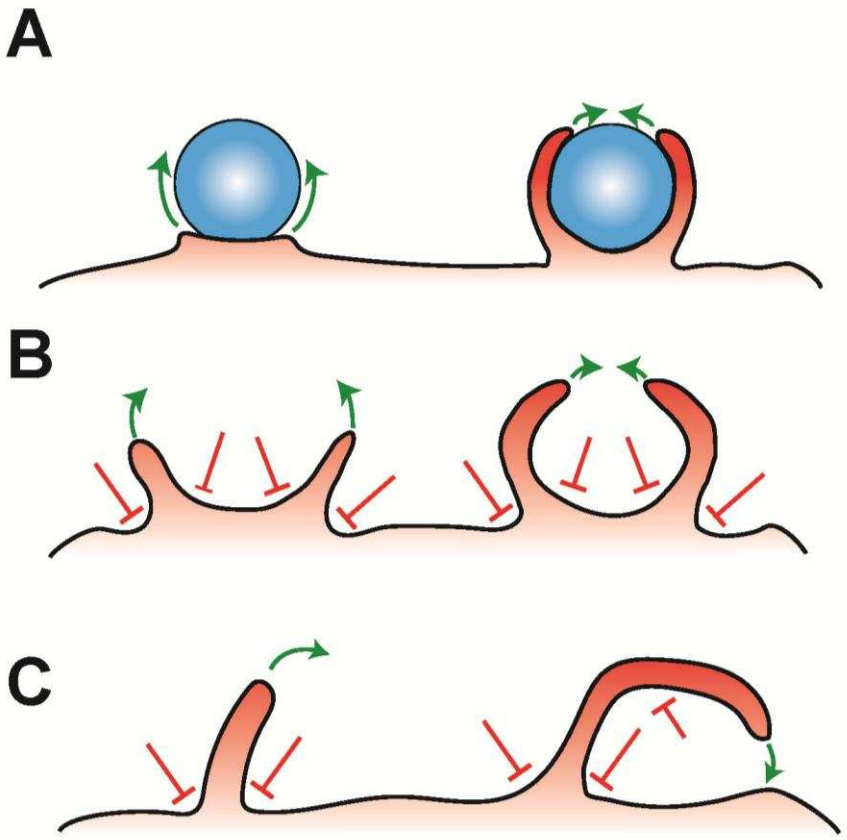

Figure 1: Membrane arrangements during macropinosome formation

Figure 1: Membrane rearrangements during macropinosome formation. (A) During Fc-mediated phagocytosis, protrusions are stimulated by, and extend around a physical particle. (B) In contrast, circular ruffles that form macropinosomes must self-assemble independently. This requires highly localised protrusion, whilst restricting extension of adjoining areas both inside, and outside the cup. (C) Linear ruffles also require similar spatial restriction of protrusions in order to generate a productive macropinosome.

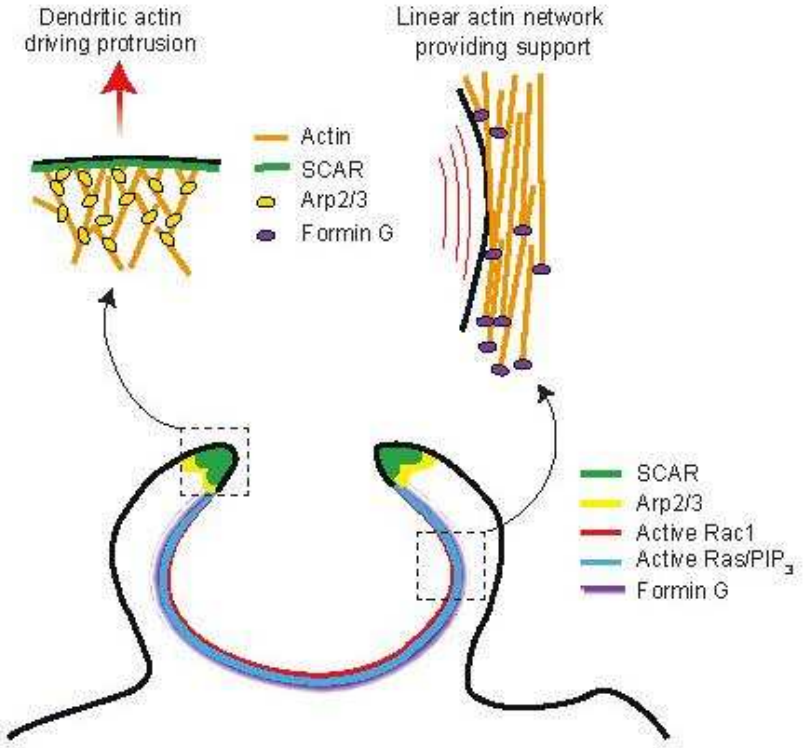

Figure 2: Model of spatial organisation and actin regulation during cup formation

Figure 2: Proposed model of actin structure and regulation in macropinocytic cups, based on studies in *Dictyostelium*. SCAR/WAVE and Arp2/3 activity are restricted to the cup rim, causing the formation of a branched, dendritic actin network perpendicular to the membrane, driving protrusion. In contrast, subsequent linear extension of these filaments driven by Formin G, causes the production of long filaments parallel to the membrane along the cup interior. This provides structural reinforcement, stabilizing the cup shape in the absence of a physical scaffold.

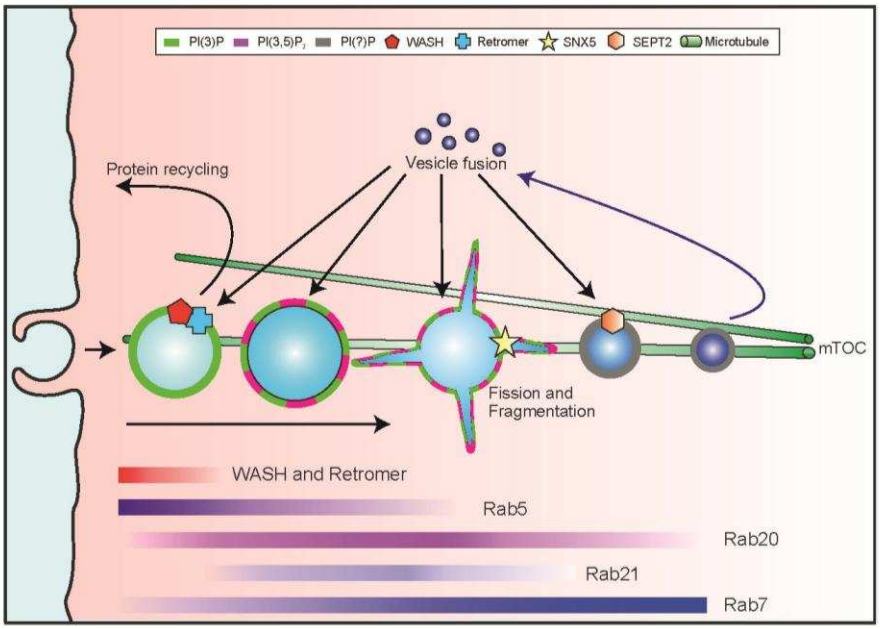

Figure 3: Regulation and progression of macropinosome maturation

Figure 3: Schematic overview of macropinosome maturation. After formation, macropinosomes undergo a series of rearrangements. This starts with the WASH/retromer driven retrieval of plasma membrane proteins, and is accompanied by gradual transitions in Rab GTPase recruitment and phosphoinositide coimposition. During this progression, there is both fusion with vesicles containing lysosomal components as well as tubulation and shrinkage, most likely assisted by microtubule interactions.