Macropinocytosis

Joel A. Swanson and Colin Watts

Macropinocytosis is a form of endocytosis that accompanies cell surface ruffling. It is distinct in many ways from the better characterized micropinocytosis, which includes clathrin-coated vesicle endocytosis and small uncoated vesicles. Because macropinosomes are relatively large, they provide an efficient route for non-selective endocytosis of solute macromolecules. This route may facilitate MHC-class-II-restricted antigen presentation by dendritic cells. Because the ruffling that leads to macropinocytosis is regulated, it has been exploited by some pathogenic bacteria as a novel route for entry into cells.

Virtually all cells use endocytosis to take up nutrients from the external milieu and to modulate the expression of cell surface molecules. Although the pathway initiated by clathrin-coated pits has received the most attention because of its importance in receptor-mediated capture of external ligands, it has recently become clear that other endocytic pathways exist, which serve additional physiological functions. One of these pathways is macropinocytosis.

Macropinocytosis was discovered by Warren Lewis in the early days of time-lapse microcinematography[1]. Speeding up the normally slow movements of macrophages revealed waving cell surface ruffles that closed back against the plasma membrane to form intracellular vesicles. Lewis named this activity pinocytosis, but it was later renamed macropinocytosis to distinguish it from endocytic processes involving smaller, clathrin-coated or uncoated vesicles[2,3]. Since then, macropinocytosis has been seen in a variety of metazoan cells, operating in macrophages and many tumour cells constitutively, and in other cells after stimulation with growth factors or phorbol esters.

Although it has been recognized for some time, the relevance of macropinocytosis to other cellular processes, such as growth or movement, has remained elusive. Recently, it has been implicated in pathogenesis, in one instance serving the host and, in another, the pathogen. Here, we summarize the regulation of macropinocytosis and its newfound relevance to host–pathogen interactions.

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Mechanics of macro- versus micropinocytosis

Macropinocytosis and clathrin-mediated endocytosis differ in several important respects. Macropinosomes form primarily at sites of ruffling, usually at the margins of spread cells, and are heterogeneous in size, sometimes as large as 5 μm in diameter[2,4,5] (Fig. 1). By contrast, coated pits appear more or less uniformly over the cell surface[6] and are restricted to a uniform size by the clathrin coat (85–110 nm diameter). This coat, together with associated proteins, also concentrates some receptors for growth factors and nutrients into vesicles. Although coated-vesicle-mediated endocytosis internalizes both non-selectively and selectively, at low ligand concentrations the selective, receptor-mediated endocytosis through coated vesicles is much more efficient than the non-selective uptake by bulk-fluid endocytosis into the small vesicle lumina. Conversely, macropinosomes have no discernible coat and do not concentrate receptors[7]. Rather, they most likely contain membrane compositionally similar to plasma membrane. Because they are generally large organelles, their surface-to-volume ratio is lower than that of smaller vesicles, and, consequently, they internalize true fluid-phase solutes more efficiently, per unit area of membrane, than smaller vesicles can[7]. They therefore provide a comparatively efficient, though non-selective, mechanism for internalizing extracellular macromolecules[8].

Little is known of the biochemistry and regulation of uncoated micropinocytic vesicles (reviewed in Ref. 9). In many respects, they may be more similar to macropinosomes than to coated vesicles. Although uncoated micropinosomes may represent the lower size-range for what is called macropinocytosis, their small size precludes them from internalizing significant volumes of extracellular fluid; and this distinguishes them functionally from macropinosomes. Until more is known of the differences and similarities between macropinosomes and uncoated micropinosomes, we would arbitrarily separate them into two groups based on their detectability by light-microscopy. Macropinosomes would, therefore, include all pinosomes larger than 0.2 μm diameter.

Different cells ruffle to different extents, and these differences contribute to the widely varying rates of macropinocytosis between cell types. The propensity for ruffling is partly related to the cellular surface-to-volume ratio; cells that can make protrusive flaps of cytoplasm are more likely to form macropinosomes. A ruffle is formed by a linear band of outward-directed actin polymerization near the plasma membrane, which lengthens into a roughly planar extension of the cell surface (Fig. 2). Ruffling can be inhibited by cytochalasins, which disrupt the actin cytoskeleton[10]. Oriented actin polymerization may provide sufficient outward force for ruffle formation, but ruffling may also require contributions from actin-based mechanochemical enzymes, such as myosin.

Ruffles take forms ranging from planar folds to circular, cup-shaped extensions of cytoplasm. In unstimulated macrophages, ruffles are relatively short, and only rarely close into macropinosomes. After stimulation by macrophage colony-stimulating factor or phorbol esters, the ruffles become longer and broader, and frequently close into large macropinosomes[4,11]. Stimulation of MDCK or PtK_2 cells with hepatocyte growth factor/scatter factor elicits circular ruffles that close into macropinosomes by a purse-string-like movement[12]. Circular ruffles also appear in stimulated macrophages (Fig. 1).

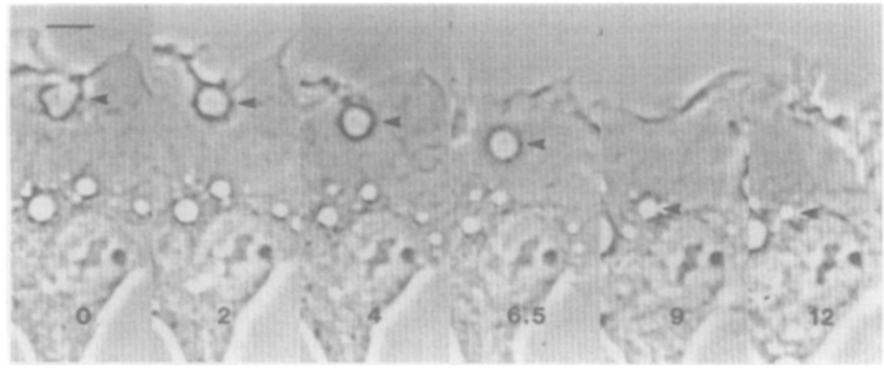

FIGURE 1

Phase-contrast microscopy of macropinocytosis in mouse macrophages, taken from a series of video images. Minutes elapsed after the left-most panel are indicated at the bottom of each panel. A circular, phase-dense ruffle closes to form a phase-bright, intracellular vesicle that migrates away from the cell margin and towards the nucleus, shrinking *en route*. Bar, 3 μm.

In some respects, macropinocytosis is much like phagocytosis. An actin-rich pseudopod that makes a phagosome is structurally similar to the ruffle that becomes a macropinosome[13]. A circular ruffle looks like a phagosome without a particle inside, and, as described below, there are some forms of phagocytosis in which bacteria appear to be caught inside developing macropinosomes. Nonetheless, in the more conventional mechanisms of phagocytosis, in which plasma membrane envelopes a particle as a close-fitting sleeve, pseudopod advance is guided by molecules on the particle surface, and this distinguishes phagocytosis from macropinocytosis. Moreover, macropinocytosis and zipper-like phagocytosis are regulated differently[13].

Because ruffling is a relatively unguided activity, closure into an intracellular vesicle does not always follow. Ruffles often recede into cytoplasm without forming vesicles, and sometimes partially formed macropinosomes abort the process. Macropinosomes that do form close, apparently, by constriction of the ruffle margin into a small connecting pore at the cell surface, after which the macropinosome pinches off as an intracellular vesicle. Thus, despite their large size, the scission of macropinosomes from the cell surface to form cytoplasmic vesicles requires only a very small membrane fusion event. Mechanistically, such fusions could be similar to those that transform coated pits into coated vesicles.

The intracellular fate of macropinosomes differs in different cell types. In macrophages, they move towards the centre of the cell, shrink by loss of water, and acidify, all within 15 minutes. During this brief life span, they change from an early-endosome-like organelle to a late-endosome-like organelle, then merge completely into the lysosomal compartment[14]. As they mature from one kind of organelle to another, they interact with other macropinosomes and other endosomes, and can receive solutes from both older and younger organelles[15]. Rarely, they fuse again with the cell surface. In contrast to this, macropinosomes in human A431 cells show little interaction with endocytic compartments apart from other macropinosomes. They appear to constitute a distinct, brefeldin-A-resistant endosome population, which eventually recycles most of its content to the cell surface[5].

Regulation

Although macropinocytosis runs constitutively in many transformed cells and in human dendritic cells cultured in the presence of interleukin-4 (IL-4) and granulocyte–macrophage colony-stimulating factor (GM-CSF; Ref. 16; see below), it is more commonly a regulated phenomenon. Microinjection of oncogenic Ras into 3T3 fibroblasts stimulates a pronounced increase in pinocytosis, evidently of macropinocytosis[17]. In some cells, treatment with growth factors or phorbol esters elicits a rapid and dramatic macropinocytotic response[3,11,18–21]. In neutrophils, phorbol myristate acetate (PMA) or diacylglycerol can stimulate pinocytosis 40-fold, and this increase is most likely attributable to macropinocytosis[22].

Although much remains to be learned, macropinocytosis appears to be regulated at several points (Fig. 3). The first is the ruffling response, which can be induced by activation of growth factor tyrosine kinases, challenge with phorbol esters, microinjection of activated Ras-family proteins or Ras-family guanine-nucleotide exchange stimulators[4,17,19,23,24]. Since microinjection of activated forms of Rac-1 can induce ruffling in the absence of any external stimulus[23], it is likely that the different external stimuli that trigger ruffling and macropinocytosis act as activators of Rac itself or of downstream targets of Rac. Current models propose that Rac regulates the assembly of an actin-nucleating complex at the plasma membrane, which then directs the formation of ruffles. Rapid assembly of such complexes could define the band of polymerizing actin beneath the plasma membrane that becomes the leading edge of the ruffle[25,26]. Phorbol esters also stimulate membrane ruffling in many cell types, again via endogenous Rac, at least in Swiss 3T3 cells[23], and macropinocytosis in neutrophils is stimulated by PMA and diacylglycerol, both potent activators of protein kinase C (Ref. 22). However, the role of protein kinase C in the regulation of macropinocytosis remains unsettled, as only some inhibitors of protein kinase C inhibit the effects of PMA (Ref. 27) and it has yet to be demonstrated that activation of protein kinase C is necessary for ruffling. It is possible that other phorbol-ester-binding proteins, such as chimaerins, are involved[26]. Many different proteins have been localized to membrane ruffles[28] and some, such as Hsp27, stimulate ruffling and pinocytosis upon overexpression[29]. The products of phosphatidylinositol 3-kinase and phospholipase A2 also modulate actin polymerization and are implicated in the ruffling response (reviewed in Ref. 26). Cytoplasmic microtubules also appear to modulate cell surface ruffling and the subsequent formation of macropinosomes[7,11,28]. Clearly, it will be important to establish which of the many responses that accompany ruffling are actually required and what stimuli are important *in vivo*.

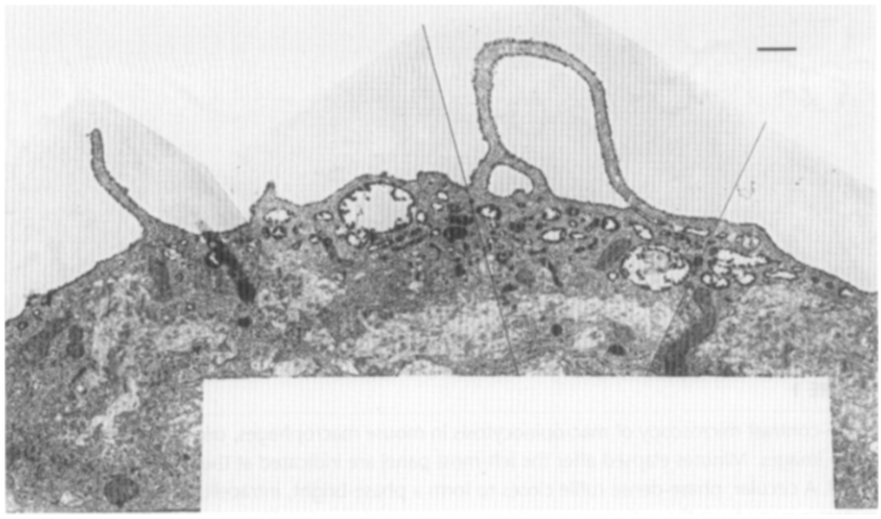

FIGURE 2

A composite, thin-section electron micrograph of a macrophage, fixed after stimulation with phorbol myristate acetate in the presence of the endocytic tracer microperoxidase. Ruffles extend from the surface of the cell; the right-most ruffle appears, in this section, to have closed back against the cell surface. Histochemical localization of microperoxidase shows labelling of both macropinosomes and smaller endocytic vesicles. Bar, 0.5 μm.

Membrane ruffling and macropinocytosis in A431 cells can be inhibited by amiloride and more potent analogues, such as dimethyl amiloride (Ref. 20, and L. J. Hewlett, PhD thesis, University of Dundee, 1995), which inhibit the Na^+/H^+ exchange protein in the plasma membrane[30]. These agents may act by lowering cytoplasmic pH, which also inhibits clathrin-mediated endocytic activity[31]. However, in A431 cells, clathrin-mediated uptake of EGF continues under conditions where macropinocytosis is inhibited by amiloride[20], suggesting that ruffling and macropinocytosis may be acutely sensitive to cytosolic acidification. Conditions that acidify cytoplasm produce a dramatic cessation of cell surface ruffling movements in macrophages and, conversely, alkalinization of cytoplasm enhances ruffling[32]. It is not yet known biochemically how cytoplasmic pH affects ruffling.

After ruffles form, they must close into intracellular vesicles; then, the actin filaments must be removed to allow interactions with other organelles. These processes may be considered the second and third points of regulation (Fig. 3), although the nature of such regulation is unknown.

Further levels of regulation are indicated by the different fates of macropinosomes in macrophages and A431 cells. In macrophages, macropinosomes lose transferrin receptors, gain and then lose Rab7, and gain the lysosomal-membrane protein Lgp-A (Ref. 14). This indicates that they undergo a series of biochemical modifications and vesicle fusion reactions during their brief lifetimes. Rab7 could contribute to the regulated maturation of the macropinosome. Similarly, other GTP-binding proteins, such as Rab4 and Rab5, may regulate the interactions between macropinosomes and other endocytic compartments. Phagosomes also undergo a sequential and, perhaps, related series of interactions with other endocytic organelles before they merge completely into the lysosomal compartment[33]. Such modifications may also influence the ultimate disposition of the macropinosome. Thus, it is possible that non-phagocytic cells lack the mechanisms that allow macropinosomes and phagosomes to mature and that this may explain why macropinosomes of A431 cells do not progress beyond the first stage and, instead, return to the cell surface.

The orderly sequence of changes in macropinosomes indicates a regulation like the checkpoint controls that regulate progress through the cell cycle[34]. For example, there may be biochemical mechanisms that prevent removal of F-actin from the macropinosome until after the macropinosome separates from the plasma membrane. Similarly, subsequent interactions between macropinosomes and other organelles may be subject to checkpoint controls that delineate a dependent series: for example, completion of Rac-dependent activities allows Rab5-dependent activities, and the completion of the latter allows Rab7-dependent activities, and so on.

Relevance

The benefits of taking in large volumes of extracellular fluids are not obvious. Indeed, it would seem counterproductive for cells to internalize ions, such as Na^+ and Cl^-, that are exported actively across the plasma membrane. It may be that macropinocytosis reflects the necessary idling of a phagocytic cell; and for a macrophage to be able to engulf particles rapidly or efficiently it must ruffle and gulp at some minimum pace. One possible benefit of macropinocytosis, albeit an imagined one, is that it could groom the cell surface by taking in plasma membrane, exposing it to an acidic pH, then returning it to the surface. This could allow membrane proteins that are usually excluded from micropinosomes to cycle occasionally through endosomes. Alternatively, as stimulated macropinocytosis also increases the flow of pinocytosed solutes into lysosomes in macrophages, it could provide a general mechanism for rerouting membrane traffic[4,35].

Recent studies have indicated more substantial roles for macropinocytosis. It may contribute to the immune response, where, prior to the expression of high-affinity antibodies, antigen-presenting cells must rely on apparently non-specific mechanisms for antigen capture, leading to antigen presentation and T-cell activation[16]. Since dendritic cells are widely believed to trigger primary T-cell responses[36], the early reports that these cells were not endocytically active were puzzling. Clarification has come from the demonstration that immature dendritic cells cultured in the presence of IL-4 and GM-CSF exhibit pronounced macropinocytosis, coincident with highly efficient antigen processing[16,37]. In such cells, the fluid-equivalent of approximately one cell-volume is taken up and concentrated each hour, and this

persists even after removal of IL-4 and GM-CSF. Importantly, markers taken up and concentrated under these conditions are targeted to a compartment rich in MHC class II molecules, which accounts for the highly efficient antigen-presenting capacity of these cells. Upon challenge with cytokines, such as TNFα, the macropinocytotic activity of dendritic cells is downregulated and T-cell stimulatory capacity is upregulated as part of a co-ordinated response, which reflects dendritic cell maturation *in vivo*[16]. It will be interesting to resolve how macropinocytosed antigens are targeted efficiently to the class II MHC compartment since, in other antigen-presenting cells, this compartment is accessed efficiently only by receptor-bound antigen[38]. The process could involve intracellular receptor-mediated delivery mechanisms, perhaps including lectin-like receptors[39].

Macropinocytosis may also serve the class I MHC response, which is normally elicited only by endogenous (i.e. cytosolic and nuclear) proteins. Surprisingly, a minority of macropinosomes in both A431 cells and bone-marrow-derived macrophages leak their contents into the cytosol, providing an access route to the conventional class I MHC pathway (C. C. Norbury and C. Watts, unpublished).

However, macropinocytosis can also do the immune system a disservice in that it can be exploited by a bacterial pathogen as a mechanism for cell invasion. *Salmonella typhimurium* is an enteric pathogen that gives susceptible, inbred mice an illness similar to typhoid fever in man. Bacteria exit the gut lumen through the epithelial cells and are subsequently phagocytosed by macrophages. Entry into epithelial cells is accompanied by stimulated cell-surface ruffling, called a splash, and the formation of phagosomes that resemble macropinosomes[40]. Entry of *S. typhimurium* into macrophages is also accompanied by stimulation of macropinocytosis[41]. Instead of entering via close-fitting 'zipper-like' phagosomes, the bacteria are caught in forming macropinosomes, then persist within these unusually spacious phagosomes. Survival inside macrophages correlates with the ability to form, or maintain, spacious phagosomes (C. Alpuche-Aranda, S. I. Miller and J. A. Swanson, unpublished). Spacious phagosomes could increase *S. typhimurium* survival by slowing the rate of acidification, or by deflecting antimicrobial defence mechanisms that work optimally in the smaller volume of a close-fitting phagosome.

For a long time, macropinocytosis was a curious sideshow of stimulated cells. Recently, membrane ruffling, the precursor to macropinocytosis, has been the focus of studies aimed at understanding the regulation of the actin cytoskeleton by GTPases and lipid metabolites. Further mechanistic insights may be expected, therefore, in this area. Macropinocytosis, itself, offers an economical means to maximize volume uptake, for example, for sampling the antigenic environment. As is the case for other endocytic pathways, pathogens have found ways of exploiting this mode of endocytosis to invade eukaryotic cells. Hopefully, they too will prove useful tools to investigate the unique character of macropinocytosis.

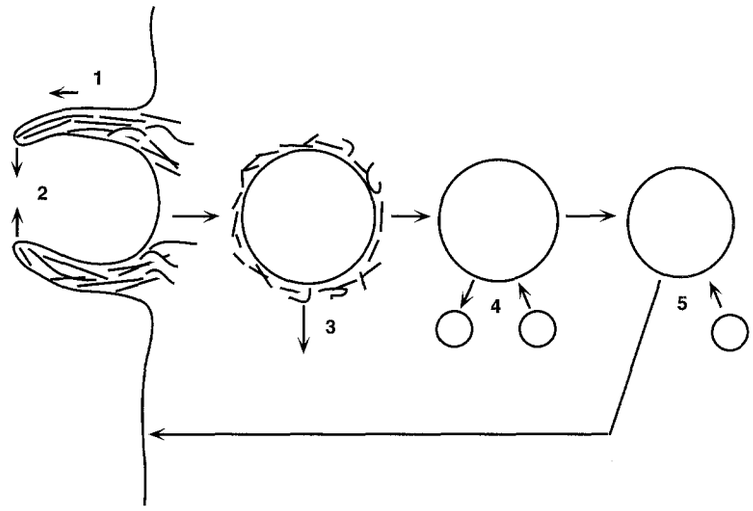

FIGURE 3

Points of regulation of macropinocytosis: (1) the initiation and formation of ruffles; (2) closure of ruffles at their outermost margins to form vesicles; (3) removal of F-actin from the macropinosome; (4) interaction with other endocytic compartments; and (5) disposal by recycling to the cell surface, as in HeLa cells, or merger with a different endocytic compartment, as in macrophages and dendritic cells.

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Make it or break it: the role of ubiquitin-dependent proteolysis in cellular regulation

Raymond J. Deshaies

Effective regulation of the concentration of a protein in the cell requires rapid protein degradation. Until recently, it was widely believed that intracellular proteolysis was largely confined to the turnover of damaged, or otherwise abnormal, proteins. Recently, however, the role of protein degradation in cellular regulation has gained centre stage, and ubiquitin/proteasome-dependent proteolysis has been shown to play a key role in processes as diverse as embryonic development, transcription and the cell cycle.

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Although several proteolytic pathways coexist in the cytosol of eukaryotic cells, the majority of cytosolic proteolysis is catalysed by the ubiquitin-dependent 26S proteasome pathway. The biochemistry and genetics of the ubiquitin–proteasome pathway have been well reviewed in recent years[1]. To briefly recap, the proteasome degrades proteins that contain covalently linked multiubiquitin chains. Ubiquitin is attached to proteins in a multistep process as diagrammed below:

$$E1 + Ub + ATP \rightarrow E1\sim Ub + AMP$$
$$E1\sim Ub + E2 \rightarrow E1 + E2\sim Ub$$
$$E2\sim Ub + substrate\ (+E3?) \rightarrow E2 + substrate\sim Ub\ (+E3?)$$

First, ubiquitin is attached via its C-terminus to the ubiquitin activating (E1) enzyme. Activated ubiquitin is then attached covalently to ubiquitin-conjugating (E2) enzymes. Most cells contain a single E1, but there are at least 12 genes in yeast that encode E2 enzymes. In many cases, ubiquitin can be transferred directly from a charged E2 to a substrate protein. Nevertheless, many physiological ubiquitination events may require the activity of a ubiquitin ligase, referred to as E3. Two different classes of E3 enzyme have been identified [hect-domain proteins (Ref. 2) and Ubr1p (Ref. 3)] and, as they are not homologous to one another, there may be other, unrecognized, E3s in the cell. Most ubiquitin conjugates are rapidly degraded to completion by the 26S proteasome. However, some ubiquitin conjugates are sufficiently stable to accumulate, suggesting that ubiquitination may regulate protein function by multiple mechanisms.

Three important discoveries during the 1980s indicated that protein ubiquitination plays a significant role in cellular regulation. First, temperature-sensitive mutant (*ts*) mammalian cell lines with defects in progression through the cell cycle were shown to have thermolabile E1 enzyme[4]. A specific role for protein ubiquitination in cell-cycle control was confirmed subsequently by the discovery that *CDC34*, which is required for progression through the G1–S transition in *Saccharomyces cerevisiae*, encodes an E2 enzyme[5]. The key event triggering the acknowledgement of protein degradation as a vital regulatory mechanism was the discovery that ubiquitin-dependent destruction of cyclin B is essential for the